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Asier Basurco Pérez

Pruebas de confirmación de la infección por Leishmania infantum: interés en la práctica clínica

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Tesis Doctoral

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Autor

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Universidad Zaragoza

Dra. **MARÍA TERESA VERDE ARRIBAS**, Catedrática de Medicina y Cirugía Animal del Departamento de Patología Animal de la Universidad de Zaragoza, y Dr. **SERGIO VILLANUEVA SAZ**, Profesor Titular de Universidad de Medicina y Cirugía Animal del Departamento de Patología Animal de la Universidad de Zaragoza, como directores,

CERTIFICAN:

Que Don **ASIER BASURCO PÉREZ** ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada: "Pruebas de confirmación de la infección por *Leishmania infantum*: interés en la práctica clínica", que se ajusta al Proyecto de Tesis presentado y que cumple las condiciones exigidas para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizan la presentación de esta Tesis, por compendio de publicaciones, para que pueda ser juzgada por el Tribunal correspondiente.

Y para que conste, firman el presente certificado

En Zaragoza, a 24 de Abril de 2025

Dra. María Teresa Verde Arribas Dr. Sergio Villanueva Saz

Índicadores de calidad de las publicaciones

Esta **Tesis Doctoral**, presentada por Don Asier Basurco Pérez, de acuerdo con el informe correspondiente, autorizado por los directores de Tesis y por el Órgano Responsable del Programa de Doctorado, se presenta como un compendio de trabajos previamente publicados. La unidad temática está directamente relacionada con el diagnóstico de *Leishmania infantum* en la práctica clínica, por lo que queda justificada su presentación por compendio de publicaciones.

Las referencias completas de los artículos que constituyen el cuerpo de la tesis son los siguientes:

Artículo 1 (artículo original. Revista Indexada en JCR (Q2), segundo autor):

Villanueva-Saz S, **Basurco A**, Martín V, Fernández A, Loste A, Verde MT. Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs. Acta Vet Scand. 2019; 61:38.

FACTOR IMPACTO 1.683
Q2 (39/141) EN CIENCIAS VETERINARIAS

Artículo 2 (artículo original. Revista Indexada en JCR (Q3), primer autor):

Basurco A, Natale A, Capello K, Fernández A, Verde MT, González A, Yzuel A, Giner J, Villanueva-Saz S. Evaluation of the performance of three serological tests for diagnosis of *Leishmania infantum* infection in dogs using latent class analysis. Rev Bras Parasitol Vet. 2020; 29:e018020.

FACTOR DE IMPACTO: 1.458
Q3 (76/146) EN CIENCIAS VETERINARIAS

Artículo 3 (artículo original. Revista No Indexada, segundo autor):

Giner J, **Basurco A**, Alcover MM, Riera C, Fisa R, López RA, Juan-Sallés C, Verde MT, Fernández A, Yzuel A, Villanueva-Saz S. First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain. Vet Parasitol Reg Stud Reports. 2020; 19:100369.

Artículo 4 (artículo original. Revista Indexada en JCR (Q1), segundo autor):

Alcover MM, **Basurco A**, Fernandez A, Riera C, Fisa R, Gonzalez A, Verde M, Garrido AM, Ruíz H, Yzuel A, Villanueva-Saz S. A cross-sectional study of *Leishmania infantum* infection in stray cats in the city of Zaragoza (Spain) using serology and PCR. Parasit Vectors. 2021; 14:178.

FACTOR DE IMPACTO 4.052
Q1 (3/24) en MEDICINA TROPICAL
Q1 (8/39) en PARASITOLOGIA

Contribución del doctorando en las publicaciones

La contribución del doctorando, Don Asier Basurco Pérez, a cada uno de los artículos que se referencían en esta tesis y que se presenta como compendio de publicaciones, es la siguiente:

Artículo 1: Villanueva-Saz S, **Basurco A**, Martín V, Fernández A, Loste A, Verde MT. Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs. Acta Vet Scand. 2019; 61:38.

Contribución del doctorando: Realización de los análisis de laboratorio, así como los análisis estadísticos, interpretación de los hallazgos, revisión y aprobación del manuscrito final.

Artículo 2: **Basurco A**, Natale A, Capello K, Fernández A, Verde MT, González A, Yzuel A, Giner J, Villanueva-Saz S. Evaluation of the performance of three serological tests for diagnosis of *Leishmania infantum* infection in dogs using latent class analysis. Rev Bras Parasitol Vet. 2020; 29:e018020.

Contribución del doctorando: Planificación y coordinación de estudio, realización de análisis de laboratorio, interpretación de resultados, redacción, así como revisión y aprobación final del manuscrito.

Artículo 3: Giner J, **Basurco A**, Alcover MM, Riera C, Fisa R, López RA, Juan-Sallés C, Verde MT, Fernández A, Yzuel A, Villanueva-Saz S. First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain. Vet Parasitol Reg Stud Reports. 2020; 19:100369.

Contribución del doctorando: Realización de análisis de laboratorio, interpretación de hallazgos, revisión del manuscrito, así como lectura y aprobación del manuscrito final.

Artículo 4: Alcover MM, **Basurco A**, Fernandez A, Riera C, Fisa R, Gonzalez A, Verde M, Garrido AM, Ruíz H, Yzuel A, Villanueva-Saz S. A cross-sectional study of *Leishmania infantum* infection in stray cats in the city of Zaragoza (Spain) using serology and PCR. Parasit Vectors. 2021; 14:178.

Contribución del doctorando: Realización de las pruebas serológicas, análisis de los datos y redacción del manuscrito, revisión del manuscrito, y finalmente lectura y aprobación del manuscrito final.

Introducción general y justificación de la unidad temática

La leishmaniosis, una enfermedad causada por el parásito *Leishmania infantum* y transmitida por la picadura de flebotomos infectados, representa una de las principales amenazas en el ámbito de las enfermedades parasitarias a nivel mundial, situándose como la segunda protozoosis más letal después de la malaria. Su impacto es especialmente relevante debido a la elevada morbilidad y mortalidad que ocasiona tanto en humanos como en animales, convirtiéndose en una problemática de interés compartido entre la medicina humana y veterinaria.

Desde la perspectiva veterinaria, la leishmaniosis afecta principalmente a perros y gatos, aunque estudios recientes alertan sobre la aparición de casos en especies no convencionales, como los mustélidos. Esta expansión de hospedadores refuerza la necesidad de un enfoque One Health, donde la salud animal, humana y ambiental se consideran interdependientes. Así, el estudio de esta enfermedad no solo reviste importancia clínica y epidemiológica, sino que también proporciona un modelo ideal para comprender las interacciones entre agentes infecciosos, vectores y hospedadores en un contexto de cambio climático y globalización.

La falta de una prueba diagnóstica universalmente aceptada como “estándar de oro” constituye uno de los principales desafíos para el control de la leishmaniosis. La identificación precisa de casos, en especial aquellos subclínicos o en especies emergentes, requiere la combinación de múltiples técnicas parasitológicas, serológicas y moleculares. Evaluar su eficacia y establecer estrategias diagnósticas integradas resulta esencial para mejorar la sensibilidad, especificidad y capacidad predictiva de los métodos empleados.

Este trabajo de tesis propone una revisión exhaustiva de los aspectos diagnósticos de la leishmaniosis en diversas especies animales, poniendo énfasis en la necesidad de adaptar las herramientas disponibles a nuevos escenarios clínicos y epidemiológicos. Asimismo, se abordará la relevancia de métodos estadísticos avanzados, como los modelos bayesianos y de clases latentes, para estimar parámetros diagnósticos en ausencia de un estándar de referencia, fortaleciendo así la robustez de los estudios comparativos.

En este sentido, la leishmaniosis no solo constituye una enfermedad de interés veterinario y humano, sino también un marco para el estudio de enfermedades infecciosas vectoriales a nivel global. Este trabajo de tesis, por tanto, busca integrar los conocimientos más actuales sobre diagnóstico, epidemiología y salud pública, fomentando una visión crítica y multidisciplinaria que permita desarrollar estrategias efectivas de prevención, control y vigilancia en distintos contextos biológicos y geográficos.

Resumen

La leishmaniosis, enfermedad zoonótica causada por el protozoo *Leishmania infantum*, supone un desafío importante tanto para la salud humana como animal. La presente tesis, compuesta por cuatro publicaciones, aborda diversos aspectos relacionados con la infección, poniendo especial énfasis en las especies canina y felina, y además presenta el primer diagnóstico y la posible implicación de especies emergentes —como el hurón doméstico (*Mustela putorius furo*)— en el ciclo epidemiológico de la enfermedad.

La **primera publicación** evaluó la eficacia de un ensayo rápido basado en inmunocromatografía, el FASTest LEISH®, en comparación con dos métodos serológicos cuantitativos: el ensayo de detección de anticuerpos mediante inmunofluorescencia indirecta (IFI) y un ensayo ELISA desarrollado internamente. La investigación tuvo como objetivo determinar la capacidad del ensayo rápido para detectar anticuerpos contra *L. infantum* en perros expuestos de manera natural al parásito vectorial.

Se analizaron 244 muestras de suero procedentes de perros de diversas edades, razas y sexos, obtenidas en distintas circunstancias diagnósticas (cribado anual, donación de sangre, sospecha clínica de leishmaniosis). Las muestras se clasificaron como seropositivas o seronegativas en función de los resultados combinados de los ensayos de referencia. De las 232 muestras con resultados concordantes, 121 se clasificaron como seropositivas y 111 como seronegativas.

El ensayo rápido demostró una alta sensibilidad (100%) y especificidad (99,1%) en comparación con los métodos de referencia. Asimismo, mostró una precisión del 99,6%, presentando un coeficiente kappa de 0,99 y un área bajo la curva ROC (AUC-ROC) de 0,995, lo que refleja su elevada fiabilidad diagnóstica. Estos resultados indican que el ensayo es particularmente útil para la identificación de perros seropositivos asintomáticos en entornos clínicos.

Los hallazgos subrayan la importancia de emplear múltiples métodos diagnósticos ante la ausencia de un estándar de oro, y resaltan la necesidad de contar con pruebas rápidas y precisas en la práctica clínica veterinaria. A pesar de las limitaciones inherentes—como la falta de muestras reactivas a otros patógenos—, los resultados consolidan la utilidad del FASTest LEISH® como herramienta diagnóstica eficiente en la leishmaniosis canina.

El **segundo estudio** investiga la eficacia de tres pruebas serológicas comerciales (FASTest LEISH®, MegaFLUO LEISH® y MegaELISA LEISH®) y de una técnica ELISA desarrollada internamente (ELISA UNIZAR) para el diagnóstico de la infección por *L. infantum* en perros, mediante un análisis de clases latentes bajo un enfoque bayesiano. Este método permite evaluar la sensibilidad y especificidad de las pruebas sin requerir un estándar de referencia perfecto, eliminando así los sesgos inherentes a pruebas diagnósticas imperfectas.

El análisis incluyó 215 muestras de suero de perros, tanto de zonas endémicas como no endémicas. Los resultados evidenciaron una alta sensibilidad en todas las pruebas, destacándose FASTest LEISH® y ELISA UNIZAR, con valores superiores al 99%,

mientras que MegaELISA LEISH® presentó la menor sensibilidad, cercana al 98,49%. En términos de especificidad, FASTest LEISH® obtuvo la mayor puntuación (98,43%), seguida de ELISA UNIZAR (97,50%), mientras que MegaFLUO LEISH® y MegaELISA LEISH® arrojaron valores alrededor del 91,9%. Además, se observó una concordancia casi perfecta entre las técnicas analizadas (valores de kappa superiores a 0,80), siendo FASTest LEISH® y ELISA UNIZAR los métodos que mostraron mayor acuerdo. El estudio también examinó las diferencias en el rendimiento de las pruebas en perros de zonas endémicas, considerando aquellos con infecciones subclínicas o asintomáticas, lo que subraya la utilidad de las pruebas rápidas en la práctica clínica veterinaria y sugiere que pueden representar alternativas válidas en ausencia de pruebas cuantitativas. Por último, se destaca la importancia de utilizar múltiples enfoques diagnósticos para optimizar la detección de infecciones por *L. infantum* en diversos contextos epidemiológicos.

El **tercer estudio** describe el primer caso documentado en España de infección natural por *L. infantum* en un hurón doméstico (*Mustela putorius furo*), identificando a esta especie como un potencial reservorio del parásito en áreas endémicas. El caso, ocurrido en Valencia, describe la presencia de una lesión papular en el pabellón auricular derecho, diagnosticada mediante histopatología como dermatitis piógranulomatosa crónica. La infección se confirmó mediante técnicas avanzadas, tales como la reacción en cadena de la polimerasa (PCR), el aislamiento del parásito en medios de cultivo específicos y diversas pruebas serológicas (incluyendo ELISA, inmunofluorescencia indirecta y Western Blot). Además, el diagnóstico se complementó con inmunohistoquímica, la cual evidenció la presencia de amastigotes de *L. infantum* en células inflamatorias del tejido afectado.

El análisis clínico reveló anormalidades como hiperfosfatasemia, elevación de la alanina aminotransferasa y de la gammaglutamiltransferasa, así como hipergammaglobulinemia policlonal, hallazgos compatibles con desórdenes hepáticos e inflamatorios. Asimismo, se asoció la posible contribución de una terapia inmunomoduladora previa (basada en prednisolona y ciclosporina A) como factor desencadenante de la manifestación clínica de la leishmaniosis. Estos hallazgos resaltan la necesidad de realizar estudios epidemiológicos más amplios para determinar el papel de los hurones como reservorios de *L. infantum* y evaluar su impacto potencial en la transmisión del parásito a humanos y otras especies. En consecuencia, se enfatiza la relevancia de incluir a los hurones en el diseño de programas epidemiológicos, así como la importancia de adaptar métodos diagnósticos y terapéuticos a esta especie.

El **cuarto estudio** consiste en un análisis transversal de la infección por *L. infantum* en gatos callejeros aparentemente sanos en Zaragoza (España), una región endémica de la enfermedad. Mediante la aplicación de métodos serológicos y moleculares, se evaluaron tanto la prevalencia de la infección como la concordancia entre las distintas técnicas diagnósticas. Se analizaron 179 gatos, obteniéndose una prevalencia global del 15,6% al considerar los resultados de la serología y de la qPCR. Entre los métodos serológicos, el Western Blot (WB) presentó la mayor sensibilidad (14,5% de positividad), seguido del ELISA (2,8%) y del IFAT (2,2%). Además, la qPCR permitió detectar ADN del parásito en el 5,6% de los gatos. Se observó un bajo grado de concordancia entre los métodos moleculares y serológicos, siendo la mayor entre IFAT y ELISA ($k = 0,89$) y la menor entre qPCR y WB ($k = 0,19$).

El estudio identificó asociaciones significativas entre el género masculino y la positividad en la qPCR, lo que sugiere un posible factor de riesgo, sin encontrar correlación con el entorno (urbano o periurbano). En lo referente a coinfecciones, se detectaron vínculos entre *L. infantum* y el virus de inmunodeficiencia felina (FIV) en los ensayos IFAT y ELISA, sin observarse asociación con el virus de leucemia felina (FeLV). Estos resultados enfatizan la importancia de combinar distintas herramientas diagnósticas para mejorar la detección de infecciones subclínicas en gatos y considerar a estos animales como posibles reservorios en la transmisión de *L. infantum*. Asimismo, se refuerza la necesidad de implementar programas de vigilancia epidemiológica en regiones endémicas y se plantean interrogantes acerca del rol epidemiológico de los gatos callejeros.

Las **conclusiones** del trabajo proponen la implementación de metodologías diagnósticas más fiables y adaptadas, que respalden la labor del médico veterinario en la práctica clínica, así como el desarrollo de estrategias orientadas a identificar reservorios emergentes que faciliten el control epidemiológico de esta zoonosis.

Summary

Leishmaniosis, a zoonotic disease caused by the protozoan *Leishmania infantum*, presents a major challenge for both human and animal health. This thesis, composed of four publications, addresses various aspects related to the infection, with a special focus on canine and feline species. It also presents the first diagnosis and potential involvement of emerging species—such as the domestic ferret (*Mustela putorius furo*)—in the epidemiological cycle of the disease.

The **first manuscript** evaluated the effectiveness of a rapid immunochromatographic test, FASTest LEISH®, in comparison with two quantitative serological methods: an indirect immunofluorescence antibody test (IFAT) and an in-house developed ELISA. The study aimed to determine the rapid test's ability to detect antibodies against *L. infantum* in dogs naturally exposed to the vector-borne parasite.

A total of 244 serum samples were analyzed from dogs of various ages, breeds, and sexes, obtained under different diagnostic circumstances (annual screening, blood donation, clinical suspicion of leishmaniasis). Samples were classified as seropositive or seronegative based on combined results from reference assays. Of the 232 samples with concordant results, 121 were classified as seropositive and 111 as seronegative.

The rapid test demonstrated high sensitivity (100%) and specificity (99.1%) compared to the reference methods. It also showed an accuracy of 99.6%, with a kappa coefficient of 0.99 and an area under the ROC curve (AUC-ROC) of 0.995, indicating its high diagnostic reliability. These findings suggest that the test is particularly useful for identifying asymptomatic seropositive dogs in clinical settings.

The results highlight the importance of using multiple diagnostic methods in the absence of a gold standard, and underscore the need for rapid and accurate tests in veterinary practice. Despite inherent limitations—such as the lack of samples reactive to other pathogens—the findings support the utility of FASTest LEISH® as an efficient diagnostic tool for canine leishmaniasis.

The **second manuscript** investigates the effectiveness of three commercial serological tests (FASTest LEISH®, MegaFLUO LEISH®, and MegaELISA LEISH®) and one in-house ELISA (ELISA UNIZAR) for diagnosing L. infantum infection in dogs, using a Bayesian latent class analysis approach. This method allows for estimating the sensitivity and specificity of tests without requiring a perfect reference standard, thus eliminating biases inherent to imperfect diagnostics.

The analysis included 215 serum samples from dogs in both endemic and non-endemic areas. Results showed high sensitivity for all tests, with FASTest LEISH® and ELISA UNIZAR exceeding 99%, while MegaELISA LEISH® had the lowest sensitivity at approximately 98.49%. In terms of specificity, FASTest LEISH® scored highest (98.43%), followed by ELISA UNIZAR (97.50%), while MegaFLUO LEISH® and MegaELISA LEISH® showed values around 91.9%. There was an almost perfect agreement between the tests (kappa values above 0.80), with FASTest LEISH® and ELISA UNIZAR showing the greatest concordance. The study also examined differences in test performance in dogs from endemic areas, particularly those with subclinical or asymptomatic infections, emphasizing the utility of rapid tests in veterinary clinical

practice and suggesting they may be valid alternatives in the absence of quantitative tests. Finally, the importance of using multiple diagnostic approaches to optimize detection of *L. infantum* infections across various epidemiological contexts was reinforced.

The **third manuscript** describes the first documented case in Spain of natural *L. infantum* infection in a domestic ferret (*Mustela putorius furo*), identifying this species as a potential reservoir of the parasite in endemic areas. The case, reported in Valencia, involved a papular lesion on the right auricular pinna, histopathologically diagnosed as chronic pyogranulomatous dermatitis. The infection was confirmed through advanced techniques including polymerase chain reaction (PCR), parasite isolation in specific culture media, and various serological tests (including ELISA, indirect immunofluorescence, and Western Blot). Additionally, the diagnosis was supported by immunohistochemistry, which revealed the presence of *L. infantum* amastigotes in inflammatory cells within the affected tissue.

Clinical analysis revealed abnormalities such as hyperphosphatasemia, elevated alanine aminotransferase and gamma-glutamyl transferase, and polyclonal hypergammaglobulinemia—findings consistent with hepatic and inflammatory disorders. A prior immunomodulatory therapy (prednisolone and cyclosporine A) may have contributed to the clinical manifestation of leishmaniasis. These findings highlight the need for broader epidemiological studies to determine the role of ferrets as *L. infantum* reservoirs and assess their potential impact on parasite transmission to humans and other species. As a result, the inclusion of ferrets in epidemiological surveillance programs is emphasized, along with the importance of adapting diagnostic and therapeutic methods for this species.

The **fourth manuscript** presents a cross-sectional analysis of *L. infantum* infection in apparently healthy stray cats in Zaragoza (Spain), an endemic region for the disease. Through serological and molecular methods, both infection prevalence and concordance between diagnostic techniques were assessed. A total of 179 cats were analyzed, revealing an overall prevalence of 15.6% based on combined serological and qPCR results. Among the serological methods, Western Blot (WB) showed the highest sensitivity (14.5% positive), followed by ELISA (2.8%) and IFAT (2.2%). Additionally, qPCR detected parasite DNA in 5.6% of the cats. A low level of agreement was found between molecular and serological methods, with the highest concordance between IFAT and ELISA ($k = 0.89$) and the lowest between qPCR and WB ($k = 0.19$).

The study identified significant associations between male gender and qPCR positivity, suggesting a potential risk factor, although no correlation was found with environment (urban vs. peri-urban). Regarding co-infections, associations were detected between *L. infantum* and feline immunodeficiency virus (FIV) in IFAT and ELISA assays, but no link was found with feline leukemia virus (FeLV). These results emphasize the importance of combining different diagnostic tools to better detect subclinical infections in cats and consider them as potential reservoirs in *L. infantum* transmission. Furthermore, the study reinforces the need to implement epidemiological surveillance programs in endemic areas and raises questions about the epidemiological role of stray cats.

The thesis concludes with a proposal for implementing more reliable and tailored diagnostic methodologies to support veterinary clinical practice, as well as strategies aimed at identifying emerging reservoirs to facilitate epidemiological control of this zoonosis.

"Para empezar un gran proyecto, hace falta valentía.

Para terminar un gran proyecto, hace falta perseverancia."

Anónimo

A mi Abuelo,

A mi compañera de vida Cris,

A mis hijos Aritz, Nahia y Ander

A todos los que me quieren

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Por fin, después de 30 años, el sueño de ser Doctor en Veterinaria ya es casi una realidad, y todo esto con la ayuda y apoyo de mis Directores, Compañeros, Familia y Amigos.

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P.D.: Aritz, Nahia, Ander, un consejo… luchar por vuestros sueños, estos se convertirán en proyectos, y con el tiempo en metas.

Maite zaituztet!

ABREVIATURAS

ADN: Ácido desoxirribonucleico (en inglés DNA)
APC: Células presentadoras de antígenos
CD4 Th1: Linfocitos T colaboradores cluster diferenciación 4
CD8: Linfocitos T citotóxicos cluster diferenciación 8
CL: Leishmaniasis cutánea
CTLA-4: Antígeno 4 asociado a los linfocitos T citotóxicos
DAT: Prueba de aglutinación directa
DC: Células dendríticas
DLA: Antígenos leucocitarios de perro
DTH: Reacción de hipersensibilidad de tipo retardado
ELISA: Ensayo por inmunoabsorción ligado a enzimas
EU: Unidades ELISA
FAST: Prueba de aglutinación directa rápida
FC: citometría de flujo
FCoV: Coronavirus felino
FeLV: Virus de la leucemia felina
FIV: Virus de la inmunodeficiencia felina
GSLC: Grupo de Estudio de la Leishmaniosis Canina
IFI: Prueba de inmunofluorescencia indirecta (en inglés IFAT)
IFN- γ: Interferón gamma
IFNB: Cuantificación del interferón gamma
Ig: Inmunoglobulina
IL: Interleucina
LPA: Estudio de la proliferación de linfocitos
LST: Test de Montenegro
LTB4: Leucotrieno B4
LV: Leishmaniasis visceral
MCL: Leishmaniasis mucocutánea
MCM: Método de cultivo microcapilar
MON: Zimodema
NK: Natural Killer
NNN: Medio de cultivo Novy, McNeal y Nicolle
NO: Óxido nítrico
OIE: Organización Mundial en Sanidad Animal
OMS: Organización Mundial de la Salud
PD-1: Proteína que se encuentra en las células T
PBS: Tampón fosfato salino
PBST: Tampón fosfato salino en Tween 20
PBSTL: Tampón fosfato salino en Tween 20 con leche en polvo desnatada
PCR: Técnica de la reacción en cadena de la polimerasa
PGE2: Prostaglandina E2
PPR: receptores de reconocimiento de patrones
qPCR: PCR cuantitativa en tiempo real
RFLP: polimorfismo en la longitud de los fragmentos de restricción
RNA: Ácido ribonucleico
ROC: receiver operating characteristic
RT-PCR: PCR de transcriptasa inversa
SIDA: Sindrome de inmunodeficiencia adquirida
Th: Linfocitos colaboradores
TNF-α: Factor de necrosis tumoral alfa
TLR: Receptores tipo Toll
Unizar: Universidad de Zaragoza
VIH: Virus de la inmunodeficiencia humana
WB: Técnica de Western Blot

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1. REVISIÓN BIBLIOGRÁFICA:

Según la Organización Mundial de la Salud (OMS), la leishmaniasis o leishmaniosis* es la segunda enfermedad protozoaria con mayor mortalidad después de la malaria (WHO 2014a); por lo que todo el conocimiento que se pueda aportar para ampliar su conocimiento, epidemiología, especies que puedan actuar como reservorios o sufrir la enfermedad, así como metodología diagnóstica y mejoras terapéuticas serán de gran interés científico (Vilas *et al.*, 2014).

En nuestras latitudes, la leishmaniosis es una enfermedad que afecta particularmente a la especie canina y constituye un reto constante de diagnóstico y tratamiento para los veterinarios, debido a las múltiples aristas que presenta desde el punto de vista de la presentación clínica, las dificultades diagnosticas de casos asociados a otros procesos, y las, cada vez más frecuentes, situaciones en las que la respuesta al tratamiento no es la esperada.

La infección provoca en los seres humanos cuatro formas clínicas de la enfermedad dependiendo del tejido afectado por el parásito: leishmaniasis visceral, leishmaniasis cutánea, leishmaniasis cutánea difusa y leishmaniasis mucocutánea (Akhoundi, et al., 2016). En la especie canina la forma preponderante es la visceral.

Con respecto a la especie humana, la gran mayoría de casos (más del 90%) se dan en India, Bangladesh, Sudán, Sudan del Sur, Etiopia y Brasil (WHO 2014b). Pero debemos tener en cuenta que en Europa es endémica en catorce países del sur, este y oeste, con unos quinientos nuevos casos cada año; de los cuales el 73% son comunicados en Albania, Italia y España (Alvar et al., 2012). La mayoría de estos casos se dan en pacientes adultos con el virus de la inmunodeficiencia humana (VIH) o sintomáticos del síndrome de la inmunodeficiencia adquirida (SIDA), de pacientes trasplantados o de pacientes, en general, inmunosuprimidos (Basset et al., 2005), y también en niños (Maroli et al., 2008).

En los últimos quince años, ha habido un aumento significativo de brotes de leishmaniosis visceral y cutánea en humanos en diversos países; siendo ejemplos cercanos el brote de Tous en la Comunidad Valencia (Roth-Damas et al., 2017), y sobre todo el ocurrido en la Comunidad de Madrid, con 446 casos entre 2009 y 2012 (Arce et al., 2013). El brote en la comunidad de Madrid, en principio inexplicable, permitió investigar y descubrir que se había producido una situación de sobrepoblación de liebres que estaban actuando de reservorio (Carrillo et al., 2013) y, por tanto, incrementando las posibilidades de transmisión de la enfermedad a los seres humanos al aumentar el porcentaje de vectores transmisores infectados a partir de las liebres.

Así pues, la leishmaniosis es uno de los mejores ejemplos para estudiar a nivel global el comportamiento de la enfermedad, tanto en animales como en seres humanos, las vías de transmisión, los reservorios del proceso, la epidemiologia animales-humanos y las medidas preventivas para todos los seres vivos afectados. Es decir que cualquier aportación científica proyectará avances a nivel de Salud Global (Palatnik de Sousa & Day, 2011).

(*) El término leishmaniosis se acepta para describir la enfermedad en los perros, mientras que leishmaniasis se utiliza con más frecuencia para hablar de la enfermedad en humanos. Sin embargo, ambos términos son utilizados indistintamente y en ocasiones han sido objeto de controversia por lo que el uso actual se basa más en la tradición educativa o preferencia personal que en la aplicación de una norma (Miró & López-Velez, 2018). En mi caso, utilizaré en todo el texto la denominación Leishmaniosis.

1.1 El parásito, el vector y el ciclo biológico

La leishmaniasis es una enfermedad vectorial compleja provocada por **parásitos protozoarios del género *Leishmania***, que se transmite a los hospedadores mamíferos a través de la picadura de flebotomos de los géneros *Phlebotomus* en lo que se considera "Viejo Mundo"; y a través de la picadura de flebotomos del género *Lutzomyia* en el denominado "Nuevo Mundo" (Moreno & Alvar, 2002).

Por el momento, han sido descritas 53 especies de *Leishmania;* de las cuales 31 especies afectan a los mamíferos, 20 son patógenas para el hombre, y 10 pueden afectar al perro. Desde los años 1970, las especies de *Leishmania* se han ido definiendo en base a diversos criterios: bioquímicos y genéticos (Rioux et al., 1990), fenotípicos a través de isoenzimas y anticuerpos monoclonales (Hide et al., 2001), genotípicos como el polimorfismo en la longitud de los fragmentos de restricción (RFLP) (Rotureau et al., 2006), la hibridación con sondas de ácidos nucleicos y las técnicas de amplificación de ácido desoxirribonucleico (ADN) (Laurent et al., 2009).

La clasificación fenotípica basada en la electroforesis de isoenzimas del parásito se considera la técnica de identificación de referencia para la clasificación del género y la identificación a nivel específico e intraespecífico, desde un punto de vista taxonómico. Las isoenzimas se consideran formas alélicas diferentes de un gen, y la variación enzimática en un determinado locus puede interpretarse como una mutación que se produjo durante la evolución. Así, los parásitos de la misma especie que poseen idéntico patrón enzimático pertenecen al mismo zimodema (Hide et al*.,* 2001), denominándose con el acrónimo "MON". *Leishmania infantum* incluye 31 zimodemas, siendo el zimodema MON-1 el responsable de la mayoría de las infecciones en el perro (Gállego et al*.,* 2001).

El concepto vector de la leishmaniosis incluye diversos artrópodos como los del género *Phlebotomus* en el Viejo Mundo y *Lutzomia* en el Nuevo Mundo (Maroli et al., 2013). De las 800 especie conocidas de artrópodos vectores, solo 98 pertenecientes a los géneros *Phlebotomus* y *Lutzomia,* son vectores demostrados o sospechosos de transmitir la leishmaniosis a los seres humanos (Maroli et al., 2013). Los flebotomos están presentes principalmente en zonas cálidas de Asia, África, Australia y el sur de Europa (Killick-Kendrick, 1999).

Los flebotomos también son vectores de otras enfermedades que afectan al ser humano como la bartonelosis (Enfermedad de Carrion) en Sudamérica, y un número de agentes víricos como los Phlebovirus o Vesiculovirus (Depaquit et al., 2010; Maroli et al., 2013).

En Europa existen nueve especies de flebotomos autóctonos *Phlebotomus ariasi, Phlebotomus perniciosus, Phlebotomus perfiliewi, Phlebotomus neglectus, Phlebotomus tobbi, Phlebotomus kandelaki, Phlebotomus balcanicus, Phlebotomus papatasi y Phlebotomus sergenti,* consideradas vectores demostrados o potenciales de *L. infantum* (Antoniou et al., 2013). Aunque varias especies de flebotomos estén implicadas en la transmisión de la *L. infantum* en Europa, solo hay dos especies de importancia en la península ibérica: *P. perniciosus* y *P. ariasi* (Lucientes et al., 2005).

Los flebotomos son artrópodos con una fase de huevo, cuatro estadios larvarios terrestres con sus correspondientes mudas, una pupa sésil y una fase adulta. En regiones de clima templado se ha descrito la presencia de al menos dos ciclos biológicos completos por temporada (Gaglio et al., 2014). La alimentación de los flebotomos, tanto machos como hembras, se basa en recursos naturales azucarados, procedentes de plantas e insectos que son su aporte energético. Tan sólo las hembras son hematófagas, obteniendo de la sangre las proteínas plasmáticas necesarias para desarrollar los huevos (Killick-Kendrick, 1999). Las hembras grávidas son atraídas a lugares con sustratos húmedos para realizar la oviposición, ya que los huevos son muy sensibles a la desecación (Killick-Kendrick, 1999). El hábitat terrestre de las larvas hace que se hayan identificado una gran variedad de biotopos como madrigueras, grietas de muros, establos, corrales, jardines, sótanos, zanjas, alcantarillas, hendiduras del terreno, ruinas, minas y vertederos (Killick-Kendrick, 1999).

La actividad de los flebotomos adultos es crepuscular y nocturna, comienza con la puesta del sol, prolongándose durante las primeras horas de la noche, cuando las condiciones de temperatura y humedad le son favorables; desde el principio de la primavera hasta el final del otoño, es decir entre mayo y octubre en la cuenca mediterránea (Lucientes et al., 2005). En cuevas y construcciones humanas donde haya poca luz pueden estar activos todo el día (Lucientes et al., 2005). La actividad de los flebotomos está muy condicionada por la temperatura. Temperaturas entre 15 y 35ºC les permitiría desarrollar sus funciones vitales. La actividad estacional de los flebótomos adultos se ve afectada principalmente por la temperatura y la pluviometría (Maroli et al., 2013).

Estas condiciones de temperatura y humedad hacen que las dos especies vectores de *L. infantum* en la Península Ibérica (*P. perniciosus* y *P. ariasi*), sean activas principalmente en los meses de mayo a octubre (Miró & Molina, 2006). Sin embargo, las variaciones climáticas asociadas al cambio climático están favoreciendo las condiciones idóneas para que los flebotomos dispongan de un margen estacional mucho mayor (De Souza & Weaver, 2024).

Las hembras de los flebotomos se pueden alimentar a partir de una gran variedad de animales vertebrados reservorio, incluyendo seres humanos, ganado, perros, conejos de campo, liebres, roedores y gatos (Killick-Kendrick, 1999), con un impacto variable sobre la epidemiologia de la leishmaniosis.

En Europa, **los principales ciclos endémicos de transmisión de leishmaniasis** humana son dos: aquel en el que el agente responsable es *L. infantum*, siendo el perro el principal reservorio de la infección; un segundo ciclo afectando a Grecia, siendo *Leishmania tropica* el agente responsable, además de ser el propio ser humano el reservorio de la infección (Ready, 2010). Recientemente se han descrito casos de *Leishmania donovani*, en Chipre, muy probablemente debido a la expansión de *L. donovani* desde la vecina Turquía, a consecuencia de los movimientos migratorios humanos en la región (Antoniou et al., 2013).

El ciclo biológico de *Leishmania* tiene lugar en dos hospedadores diferentes: uno intermedio que actúa como vector y un hospedador definitivo. Se trata de un ciclo de vida digenético, con **una forma de promastigote móvil y flagelada**, localizada en el tubo digestivo del hospedador invertebrado (Killick-Kendrick, 1990) y **una forma inmóvil y aflagelar o amastigote**, que afecta al hospedador vertebrado, localizándose principalmente en los macrófagos. Para que el ciclo de transmisión se lleve a cabo es necesario que la hembra del vector contenga formas infectantes del parásito (Desjeux, 2004) y se alimente de la sangre de un mamífero, depositando así al parásito en la dermis del hospedador.

La hembra del flebotomo, infectada al haber alimentado de sangre de un animal infectado, inocula junto con su saliva los promastigotes metacíclicos presentes en su proboscis. Una vez en los capilares cutáneos del punto de inoculación del vertebrado, múltiples células del sistema inmunitario, principalmente macrófagos y neutrófilos migran hacia el foco inflamatorio, fagocitando a los parásitos presentes. Estos son incluidos en una vacuola parasitófora con el fin de eliminarlos mediante la síntesis y liberación de diversas moléculas, destacando el papel leishmanicida del óxido nítrico (NO) (Holzmuller et al., 2006; Zafra et al., 2008). Este mecanismo se da cuando **la respuesta fagocítica es efectiva.**

Si **la respuesta fagocítica no es efectiva,** el parásito pone en marcha diversos mecanismos de evasión (Bogdan, 2008) destacando la alteración del sistema complemento (Bogdan & Röllinghoff, 1998; Olivier et al., 2005; Ritter et al., 2009), la inhibición del procesamiento y presentación antigénica (Bogdan & Röllinghoff, 1998; De Almeida et al., 2003), y la modulación del ambiente inmunológico (Shio et al., 2012). Una vez en el interior del macrófago, los parásitos se multiplican por sucesivas fisiones binarias (Antoine et al., 1990; Moradin & Descoteaux, 2012) haciendo que el macrófago sea incapaz de destruir al parásito (Solano-Gallego et al., 2009).

Tras la multiplicación en el interior de los macrófagos infectados, estos se lisarán y permitirán la liberación del parásito en forma de amastigotes, pudiendo ser fagocitados por macrófagos o neutrófilos próximos y favorecer, por tanto, la diseminación del parásito y alcanzar diversos órganos del sistema linfohematopoyético, como médula ósea, linfonodos, bazo e hígado, entre otros. La progresión de la enfermedad dependerá de la virulencia del parásito, de la dosis infectante inoculada por el vector y de las condiciones del hospedador infectado (eficacia de la respuesta inmune, condiciones socioeconómicas y predisposición genética) (Alvar et al., 2004; Reithinger et al., 2007).

Las células parasitadas circulantes pueden ser ingurgitadas por otros flebotomos, en cuyo aparato digestivo los amastigotes se liberan de las células en las que se encontraban alojados, y desarrollan un flagelo para transformarse en promastigotes procíclicos, tras 24-48 horas. Estos se multiplican por fisión binaria longitudinal, alcanzando, después de varios días, la capacidad infectante (promastigotes metacícliclos), proceso denominado metaciclogénesis. En este momento, los parásitos se encuentran en la probóscide de la hembra de flebotomo infectante, pudiendo ser inoculados a un nuevo hospedador vertebrado, cerrándose así el ciclo biológico del parásito.

Para asegurar la transmisión de la enfermedad a los hospedadores, el parásito ha desarrollado mecanismos capaces de modificar el comportamiento del vector infectado con promastigotes metacíclicos (fase infectante). Es este sentido, se ha comprobado que el vector es capaz de aumentar la frecuencia y el tiempo de ingestión de sangre, lo que optimiza la transmisión (Rogers & Bates, 2007).

1.2. La patogenia y el desarrollo de la enfermedad

La leishmaniosis es una enfermedad de la que todavía no conocemos completamente todos los mecanismos inmunopatogénicos involucrados, ni la medida en que diversas especies animales pueden infectarse por otras vías no vectoriales de transmisión y ser reservorios activos del ámbito doméstico y salvaje. Por ello, numerosos investigadores siguen aportando información sobre los hallazgos clínico-patológicos, principalmente en la especie canina, pero también en otras especies, fundamentalmente animales de compañía como gatos y hurones en los que se está manifestando una enfermedad con la que no se contaba en los protocolos de diagnóstico.

En la **especie humana**, las manifestaciones de la leishmaniasis pueden variar desde autolimitantes hasta fatales, dependiendo de la especie del parásito y del estado del sistema inmunológico del huésped. Clínicamente se presenta como: **leishmaniasis visceral** (LV), generalmente causada por *L. donovani* y *L. infantum* y también conocida como *Leishmania chagasi*) ; **leishmaniasis cutánea** (CL), causada por *L. tropica*, *Leishmania major*, *Leishmania aethiopica*, *L. infantum*, *L. donovani* en el Viejo Mundo y en el Nuevo Mundo por especies del complejo *L. mexicana* o del subgénero *Viannia*, y también *L. infantum/L.chagasi*; **leishmaniasis mucocutánea** (MCL) causada por *Leishmania amazonensis* y por especies del subgénero *Viannia*) (Mann et al., 2021). La forma más grave es la visceral, localizándose el parásito en la médula ósea, el bazo y el hígado (Alvar et al., 2004).

Los **cánidos** pueden presentar cuadros cutáneos, pero salvo la forma pápulo-pustular que podría corresponder solo a la reacción local en el punto de la picadura de los flebotomos, la forma clínica preponderante es la visceral con una distribución prácticamente generalizada del parasito por todo el organismo, lo que dará lugar a signos clínicos muy diversos, en función de los órganos más afectados: bazo, hígado, linfonodos, médula ósea, riñón, ojos, articulaciones o piel (Alvar et al., 2004; Solano-Gallego et al., 2011; Maia & Campino, 2018).

En los perros, potencialmente, cualquier órgano o tejido puede verse afectado por el parásito y por la reacción inmunomediada desencadenada, lo que da lugar a una creciente lista de presentaciones frecuentes y también inusuales o de nueva aparición; por lo que la leishmaniosis canina se considera “una gran imitadora” y supone un importante desafío para veterinarios en su práctica clínica (Pennisi, 2015).

De **forma resumida,** sabemos que **el proceso de infección en el perro** tiene lugar cuando los flebotomos inoculan promastigotes metacíclicos de *L. infantum* a un individuo y sus macrófagos los fagocitan, comenzando la transformación en amastigotes, a la vez que se van multiplicando en el interior del macrófago. Los parásitos son capaces de eludir la respuesta inmunitaria innata del perro a través de la remodelación de la vacuola parasitófora, lo que dificulta las vías de señalización de los macrófagos en su beneficio (Koutinas & Koutinas, 2014). Una vez que la carga parasitaria es demasiado grande, los macrófagos estallan y liberan amastigotes que son fagocitados por otras células del sistema fagocítico mononuclear, principalmente en la sangre, el hígado, el bazo y la médula ósea (Morales-Yuste et al., 2022). Si el sistema inmune innato del hospedador está en estado de alerta máxima los amastigotes son reducidos dentro de los macrófagos, evitándose la diseminación.

Por tanto, la presentación clínica de la leishmaniosis canina va a depender de un continuo juego de equilibrios entre el ataque de los flebotomos con promastigotes y las vías útiles de respuesta inmune por parte del hospedador, que cuando son ineficaces dan lugar a una serie de mecanismos inflamatorios nocivos para el funcionamiento de los órganos afectados.

1.2.1 Especie canina

En el perro, el parasito *Leishmania* spp. dispone de diversos mecanismos para evadir y entorpecer la respuesta inmunitaria del huésped, lo que le permite persistir y establecer una infección crónica en el paciente (Srivastava et al., 2016). Hasta el momento, de los estudios publicados, podemos resumir como aspectos inmunológicos importantes que intervienen en el desarrollo de la enfermedad los siguientes:

- La **respuesta inmune innata** a nivel cutáneo tiene un importante papel en el mecanismo de inicio y desarrollo de la infección, siendo las células de Langerhans y los macrófagos los actores principales; pero también intervienen neutrófilos, fibroblastos y queratinocitos (Papadogiannakis & Koutinas, 2015).
- **El desequilibrio en la respuesta de linfocitos T helper tipo 1 (Th1) y linfocitos T helper tipo 2 (Th2)**, en favor de Th2, ha sido la teoría clásica para explicar el desarrollo de la leishmaniosis en la especie canina; sin embargo, no ha permitido justificar el desarrollo y la gravedad de la leishmaniosis humana.
- El **agotamiento de las células T** es una disfunción, resultado de la exposición prolongada al antígeno *Leishmania*. Se ha demostrado que el **agotamiento de las linfocitos T con cluster de diferenciación (CD) tipo 4 positivos (CD4+)** está asociado con la expresión de la muerte programada-1 (PD-1) y el antígeno 4 asociado a los linfocitos T citotóxicos (CTLA-4) (Sharpe et al., 2007). Boggiatto et al. (2010) observaron que, en perros con leishmaniosis, la proliferación de células T CD4+ se reducía a medida que la enfermedad progresaba, junto con un aumento de IL-10 y una disminución de IFN-γ (Boggiatto et al., 2010). Así mismo, Esch et al. (2013) demostraron que las células T CD4+ de perros con signos clínicos de leishmaniosis tenían un aumento de 4 veces en la expresión de PD-1 en las células T CD4+ en comparación con las células T de perros control. Los perros con leishmaniosis canina también tenían células T CD8+ agotadas con un aumento de PD-1 (perros clínicos con un aumento de 2 veces en PD-1 y perros subclínicos con un aumento de 1,5 veces en la expresión de PD-1 de superficie sobre el control) (Esch et al., 2013).

- **Las citocinas implicadas** en la vía de una respuesta inmune protectora que permite controlar la infección y la eliminación del parásito incluyen: IL-2, IL-18, IL-10, el factor de necrosis tumoral alfa (TNF-α) y el interferón gamma (IFN-γ) (Barbiéri 2006; Solano-Gallego et al., 2009; Carrillo & Moreno 2009; Hosein et al., 2017; Samant et al., 2021; García-Castro et al., 2022). De hecho, niveles séricos elevados de IFN-γ se relacionan con recuentos bajos de parásitos y pocos o ningún signo clínico en comparación con los perros enfermos (Ordeix et al., 2020), y estos niveles aumentan con el tratamiento *anti-Leishmania* junto con la mejoría clínica y la parasitemia sanguínea baja (Priolo et al., 2019, Solano-Gallego et al., 2016). En este sentido, Abbehusen et al. (2017) observaron, en perros infectados de forma experimental y de forma natural que se mantenían asintomáticos, con aumentos en los niveles séricos de IFN-γ, IL-6 e IL-18 y disminuciones en TNF-α, IL-2 e IL-8 (Abbehusen et al., 2017). Por otra parte, Turchetti et al. (2015) demostraron que la disminución de la supervivencia intracelular de *L. infantum* en macrófagos caninos se podía asociar con una mayor producción de TNF-α e IFN-γ y una menor producción de IL-10 (Turchetti et al., 2015).
- **Perfil inflamatorio de leucotrienos y prostaglandinas**. Se ha observado una disminución del leucotrieno B4 (LTB4) y de la prostaglandina E2 (PGE2) así como un aumento gradual de lasquimiocina CXCL1 al aumentar el grado de enfermedad de la leishmaniosis en perros (Solcà et al., 2016). Los análisis discriminantes revelaron que la evaluación combinada de LTB4, PGE2 y CXCL1 fue capaz de diferenciar perros con diferentes puntuaciones clínicas. Los perros con los valores de puntuación clínica más elevados también exhibieron cargas parasitarias más altas y concentraciones más altas de anticuerpos anti-saliva flebotomo. Estos hallazgos sugieren que la gravedad clínica de la leishmaniosis visceral canina está estrechamente asociada con un perfil inflamatorio diferente, marcado por una expresión diferencial de eicosanoides y quimiocinas circulantes (Solcà et al., 2016).
- **Las células linfocitarias tipo B** de la médula ósea juegan un papel importante en la respuesta humoral del sistema inmunitario, activando las células T CD4+ y produciendo anticuerpos contra el parásito *Leishmania*. Durante la infección, las células B actúan como células presentadoras de antígeno adicionales (APC adicionales) y muestran el antígeno a las células auxiliares Th1 CD4+ que, a su vez, activan las células B para producir anticuerpos (Rodriguez-Pinto et al., 2014). Al inicio de la enfermedad, los anticuerpos producidos por las células B son específicos para el parásito y están preparados para inducir opsonización y fagocitosis inducida por anticuerpos, y también neutralización de factores de virulencia específicos de la *Leishmania* (Gibson-Corley et al., 2014).

Conforme el parásito continúa propagándose, aumenta el nivel de anticuerpos IgG no específicos, de tal forma que en los perros con enfermedad clínica aparece hipergammaglobulinemia (Pennisi 2015). Por otra parte, el aumento de la producción de anticuerpos dará como resultado el depósito de immunocomplejos, a diferentes niveles tisulares como riñones, articulaciones, hígado, bazo, entre otros; dando lugar a signos clínicos como glomerulonefritis, insuficiencia renal o artritis (Esch et al., 2015). También han sido estudiados los niveles de IgM, IgA e IgE sin haberse hallado resultados concluyentes sobre su efecto inmunoprotector en la leishmaniosis canina (Coura-Vital et al., 2011, De Freitas et al., 2012).

- **Los receptores tipo Toll (TLR)** constituyen un tipo de receptores de reconocimiento de patrones (PPR) en la inmunidad innata y actúan como vinculo de unión entre el sistema inmune innato y el adaptativo. Durante la infección por *Leishmania*, la activación de los TLR influye sobre las respuestas inmunitarias especificas frente al patógeno, pudiendo desarrollar un papel decisivo en la determinación del resultado de la infección: hacia la eliminación del parásito, o hacia la supervivencia del patógeno. Las APC del sistema inmunitario innato, como los macrófagos, las células dendríticas (DC), los neutrófilos, las células asesinas naturales (NK), expresan TLR2, que desempeñan un papel crucial en el reconocimiento del parásito y el desarrollo de respuestas inmunitarias frente a la infección por *Leishmania*. Dependiendo de la especie de *Leishmania* que infecte, las vías TLR2 pueden dar lugar a una respuesta protectora del huésped o a una respuesta que agrave la enfermedad (Gazzinelli & Denkers 2006).

1.2.2 Especie felina

En la especie felina, recientemente, se ha concluido que la inmunopatogénesis de la infección por *L. infantum* muestra similitudes con la respuesta canina, y los gatos pueden generar una respuesta inmunitaria Th1 específica contra *L. infantum* (Priolo et al., 2022a). Los gatos con niveles elevados de anticuerpos o con PCR en sangre positiva son menos capaces de producir IFN-γ específico (Priolo et al 2019). Sin embargo, los gatos parecen tener un nivel más bajo de respuesta inmune tanto humoral como mediada por células en comparación con los perros (Priolo et al., 2019).

Akhtardanesh et al. (2018) sugieren que los gatos podrían ser resistentes a la leishmaniosis visceral, ya que la dosis de inoculación que induce las características clínicas patognomónicas en los perros simplemente crea una parasitemia asintomática en los gatos. Sin embargo, debido al mantenimiento de parasitemia durante largos periodos de tiempo, estos gatos podrían actuar como reservorios apropiados para la transmisión de leishmaniosis visceral a la población humana.

En otras especies como bovinos (Lobsiger et al. 2010), ovinos (Villanueva-Saz et al., 2023), équidos (Gazzonis et al., 2020), y animales de compañía exóticos (Alcover et al., 2022) y también en animales salvajes (Alcover et al., 2020), están apareciendo publicaciones en las que se constatan niveles de anticuerpos *anti-Leishmania*, pero con sintomatología escasa o nula.

1.3 ASPECTOS EPIDEMIOLÓGICOS DE LA LEISHMANIOSIS

La epidemiologia de la leishmaniosis se ve afectada por los hospedadores, los reservorios, el vector y el medio ambiente (Oryan & Akbari, 2016). El perro juega un papel fundamental en la epidemiología de la infección, dado que es el principal reservorio peridoméstico para el hombre (Gramiccia & Gradoni, 2005).

La infección por *L. infantum* en perros es endémica en más de 70 países del mundo, pero se desconoce la prevalencia global en perros domésticos (Solano-Gallego et al., 2011). En los países de la cuenca mediterránea, la enfermedad se distribuye en focos endémicos con una prevalencia de infección muy variable entre focos hipoendémicos e hiperendémicos (Pennisi, 2015).

La situación epidemiológica de la leishmaniosis canina es difícil de evaluar debido a la dificultad en su diagnóstico y a que los animales enfermos solo son una parte de animales infectados. Existe un número importante de animales que son diagnosticados por diferentes pruebas laboratoriales, pero también hay otro grupo de animales que están sanos, son negativos a pruebas laboratoriales, pero han estado en contacto con la enfermedad (Pennisi 2015).

El número de portadores asintomáticos se considera que tiene un rango del 25% a más del 80%, dependiendo del área geográfica estudiada y de la prueba de detección utilizada (Michel et al., 2011). La detección de estos animales es crucial para el control de la enfermedad tanto en áreas endémicas como no endémicas (Maia & Cardoso 2015).

En España se han estudiado las seroprevalencias de la población canina desde 1985 hasta 2019 (Gálvez et al., 2020), mostrando un amplio rango, desde 0% en Vizcaya hasta el 57,1% en las islas Baleares; lo que pone de manifiesto su distribución parcheada y la existencia de zonas no endémicas, hipoendémicas, endémicas e hiperendémicas. Las diferencias halladas entre las diferentes comunidades autónomas pueden deberse al tamaño de la muestra y las técnicas serológicas empleadas.

La **transmisión de la enfermedad** se produce fundamentalmente a través de flebotomos, que son los únicos artrópodos adaptados para la transmisión biológica de la infección por *Leishmania* spp. en zonas donde es endémico (Killick-Kendrick, 1990). Sin embargo, con técnicas de biología molecular se ha podido detectar ADN del parásito en otros artrópodos hematófagos, como ixódidos de la especie *Riphicephalus sanguineus*, pulgas de la especie *Ctenocephalides felis felis* y, más recientemente, en la mosca del caballo, *Tabanus importunus y Culicoides spp*, sin haberse demostrados su papel en la transmisión natural de *Leishmania* y tampoco su capacidad infectante (Lucientes, 2017).

También se han descrito y confirmado modelos de transmisión de la leishmaniosis sin la participación del flebotomo (Solano-Gallego et al., 2011). Estos incluyen la transmisión vía productos sanguíneos de animales donantes infectados con *Leishmania spp.* utilizados en donaciones de sangre (Owens et al., 2001, de Freitas et al., 2006). La infección por *L. infantum* es común en los donantes de sangre caninos y sus hemoderivados en un área endémica, a pesar de un cribado serológico comercial negativo para enfermedades infecciosas (Tabar et al., 2008). Esto hace necesario que los donantes de sangre sean testados de manera periódica y rigurosa para detectar la infección por *Leishmania*, para prevenir la diseminación de la enfermedad a través de transfusiones de sangre. Por lo tanto, la detección mediante PCR debe incluirse en un enfoque integrado para evaluar la infección por *L. infantum* entre los posibles donantes de sangre (Tabar et al., 2008).

La transmisión vertical de madre a cachorros ha sido demostrada experimentalmente en un perro infectado (Rosypal et al., 2005) y de forma natural (Boggiatto et al., 2011). La alta frecuencia, detección de infección activa y similitud de cargas de *L. infantum* en el tracto genital de machos y hembras infectados sugiere el potencial de transmisión venérea de este parásito por ambos sexos y de transmisión vertical por hembras. Además, la transmisión vertical puede ser frecuente ya que la infección activa por *L. infantum* fue una observación común en el útero. (Boechat et al., 2020).

Existen sospechas no confirmadas de la transmisión de la leishmaniosis entre perros en casos de mordeduras (Naucke at al., 2016) o contacto con heridas de perro infectados (Daval et al., 2016). Recientemente se ha descrito la transmisión de la infección de perro a perro en la misma casa en Inglaterra (McKenna et al., 2019). Sin embargo, el impacto que pueden tener estas formas de transmisión sin participación del vector en regiones donde *Pheblotomus* se encuentre presente, sería de menor importancia que la forma habitual de transmisión. No obstante, estas otras vías de transmisión deben ser tenidas en cuenta en aquellas regiones en las que el vector se encuentra ausente (Pennisi, 2015).

El reservorio principal de la *L. infantum* son los perros domésticos, siendo capaces de mantener durante largos periodos de tiempo al parasito como único hospedador (Ashford, 1996). Sin embargo, es importante considerar la importancia potencial de otros huéspedes y sus implicaciones en el control de leishmaniosis, de hecho, se han encontrado zonas con una especialmente alta incidencia de leishmaniasis que puede asociarse a la intervención de otros hospedadores (Martín-Sánchez et al., 2020). Un número creciente de comunicaciones sugiere que la leishmaniosis no se limita a los perros, sino que también afecta a muchas otras especies de mamíferos y aves. Como consecuencia, se expandiría el reservorio potencial, lo que supone preocupación sanitaria en el ámbito de la salud pública global (Cardoso et al., 2021).

Todavía no se conoce el papel epidemiológico que pueden tener en la infección diferentes mamíferos silvestres o domésticos distintos de los mencionados; bien como reservorios secundarios, como meros hospedadores ocasionales o el grado de interacción entre los ciclos de vida domésticos y silvestres de *L. infantum* (Millán et al., 2014; Tomassone et al., 2018; Cardoso et al., 2021). De la misma forma, los mamíferos silvestres han sido considerados como potenciales reservorios silenciosos de *L. infantum* en el área mediterránea, sugiriendo la presencia de infección subclínica crónica sin evidencia de signos clínicos y pueden contribuir a mantener el ciclo zoonótico en una zona donde la presencia del perro es limitada (Alcover et al., 2020). Sin embargo, existen pruebas sólidas de que la alteración antropogénica del entorno natural de los vectores y la vida silvestre puede provocar la acumulación y el contagio de infecciones, lo que da lugar a epidemias en seres humanos susceptibles (Tomassone et al., 2018). El estudio de la vigilancia de la infección en la vida silvestre ayuda evaluar los riesgos potenciales en el entorno doméstico y su papel en la propagación de infecciones en áreas no endémicas (Del Río et al., 2014).

Se ha informado de evidencia de infección por *L. infantum* en la vida silvestre en carnívoros, insectívoros, lagomorfos y roedores, así como en marsupiales, primates, desdentados (**osos hormigueros**, **armadillos** y **perezosos**) y **murciélagos** (De Lima et al., 2008; Quinnell & Courtenay, 2009; Malta et al., 2010; Antoniou et al., 2013; De Araújo et al., 2013; Cardoso et al., 2021).

En fauna silvestre europea, se han reportado estudios epidemiológicos en **zorros rojos** (Criado-Fornelio et al., 2000; Verin et al., 2010; Del Río et al., 2014; Ortuño et al., 2019), **lobos** (Sobrino et al., 2008; Oleaga et al., 2015), **chacal dorado** (Cirović et al., 2014), **gato montés** (Del Río et al., 2014), **nutria** (Oleaga et al., 2018), **garduña** (Alcover et al., 2020), **martas** (Oleaga et al., 2018), **tejón** (Battisti et al., 2020), **visón europeo** (Del Río et al., 2014), **turón** (Del Río et al., 2014), **visón americano** (Tsakmakidis et al., 2019), **foca monje** (Toplu et al., 2007), **oso pardo** (Ortuño et al., 2019), **ginetas** (Del Río et al., 2014, Millán et al., 2015), **linces** (Alcover et al., 2020), **meloncillo** (Gomes et al., 2020), **murciélagos** (Azami-Conesa et al., 2020), **erizo** (Alcover et al., 2020), **musaraña** (Millán, 2018), **liebre** (Ruiz-Fons et al., 2013; Tsakmakidis et al., 2019; Ortega-García et al., 2019), **conejos** (Ortuño et al., 2019; Tsakmakidis et al., 2019; Abbate et al 2019), **ratones** (Risueño et al., 2018; Ortuño et al., 2019; Alcover et al., 2020; Martín-Sánchez et al., 2020), **ratas** (Galán-Puchades et al., 2019), y **ardillas** (Alcover et al., 2020). La ausencia o baja prevalencia de lesiones es una característica común a todos estos estudios.

Se han reportado en Europa infecciones en animales domésticos como **ganado vacuno** (Lobsiger et al., 2010), **caballos** (Fernández-Bellon et al., 2006; Gazzonis et al., 2020), **burros** (Nardoni et al., 2019), **conejos de granjas y de compañía** (Tsakmakidis et al., 2024). También se han descrito infecciones en animales mantenidos en cautiverio como **primates** (Malta et al., 2010; Miró et al., 2018), **leones** (Libert et al., 2012), **tigres** (Cavalera et al., 2020; Iatta et al., 2020) y en **marsupiales** como un **ualabí de Bennett** (Ramírez et al., 2012; Montoya et al., 2016).

Además de los perros domésticos, la capacidad de transmitir infecciones ha sido confirmada por xenodiagnóstico en ratas negras y gatos domésticos (Quinnell & Courtenay, 2009), liebres (Molina et al., 2012) y conejos (Jiménez et al., 2014), lo que sugiere que pueden representar un reservorio secundario de *L. infantum*. La constatación de si estos hospedadores, son reservorios primarios o secundarios, requiere más estudios de xenodiagnóstico a nivel poblacional (Quinnell & Courtenay, 2009; Millán et al., 2014).

Los factores de riegos de la leishmaniosis que han sido estudiados son numerosos y su análisis resulta interesante para el desarrollo de estrategias del control de la enfermedad (Barbosa et al., 2022). Sin embargo, no forman parte de los objetivos de estudio del este trabajo. De forma resumida, mencionar que existen factores de riesgo individuales como la raza, la edad, el sexo, el tamaño, la longitud del pelo, y sobre todo las características genéticas; factores de riesgo medioambientales como el tipo de propietario de los perros, la convivencia con otros perros en guarderías o zonas abiertas, si recibe tratamientos repelentes y el tipo de actividad de trabajo del perro; factores geográficos asociados a altitud, latitud (zona geográfica) y tipo de hábitat (campo, forestal, ciudad) (Solano-Gallego et al., 2000; Velez et al., 2019; Rombolà et al., 2021).

La leishmaniosis en los gatos se detectó por primera vez en Argelia en 1912 cuando se encontraron amastigotes en la médula ósea de un gato que vivía en la misma casa que un niño que fue diagnosticado con leishmaniosis visceral (Pennisi, 2013). En 1933 se describió la primera leishmaniosis en un gato en España (Ondovilla, 1933), y la información sobre el número de publicaciones relacionadas con casos clínicos sobre leishmaniosis felina ha ido en aumento (Garcia-Torres et al., 2022).

Al comparar poblaciones de gatos y perros expuestos a flebotomos y *L. infantum* en las mismas condiciones, se observó que, aunque se detecta una alta tasa de exposición en gatos, manifestada por un grado significativamente mayor de seropositividad; los perros tenían cargas de parásitos en sangre significativamente más altas (Baneth et al., 2020), por lo que parece que la inoculación de los flebotomos induce una infección mayor en los perros que en los gatos.

Algunas características del comportamiento felino, como el hábito de caza nocturna, el que recorren distancias medias de 1,5 km desde sus hogares, así como la incursión en zonas forestales, actuando como enlace entre el ambiente doméstico y salvaje, podría favorecer la diseminación del parásito en esta especie (da Silva *et al.*, 2008).

La primera evidencia demostrada de transmisibilidad de la *L. infantum* de un gato a un vector (*P. perniciosus*) por xenodiagnóstico fue comunicada por Maroli et al., en 2006, lo que sugiere que los gatos pueden representar un reservorio doméstico adicional de leishmaniosis (Maroli et al., 2007; da Silva *et al.*, 2010).

Si bien las infecciones felinas subclínicas son comunes en áreas endémicas de leishmaniosis canina, la enfermedad clínica debida a *L. infantum* en gatos es rara (Pennisi et al., 2015). Las tasas de prevalencia de la infección felina por *L. infantum* en estudios serológicos o de base molecular oscilan entre el 0% y más del 60%. En general, la prevalencia descrita en gatos tanto mediante PCR como por serología es menor que en perros de áreas geográficas similares (Pennisi, 2015).

Los gatos pueden infectar a los flebotomos, pudiendo actuar como reservorio secundario. Debido a la parasitemia de larga duración, los gatos pueden actuar como reservorios apropiados para la transmisión de leishmaniasis a la población humana y canina (Akhtardanesh et al., 2018; Asfaram et al., 2019; Morelli et al., 2020). Debido a que los gatos parece que juegan un papel como reservorio adicional de *L. infantum*, se deberían tener en consideración desde la perspectiva de la toma de decisiones preventivas en el marco de una salud global (Pennisi & Persichetti, 2018).

La leishmaniosis felina causada por *L. infantum* se presenta sobre todo en gatos domésticos de pelo corto, adultos, que viven o viajan a países endémicos del sur de Europa y Brasil. La enfermedad tiene un curso crónico y puede manifestarse por una gran cantidad de signos clínicos y/o anomalías clínico-patológicas (Pereira & Maia, 2021). En general, el estado clínico y los signos clínicos no pueden asociarse a un determinado estado infeccioso porque la mayoría de los gatos infectados son asintomáticos (Solano-Gallego et al., 2009; Spada et al., 2023). Por otra parte, aproximadamente un tercio de los casos felinos positivos a leishmaniosis, mostraron infecciones de enfermedades concomitantes, incluido virus de la inmunodeficiencia felina (FIV), virus de la leucemia felina (FeLV), coronavirus felino (FCoV), *Toxoplasma. gondii*, *Bartonella henselae*, diabetes mellitus, pénfigo foliáceo, neoplasia y/o estaban bajo terapias inmunosupresoras en el momento del diagnóstico (Pereira & Maia, 2021).

Las anomalías laboratoriales más frecuentes encontradas en los casos de leishmaniosis felina incluyen anemia normocrómica, normocítica, hiperproteinemia con hipergammaglobulinemia, trombocitopenia y azotemia. En los gatos positivos a leishmaniosis no se disponen de guías científicas sobre el diagnóstico y manejo terapéutico de la enfermedad en comparación a la especie canina, en las que se dispone de directrices publicadas tanto por el Grupo de Trabajo sobre Leishmaniasis canina (Paltrinieri et al., 2010) como por el Grupo científico LEISHVET (Solano-Gallego et al., 2009).

En un estudio realizado en Brasil concluyeron que las pruebas hematológicas y bioquímicas en gatos con leishmaniasis no aportaban valores concluyentes y ningún estudio publicado ha analizado una posible diferencia entre grupos de animales infectados y sanos utilizando estos parámetros clínicos (Batista et al., 2023). En otro estudio realizado por Chatzis et al. (2020) con 50 gatos, todos con signos clínicos, observaron que la única anomalía laboratorial detectada fue un aumento del fosforo inorgánico sérico, por lo que este hallazgo puede sugerir una asociación entre la infección por *L. infantum* y la enfermedad renal en los gatos (Chatzis et al., 2020). Por su parte, Silva et al. (2023) observaron recuentos bajos de plaquetas e hiperproteinemia con hipoalbuminemia en gatos con leishmaniosis (Silva et al., 2023). La panleucopenia, así como la lesión renal aguda están asociados con un mal pronóstico de la leishmaniosis felina (Pereira & Maia, 2021).

1.4 LA METODOLOGÍA PARA ESTABLECER EL DIAGNOSTICO

Una prueba diagnóstica eficaz debería poder confirmar la sospecha clínica en un solo paciente, detectar infección en individuos asintomáticos, detectar todos los individuos infectados por *Leishmania*, debería poder realizarse en muestras de recogida poco o nada invasiva, debería tener una alta sensibilidad, especificidad y reproducibilidad; y, además debería ser simple, fácil de realizar, económica, factible en laboratorios regionales o adaptable a las condiciones de campo.

En los perros, los signos clínicos y/o alteraciones clínico-patológicas compatibles con la enfermedad, en un elevado porcentaje de casos son sugestivos de que el animal padece leishmaniosis canina. La confirmación de la etiología de la infección se puede alcanzar mediante diferentes métodos laboratoriales directos e indirectos: parasitológicos, moleculares y serológicos (Morales-Yuste et al., 2022). A pesar de la gran cantidad de técnicas diagnósticas disponibles para detectar la presencia del agente o anticuerpos frente al mismo, por el momento, no se dispone de una técnica “estándar de oro” que aporte resultados inequívocos. El diagnostico laboratorial de la leishmaniosis continúa siendo un desafío a pesar de los avances en el desarrollo de métodos directo e indirectos; por ello, en determinadas situaciones, es necesario utilizar diversas pruebas para la confirmación de la enfermedad.

Las pruebas laboratoriales pueden emplearse, también, en el seguimiento de la infección durante el tratamiento, en estudios clínicos para la validación de vacunas (Chagas et al., 2021), en el cribado de perros donantes de sangre (Correia et al., 2024), en animales reproductores o previamente a la vacunación contra la leishmaniosis (Solano-Gallego et al., 2017).

1.4.1 DIAGNOSTICO PARASITOLÓGICO

El diagnóstico parasitológico permite visualizar al microscopio óptico amastigotes de *Leishmania spp.* en tejidos o fluidos. Se puede realizar mediante estudios citológicos, histológicos, cultivos del parásito, xenodiagnóstico y por inoculación a ratones o hámsters.

El **exámen citológico** de aspirados de médula ósea, de bazo, de linfonodos o de lesiones cutáneas, así como impresiones de piel u otros tejidos permite la visualización directa de los amastigotes. Los portaobjetos se pueden teñir con tinciones de tipo Romanoswsky, May Grunwald-Giemsa o cualquier otra tinción comercial rápida.

Los amastigotes de *Leishmania* generalmente se detectan intracelularmente en macrófagos y, más raramente, en los neutrófilos, aunque también de forma extracelular (Baneth & Solano-Gallego, 2022). En principio cualquier lesión susceptible de ser aspirada u obtener material por impronta es válida para realizar el examen citológico. El parásito es posible detectarlo en otras localizaciones atípicas como lengua, testículos, masas orales o nasales (Paltrinieri et al., 2016).

El **estudio histopatológico**puede demostrar la presencia de *Leishmania* en secciones teñidas habitualmente con hematoxilina-eosina pero frecuentemente precisa de tinciones inmunohistoquímicas para poder en evidencia los parásitos (Toplu & Aydogan, 2011), ya que la mayoría de las veces se observa un patrón histológico compatible con reacción inflamatoria asociada con *Leishmania* pero no se observa directamente los amastigotes. En comparación con la reacción en cadena de la polimerasa (PCR) y la citología, la histología tiene dos inconvenientes principales: es más laboriosa requiriendo más tiempo y la identificación de amastigotes puede ser más difícil que en las muestras citológicas (Paltrinieri et al 2016). Por otro lado, la histología tiene la ventaja de proporcionar información adicional sobre el patrón histológico de las lesiones. La interpretación de los resultados histológicos está bien documentada por numerosas publicaciones que describen las lesiones típicas y la distribución de los parásitos asociados con la enfermedad activa, caracterizada principalmente por inflamación linfoplasmocitaria o granulomatosa-piogranulomatosa y/o por vasculitis en órganos generalmente afectados por *Leishmania* (médula ósea, bazo, piel, linfonodos, riñón). También en tejidos de localización inusual como corazón, pulmón, glándula suprarrenal, tracto genital, sistema nervioso central, músculo esquelético, tracto gastrointestinal, uñas, glándulas lagrimales y músculos oculares (Paltrinieri et al., 2016).

El **aislamiento y cultivo del parásito** no se utiliza de forma rutinaria en un entorno clínico, sin embargo, el cultivo puede mejorar la sensibilidad de detección y los parásitos aislados pueden identificarse a nivel de genotipo y especie (Hong et al., 2020). Las cepas de *Leishmania* se pueden mantener como promastigotes en medios de cultivo. Los medios de cultivo utilizados pueden ser bifásicos (medio Novy-McNeal Nicolle (NNN)) para la transformación de amastigotes en promastigotes; mientras que se prefiere el medio monofásico (medio de Schneider, M199 o medio de Grace) para el crecimiento exponencial de los parásitos (Srivastava et al., 2011). En las últimas décadas ha surgido un método de cultivo microcapilar (MCM) para aumentar la sensibilidad de las técnicas de cultivo de parásitos mediante el uso de células mononucleares de sangre periférica y capas leucocitarias. Cabe destacar que el MCM sólo requiere un pequeño volumen de medio de cultivo y es más barato que otros medios (Thakur et al., 2020).

El **xenodiagnóstico** es una técnica que puede utilizarse para identificar especies reservorio supuestas de *Leishmania*, ya que permite determinar si un hospedador particular, que está infectado, es capaz de transferir naturalmente el patógeno a un vector potencial y evaluar el desarrollo de promastigotes en el intestino medio de los flebotomos (Vioti et al., 2022). Se ha realizado con éxito en perros, gatos, liebres (*Lepus granatensis*), ratas (*Rattus rattus*) (Millán et al., 2014; Magalhães-Junior et al., 2016; Vioti et al., 2022) y en seres humanos (Singh et al., 2020).

La **inoculación experimental en ratones o hámsteres** se limita al campo de estudios de investigación o cuando la inoculación del medio de crecimiento corre el riesgo de contaminación. Los animales inoculados deben ser sacrificados transcurridos dos meses y sus bazos examinados en busca de parásitos (Alvar et al., 2004).

1.4.2 DIAGNÓSTICO MOLECULAR

Las técnicas moleculares incluyen PCR convencional, PCR anidada y la PCR cuantitativa en tiempo real (qPCR) (Maurelli et al., 2020). La qPCR es la modalidad más utilizada para la cuantificación de la carga parasitaria en diferentes tejidos de perros infectados por *Leishmania* con o sin manifestaciones clínicas. Se emplea en el diagnóstico, en el seguimiento de la infección durante el tratamiento y en estudios clínicos para la validación de vacunas (Chagas et al., 2021).

La **PCR,** actualmente, se considera una técnica molecular sencilla y válida para detectar *Leishmania* spp. en diferentes muestras clínicas, así como para identificar las especies y genotipos del parásito. Puede ser aplicada, no solo en casos activos, sino también para monitorizar la curación parasitológica después de la terapia (Maia & Campino, 2008). Existen muchos protocolos con sensibilidades y especificidades que dependen de diferentes factores como el método de extracción de ADN, el material clínico, los cebadores, el número de copias objetivo y las condiciones técnicas. Con la PCR, el diagnóstico de la leishmaniosis canina ha mejorado considerablemente, alcanzando sensibilidades del 90-100% en casos clínicamente sospechosos o confirmados parasitológicamente. Sin embargo, cuando se trata de un diagnóstico precoz y/o de la detección del parásito en casos subclínicos, lo que puede suceder en el 80% de los perros, la sensibilidad puede ser mucho menor (Albuquerque et al., 2017).

La detección de ADN de *Leishmania* se puede realizar con una amplia gama de muestras: sangre completa, capa leucocitaria, médula ósea, linfonodo, piel o hisopos de conjuntiva (Maia et al., 2009; Ferreira et al., 2008; Lombardo et al., 2012; Leite et al., 2015; Chagas et al., 2021; Bento et al., 2023). También se ha encontrado ADN de *Leishmania* en varias otras muestras biológicas que normalmente no se utilizan para el diagnóstico de rutina, como bazo, hígado, pulmón, corazón, pene, vagina, testículos, semen, útero, placenta, riñón, intestino, leche y orina (Andrade et al., 2002; Diniz et al., 2005; Barrouin-Melo et al., 2006; Franceschi et al., 2006; Goncalves et al., 2018; Pappa et al., 2021; Bento et al., 2023).

En los últimos años se está planteando el uso de muestras biológicas no obtenidas por procedimientos invasivos entre las que destacan el pelo, en citologías bucales, así como en el cerumen en el que se ha localizado ADN de *Leishmania* spp. (Lombardo et al., 2012; Belinchón-Lorenzo et al., 2013; Belinchón-Lorenzo et al., 2016; Merino Goyenechea et al., 2023). Se ha demostrado la utilidad del pelo para el diagnóstico de la leishmaniosis en lobo ibérico, lo que puede ayudar a diagnosticar la infección sin necesidad de cazar al animal, ya que se pueden utilizar los restos de pelo hallados en los hábitats de estos animales (Merino Goyenechea et al., 2023).

Desde un punto de vista diagnóstico se recomienda la PCR de linfonodo. Sin embargo, la PCR de médula ósea es una alternativa en perros sin adenomegalia detectable. En estudios epidemiológicos de campo a gran escala, la detección de anticuerpos es apropiada y se puede utilizar la PCR en sangre total para complementar los resultados serológicos (Maia et al., 2009).

Según un estudio realizado por Solcà et al. (2012), tanto en perros oligosintomáticos como polisintomáticos, la **PCR convencional** reveló un mejor análisis de rendimiento en comparación con la qPCR y el ensayo por inmunoadsorción ligado a enzimas (ELISA). Mas recientemente, Castelli et al. (2021) con nuevas técnicas de **PCR cuantitativa** han permitido la detección del ADN de *Leishmania spp.* y la cuantificación de cargas parasitarias considerablemente bajas en muestras que habían sido diagnosticadas como negativas mediante técnicas convencionales. Por lo tanto, este estudio concluyó que la **qPCR** se puede utilizar para el diagnóstico de leishmaniasis humana, canina y felina con alta sensibilidad y especificidad, pero también para evaluar el tratamiento y la determinación del criterio de valoración de la leishmaniasis.

Es importante resaltar que la información proporcionada por la PCR debe valorarse junto con los datos obtenidos en las evaluaciones clínico-patológicas y serológicas del paciente. Todos estos deben combinarse para realizar una evaluación integral (Solano-Gallego et al., 2009). Un único resultado negativo de la PCR en un perro clínicamente sospechoso no es suficiente para descartar la infección. Por otro lado, durante la temporada de transmisión también pueden ocurrir falsos positivos debido a una contaminación natural o una infección transitoria (Maia & Campino, 2008).

En el caso de la **especie felina**, la PCR ha permitido la detección del ADN de *Leishmania* en muestras de sangre completa, capa leucoplaquetaria, hisopos conjuntivales y orales, cabello, piel, tejido nasal, hígado, bazo, riñones, linfonodos, médula ósea, y fluido mamario (Chatzis et al., 2014a, Pereira & Maia, 2021).

1.4.3 DIAGNOSTICO INMUNOLOGICO

Las técnicas inmunológicas utilizadas en el diagnóstico de la leishmaniosis permiten valorar la respuesta inmunitaria humoral y celular.

1.4.3.1 Evaluación de la respuesta inmune humoral

Existen diversas pruebas serológicas para el diagnóstico de la leishmaniosis a través de la cuantificación de anticuerpos específicos *anti-Leishmania*, pero las más utilizadas son, la inmunofluorescencia indirecta (IFI), el test de aglutinación directa (DAT) y el ELISA. La sensibilidad y la especificidad de cada una de ellas no dependen únicamente de las características inherentes a la propia técnica, sino, en gran medida, del antígeno utilizado (Maia & Campino, 2008, Barbosa de Castro et al., 2022).

La muestra más habitual para la detección de estos anticuerpos específicos *anti-Leishmania* es el suero, ya que sus concentraciones se correlacionan positivamente con el cuadro clínico y el nivel de parasitemia (Teixeira Neto *et al.*, 2010; García-Castro et al., 2022). También se han encontrado en otras muestras como la orina (Todolí *et al.*, 2009), el líquido cefalorraquídeo (Melo et al., 2015), o la saliva (Cantos-Barreda et al., 2017).

Recientemente, Baxarias et al. (2022a) demostraron una buena concordancia entre saliva (muestra no invasiva) y el suero (muestra invasiva) y que fueron analizadas mediante ELISA. Estos resultados son particularmente de interés clínico en perros enfermos con niveles altos de anticuerpos, mientras que los resultados parecen menos óptimos en perros aparentemente sanos con niveles bajos de anticuerpos.

La respuesta humoral específica en la leishmaniosis canina es, en general, muy intensa, con altos niveles de inmunoglobulinas específicas que permiten el diagnóstico serológico. Además, la seroconversión ocurre a los pocos meses de la infección (Morales-Yuste et al., 2022) con una media de 5 meses (rango 1-22 meses) para infecciones naturales y de 3 meses (rango 1-6 meses) para infecciones experimentales (Moreno & Alvar, 2002). Debido a la respuesta inmune humoral exagerada (no protectora) en perros enfermos, la sensibilidad del diagnóstico serológico es muy elevada.

En el caso de las pruebas serológicas, pueden darse fenómenos de reacciones cruzadas con otros agentes patógenos cuando dan títulos bajos. Los principales agentes infecciosos que producen este fenómeno son *Trypanosoma spp.* y otras especies de *Leishmania* presentes en el continente americano (Viol et al., 2012; Meyers et al., 2021). Sin embargo, también se han descrito reacciones cruzadas con *T. gondii, Ehrlichia canis, Neospora caninum, Babesia canis* (Oliveira et al., 2008; Solano-Gallego et al., 2014; Zanette et al., 2014) y *Leptospira interrogans* (Porrozzi et al., 2007). Por otro lado, Krawczak et al. (2015) concluyeron en su estudio que existe coinfección de *Leishmania spp*. con *Ehrlichia spp*. y *Babesia spp*, pero también que las pruebas serológicas que se utilizaron no tuvieron reacciones cruzadas, por lo que la reacción cruzada depende de la prueba utilizada (Krawczak et al., 2015).

Las **técnicas serológicas cuantitativas** son apropiadas para el diagnóstico en la clínica veterinaria y el seguimiento del tratamiento, aunque debe llevarse a cabo en laboratorios especializados (Bourdeau *et al.*, 2014). Estas técnicas serológicas permiten la cuantificación de los niveles de anticuerpos *anti-Leishmania* circulantes.

La técnica ***IFI*** ha sido considerada tradicionalmente “la prueba estándar de oro” para el diagnóstico serológico de la infección por *L. infantum*, con medidas de rendimiento óptimas en cuanto a sensibilidad y especificidad (Zaffaroni et al., 1999) y algunos autores todavía consideran que esta prueba es una referencia técnica en las prácticas de laboratorio de diagnóstico (Paltrinieri et al., 2016). Sin embargo, su interpretación puede ser subjetiva dependiendo de las habilidades y experiencia del operador al interpretar los resultados (Solano-Gallego et al., 2014).

Esta técnica se basa en la utilización, como fuente antigénica, de una suspensión de promastigotes completos de *Leishmania* fijados en un portaobjetos de cristal al que se añaden diluciones seriadas del suero problema. La fijación de los anticuerpos específicos sobre el parásito se visualiza mediante un conjugado de anti-inmunoglobulinas caninas marcadas con isotiocianato de fluoresceína a través de un microscopio de fluorescencia (Basurco et al., 2020). No existe un consenso entre los laboratorios sobre el punto de corte de la técnica de IFI, pudiendo oscilar entre 1:40 y 1:320 (Otranto et al., 2005; Gramiccia, 2011; Morales-Yuste et al., 2012; Solano-Gallego et al., 2014). En general, se consideran títulos elevados todos aquéllos que superen de 2 a 4 veces el punto de corte indicado por el laboratorio de referencia (Paltrinieri *et al.*, 2010).

La técnica **ELISA** permite examinar una gran cantidad de muestras utilizando microplacas recubiertas de antígeno y un espectrofotómetro que determina los títulos de anticuerpos mediante la medición de la densidad óptica. El potencial para la cuantificación absoluta de anticuerpos convierte a ELISA en una herramienta poderosa que es menos susceptible al sesgo del operador. Uno de sus puntos fuertes es la posibilidad de utilizar combinaciones de múltiples antígenos, aumentando así la sensibilidad y/o especificidad del método (Maia & Campino, 2008, Travi et al., 2018). En algunos artículos, se demuestra que la sensibilidad diagnóstica es superior a la técnica de IFI (Solano-Gallego *et al.*, 2014).

Los antígenos utilizados pueden clasificarse en cuatro grupos según su naturaleza: extractos de promastigotes solubles o completos, proteínas recombinantes y proteínas purificadas (Solano-Gallego et al., 2014). La utilización de amastigotes como antígeno en la técnica ELISA hace que la técnica tenga una mayor sensibilidad en comparación a la utilización de promastigotes (Santarém et al., 2010).

Uno de los antígenos más empleados es el antígeno recombinante rK39 de *L. chagasi*, un epítopo inmunodominante repetitivo en una proteína relacionada con la cinesina que está altamente conservada entre las especies viscerotrópicas de *Leishmania*, lo que ha sido aprovechado para desarrollar técnicas diagnósticas a partir del antígeno rK39 (Mettler et al., 2005; Scalone et al., 2002). Diversos estudios serológicos muestran su elevada sensibilidad en el diagnóstico de la leishmaniosis (Morales-Yuste et al., 2022).

La técnica **DAT** es una prueba que requiere de incubaciones largas (18 horas) antes de la lectura de los resultados y se requiere realizar diluciones seriadas (Srividya et al., 2012). El fenómeno de aglutinación se detecta visualmente como difuminación total o parcial del botón, mientras que si aparece un botón compacto, no se ha producido fenómeno de aglutinación.La DAT se puede realizar en suero, plasma y sangre completa (Moody & Chiodini, 2000).

Para superar el largo periodo de incubación, se han puesto a punto otras pruebas como la Easy-DAT, que reduce el tiempo de la prueba a 5 horas (Gómez-Ochoa et al., 2003), o el test de aglutinación rápida (FAST), que combina una concentración mayor de parásito con un volumen de ensayo menor, por lo que requiere una única dilución de la muestra y una lectura en menor tiempo (3 horas), siendo muy útil para estudiar poblaciones grandes de perros (Schallig *et al.*, 2002).

La técnica de *WB* es un método serológico cualitativo que es capaz de distinguir el peso molecular de los antígenos de *L. infantum* que estimulan la producción de anticuerpos (Persichetti et al., 2017). Debido a su complejidad, esta técnica es utilizada con menos frecuencia en la práctica veterinaria para el diagnóstico de la leishmaniosis.

Este procedimiento permite determinar las fracciones antigénicas responsables de algunas de las reacciones cruzadas que probablemente afectan a las pruebas de IFI y ELISA. Mediante el WB se logra detectar anticuerpos específicos que aparecen a partir de las 10 semanas post infección, permitiendo realizar diagnósticos tempranos de la leishmaniosis (Olías-Molero et al., 2019). Así mismo, un posible campo de aplicación del método WB es la discriminación entre infecciones subclínicas y animales enfermos (Iniesta et al., 2007).

La **citometría de flujo (FC)** es una nueva herramienta inmunológica que se ha explorado en el diagnóstico de la leishmaniosis visceral canina mediante la detección de anticuerpos anti-fijados de *L. infantum*. Los investigadores han demostrado una alta especificidad, sensibilidad, valores predictivos y precisión, incluso cuando los animales estaban infectados con otros patógenos como *Trypanosoma cruzi* y *Babesia canis*; así como la ausencia de falsos positivos en perros vacunados (Ker et al., 2013). Por lo tanto, aunque todavía la FC es una técnica experimental costosa, se está convirtiendo en una herramienta cada vez más útil en los laboratorios de investigación y atención médica debido a su precisión y reproducibilidad (de Paiva-Cavalcanti et al., 2015).

Las **técnicas serológicas cualitativas o pruebas de inmunocromatografía** son más rápidas, más baratas, se pueden realizar en cualquier centro clínico, pero solo informan de un resultado positivo o negativo (Baxarias et al., 2022b). Las pruebas rápidas no pueden identificar perros con niveles bajos de anticuerpos, por lo que un resultado positivo mediante una prueba cualitativa es sólo un primer paso y debe ir seguido de un método serológico cuantitativo (Bourdeau *et al.*, 2014).

Este tipo de pruebas son de fácil ejecución, dando un resultado dicotómico después de un breve periodo de tiempo (Villanueva-Saz et al., 2019). Su rendimiento diagnóstico puede no ser óptimo en algunos prototipos comerciales, aunque generalmente tienen alta especificidad, pero una sensibilidad muy variable (Mettler et al., 2005). En general, se recomienda después de obtener el resultado de una prueba rápida, realizar una prueba cuantitativa que proporcione una titulación de anticuerpos (Solano-Gallego et al., 2011; Paltrinieri et al., 2016). Tienen la ventaja de encontrarse disponibles para su empleo en estudios epidemiológicos de campo (Villanueva-Saz et al., 2022).

Existen diversas pruebas inmunocromatográficas y cada una difiere en el antígeno y reactivo empleado (Paltrinieri *et al.*, 2010). El antígeno rK39 también se utiliza en estas pruebas y está disponible comercialmente en forma de tiras de papel de nitrocelulosa impregnadas con antígeno adaptadas para su uso en condiciones de campo. La aparición de dos líneas (una de control y otra de prueba, independientemente de la intensidad de la tinción) indica un resultado positivo (Maia & Campino, 2008).

1.4.3.2. Evaluación de la respuesta inmune celular

Los análisis para evaluar la respuesta inmune celular de un perro frente a *Leishmania* están muy poco estandarizados y generalmente no se utilizan como herramientas de diagnóstico clínico (Maia & Campino, 2008). La determinación de la respuesta inmune celular específica a *L. infantum* en perros constituiría un indicador crucial, similar a la valoración del tipo de respuesta Th1/Th2, ya que iría asociada a que el paciente realice un control eficaz de la infección y se acompañe de la supervivencia de este (Fernández-Bellon et al., 2005).

Un ensayo práctico y estandarizado para evaluar la inmunidad celular frente a la infección por *Leishmania* en entornos clínicos tendría una gran utilidad práctica para ayudar a monitorizar la leishmaniosis y el resultado del tratamiento (Maia & Campino, 2008). Existen varias pruebas para valorar la respuesta inmune celular frente a leishmaniosis como la prueba de la leishmanina (LST), test de linfoproliferación (LPA) y la cuantificación del interferón gamma (IFN- γ).

La prueba de la **leishmanina o test de Montenegro** es una prueba cutánea que mide la reacción de hipersensibilidad de tipo retardado (DTH) a una inyección intradérmica de una suspensión de promastigotes de *Leishmania* muertos. Es una herramienta útil para el diagnóstico de leishmaniosis debido a su simplicidad, sensibilidad y especificidad. Sin embargo, no permite la identificación de especies de *Leishmania* y no discrimina entre infecciones pasadas y actuales (Akhoundi et al. 2017). Tiene interés sobre la serología porque la inmunidad mediada por células es más duradera que la inmunidad humoral después de la curación del proceso. También tiene interés en la leishmaniosis visceral ya que el mantenimiento de inmunidad celular positiva es más indicativa de protección frente a las reinfecciones (Pennisi 2015, Carstens-Kass et al., 2021).

El **LPA** es una prueba *in vitro* para evaluar la inmunidad mediada de tipo celular frente a *Leishmania*. Los perros asintomáticos y resistentes presentan una fuerte respuesta proliferativa a los antígenos de *L. infantum*, mientras que los animales susceptibles y enfermos no responden al mitógeno (Strauss-Ayali et al., 2005). Permite valorar si se ha restablecido la capacidad de linfoproliferación después de un tratamiento leishmanicida exitoso (Fernández-Pérez et al., 2003).

La **cuantificación IFN-γ** permite valorar el nivel de estimulación de una respuesta de memoria inmune de polaridad Th1 específica al antígeno de *Leishmania* (Fernández-Bellón et al., 2005; Zribi et al., 2017).

2. JUSTIFICACIÓN Y OBJETIVOS:

2. JUSTIFICACIÓN Y OBJETIVOS:

En la clínica generalista de animales de compañía, al igual que los médicos de familia, tenemos que estar preparados para hacer frente a un número importante de diferentes patologías, para diagnosticarlas y tratarlas, o bien saber enfocarlas y derivarlas a otros veterinarios expertos en diferentes especialidades. Los procesos clínicos habitualmente más frecuentes en animales de compañía son las patologías cutáneas, digestivas y urinarias. Por otra parte, teniendo en cuenta que nuestra área de influencia se halla en la cuenca mediterránea, existen procesos que son endémicos en esta zona geográfica y por tanto también muy frecuentes. En este sentido, tenemos un especial interés por las enfermedades vectoriales y en concreto por la leishmaniosis, por tratarse de un proceso muy frecuente, que además se caracteriza por cursar con sintomatología muy diversa al afectar a prácticamente a todos los sistemas orgánicos y cursar, con elevada frecuencia, con signos cutáneos, oftalmológicos, urinarios y digestivos.

Para los clínicos generalistas es fundamental disponer de técnicas diagnósticas lo suficientemente sensibles y específicas como para tener una alta certeza de que un resultado es positivo o negativo; ya que de otra forma es muy difícil avanzar hasta el diagnóstico definitivo. En este sentido, la leishmaniosis, que es una enfermedad gran imitadora por la variedad de síntomas que puede inducir en el hospedador, se halla incluida en multitud de listados de diagnósticos diferenciales, muy especialmente en los cuadros cutáneos, en los urinarios y en los digestivos. La indicación para confirmarla/descartarla es realizar un test serológico, pero hay muchos casos en los que los resultados no son suficientemente claros.

Por ello en nuestro grupo de trabajo tenemos interés especial en el desarrollo de pruebas serológicas con elevada capacidad diagnóstica frente a la leishmaniosis, en el estudio comparativo de resultados con diferentes pruebas diagnósticas y en el análisis de resultados para la propuesta de metodologías diagnósticas que den confiabilidad a los clínicos.

Por otra parte, somos conscientes de que la situación epidemiológica de la leishmaniosis es cambiante por diversos factores, pero especialmente consecuencia de las variaciones que el cambio climático está suponiendo para una mejor adaptación de los vectores flebotomos. Éstos se están aclimatando a zonas geográficas nuevas. Además, se está describiendo la presencia de otros potenciales hospedadores, diferentes al perro, que pueden estar ampliando el problema endémico y por tanto los riesgos para la salud de los seres humanos. Otro foco de interés de nuestro equipo es la evaluación de la situación de *L. infantum* en otras especies de animales de compañía como el gato, a la vez que estamos atentos a la detección de la enfermedad en otras especies en las que se observen signos sospechosos de la enfermedad.

Por todo ello, para realizar el trabajo que presentamos, nos hemos marcado los objetivos que a continuación se resumen, habiendo sido, los resultados de cada uno de ellos, objeto de una publicación internacional.

Objetivos focalizados en el estudio de la calidad diagnóstica de pruebas para cuantificar anticuerpos *anti-Leishmania* en perros:

1. Evaluar los resultados de un test de inmunocromatografía rápida cualitativa en perros con diferentes niveles de anticuerpos *anti-*Leishmania y comparar los resultados con otras dos pruebas serológicas cuantitativas, IFI y ELISA, como pruebas de referencia, a través del cálculo de la sensibilidad (probabilidad de un resultado positivo entre aquellos que tienen con seguridad la enfermedad), de la especificidad (probabilidad de un resultado negativo entre aquellos que, con seguridad, no tienen la enfermedad), de la precisión (la capacidad de la prueba para diferenciar correctamente el paciente sano del enfermo), y de la curva qué mide la utilidad de una prueba a través de la relación entre sensibilidad y especificidad.

2. Evaluar estadísticamente la sensibilidad y especificidad de una prueba ELISA interna en comparación con tres pruebas serológicas comerciales diferentes, en dos grupos de muestras de suero canino; uno procedente de áreas no endémicas y el otro originario de áreas endémicas; asumiendo condiciones epidemiológicas y seroprevalencias significativamente diferentes entre ambos dos grupos, mediante modelos de clase latente en un análisis bayesiano.

Objetivos enmarcados en la valoración de la situación de la leishmaniosis en otras especies de animales de compañía:

3. Investigar la prevalencia de la infección por *Leishmania* spp. en gatos, aparentemente sanos, en la ciudad de Zaragoza, que es una región endémica de España, comparando el rendimiento de tres métodos serológicos: una prueba de anticuerpos inmunofluorescentes (IFI), una prueba de tipo ensayo inmunoabsorbente ligado a enzimas (ELISA) y un test Western Blot. (WB) para la detección de anticuerpos contra *L. infantum*; así como una prueba de reacción en cadena de la polimerasa (PCR) en tiempo real para la detección de ADN de *L. infantum*.

4. Realizar el primer diagnóstico y aislamiento de *L. infantum* en un hurón doméstico infectado de forma natural en una región endémica (España) donde se detecta con frecuencia leishmaniosis canina y felina.

3. RELACIÓN DE PUBLICACIONES:

ARTÍCULO 1

Villanueva-Saz S, **Basurco A**, Martín V, Fernández A, Loste A, Verde MT. **Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs**. Acta Vet Scand. 2019; 61:38.

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BRIEF COMMUNICATION Open Access

Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs

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Abstract

Canine leishmaniosis is a disease caused by *Leishmania infantum,* a vector-borne parasite. Due to the zoonotic potential of canine leishmaniosis, infected dogs must be identified. Serological assays are the most common methods for the detection of *L. infantum* infection in dogs used in veterinary practice. The aim of the study was to assess the performance of a rapid immunochromatographic test (*FAST*est LEISH®, MEGACOR Diagnostik) for the detection of specific antibodies to that of the *L. infantum* in dog sera. The results were simultaneously compared using a commercial brand of indirect immunofluorescence antibody test and an in-house enzyme-linked immunosorbent assay as references. Between the two reference tests, 232 serum samples out of 244, produced concordant results while 12 exhibited discordant results. Of the 232 concordant samples, 121 were classified as *L. infantum* seropositive, and 111 samples were previously classified as *L. infantum* seronegative by a combination of the reference assays. All samples that were seropositive by the reference tests were also positive according to the rapid test, and only one sample that was seronegative according to the two reference assays was positive according to the rapid test. Compared with the reference tests, the rapid test sensitivity was 100%, specificity was 99.1%, accuracy was 99.6%, Cohen's kappa coefficient was 0.99, and the area under receiver operating characteristic curve was 0.995. The *FAST*est LEISH® is a rapid, qualitative in-clinic test with high sensitivity and specificity.

Keywords: Diagnostic techniques and procedures, Immunoglobulins, Canine leishmaniosis

Findings

Canine leishmaniosis is zoonotic disease caused by *L. infantum,* a vector-borne protozoan parasite transmitted by phlebotomine sand flies under natural conditions. Clinical manifestations of infection may range from mild to absent to mild, several and even fatal disease. This variability is thought to be the result of cell-mediated immune response from the host which may be influenced by the genetic background of the dog [1]. Individual incubation times range from a few months to several years [2]. The detection of *L. infantum* antibodies indicates existing or past infections. High antibody levels are often associated with a high parasitic load and disease [3]. Conversely, low antibody levels in clinically normal dogs with a negative result on molecular and/or parasitological tests may indicate exposure or early stages of *Leishmania* infection [4]. Different confirmatory techniques were used and the diagnosis was based on the clinical manifestation and/or the laboratory abnormalities that were compatible with the disease as well as by the confirmation of *L. infantum* infection [5].

Serological assays, including enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), and rapid tests, are the most common methods for the detection of infection in dogs [6].

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Table 1 Details of dogs including purpose of diagnosis and signalment

	Description	Number of dogs (%)
Purpose of diagnosis	Annual screening program for clinically healthy dogs	78 (32.0)
	Cases of suspected clinical leishmaniosis	103 (42.2)
	Blood donor screening program	23 (9.4)
	Pre vaccination screening for *L. infantum* infection	40 (16.4)
Signalment		
Sex	Male	115 (47.1)
	Female	129 (52.9)
Age	12 months of age	68 (27.9)
	≥ 12 months of age and < 8 years of age	135 (55.3)
	≥ 8 years of age	41 (16.8)
Race	Purebred	94 (38.5)
	Mixed-bred	150 (61.5)

Rapid tests have been used as an important first step in diagnostic algorithms, enabling results to be obtained within a short time [7].

The present study evaluated the diagnostic performances, including the sensitivity (probability of a positive test result among those having the target condition), specificity (probability of a negative test result among those without the target condition), accuracy (the ability of a test to differentiate the patient and healthy cases correctly) and area the receiver operating characteristic curve (measure of the usefulness of a test showing the relationship between sensitivity and specificity) of a rapid qualitative immunochromatographic in dogs with different anti-*Leishmania* antibody levels and compared the results from a combination of two quantitative serological tests, IFAT and ELISA, as reference tests.

Blood samples from 244 dogs of different breeds, ages, and genders that were naturally exposed to *L. infantum* infection were included in the study from May 2017 to May 2018. These dogs presented at the Veterinary Teaching Hospital of the University of Zaragoza (Spain) for different diagnostic purposes, including the annual screening program for clinically healthy dogs, cases of suspected clinical leishmaniosis, the blood donor screening program and pre vaccination screening for *L. infantum* infection (Table 1). The serological status was recorded by a retrospective review of sample files through the laboratory database (Tables 2 and 3). Two aliquots of each obtained serum sample were stored at -20 °C until testing. Because samples were collected for the sole intention of determining a diagnosis, ethical approval was not needed. However, the owners were required to sign an informed consent.

The rapid test (*FAST*est Leish®, MEGACOR Diagnostik, Hörbranz, Austria) was performed following the

Table 2 Frequency of clinical signs in diseased dogs (n = 121, with IFAT and ELISA result)

Lymphadenomegaly	98 (80.1)
Cutaneous involvement	86 (71.1)
Weight loss	67 (55.4)
Anorexia	54 (44.6)
Exercise intolerance	38 (31.4)
Ocular involvement	25 (20.7)
Pale mucous membranes	19 (15.7)
Fever	15 (12.4)
Lameness	11 (9.1)
Vomiting, diarrhea	7 (5.8)
Polyuria and polydipsia	4 (3.3)
Muscle atrophy	3 (2.5)
Splenomegaly	2 (1.7)

Table 3 Frequency of hematologic and biochemical alterations in diseased dogs (n = 121, with IFAT and ELISA result)

Hematological parameters	
Anemia	67 (55.4)
Other hematological abnormalities	
Neutrophilia	17 (14.0)
Lymphopenia	22 (18.2)
Lymphocytosis	4 (3.3)
Thrombocytopenia	10 (8.3)
Biochemical parameters	
Renal azotemia	9 (7.4)
Hyperproteinemia with hypoalbuminemia and inverted albumin: globulin ratio	75 (62.0)
Urinalysis	
Proteinuria	43 (35.5)
Decreased urine specific gravity	36 (29.8)

instructions of the manufacturer. All tests were stored at room temperature and were performed as described in the instructions supplied with the test kit. This rapid test employs a combination of monoclonal antibodies conjugated with colloidal gold particles and recombinant *L. infantum* antigens bound to the solid phase of a nitrocellulose membrane that detect anti-*L. infantum* antibodies in whole blood, plasma or serum from the dog. The antibodies against *L. infantum* present in the sample react in the conjugate pad with mobile monoclonal antibodies to form antibody complexes. These antibody complexes migrate along the nitrocellulose membrane and bind to fixed *Leishmania* spp. antigens, producing clear pink-purple colored test line. Particles that do not bind to the conjugate continue their course along the membrane and pass through the control line with membrane-fixed control antibodies. The control line shows that the sample and reagents have been properly applied and migrated through the device. The buffer diluent facilitates the migration and promotes the binding of antibodies to antigens. Samples with a clear test and control line are classified as positive, and samples that show a control line are classified as negative. Prior to the study, technicians were trained to perform and interpret results. All tests were read by two laboratory technicians after 15 min. If discrepancies arose between results, a third observer participated. The examiners were blinded to the results of the quantitative serological tests.

The commercial IFAT (MegaFLUO LEISH test®, MEGACOR Diagnostik, Hörbranz, Austria) was performed as described in the instructions supplied with the test kit. This commercial IFAT has been employed in other comparative studies of diagnostic tests [8]. All samples were examined by two different investigators. They were blinded to the results of the other serological tests. Samples negative at 1:100 were considered negative and the endpoint titer of positive samples was determined by preparing serial twofold dilutions of the serum starting from the cut-off value (1:100).

As a second quantitative serological reference method, an in-house ELISA by *L. infantum* was performed on all serum samples as previously described, with some modifications [9]. The in-house ELISA test was performed by a different researcher who had no knowledge of the rapid test and IFAT results. The cut-off value was 30 ELISA unit (EU). Sera with an EU ≥ 200 were classified as high positive, with an EU ≥ 100 and < 200 as moderate positive, and with an EU > 30 and < 100 as low positive (Additional file 1).

Data obtained were checked for normal distribution using the Kolmogorov–Smirnov test ($P > 0.05$). A regression analysis between the two quantitative tests and Pearson´s correlation coefficients were calculated for all results that were concordant among the three serological tests. Statistical analyses were performed using IBM SPSS statistics software version 22 (SPSS Inc., Armonk, NY, USA). Because a single widely accepted reference standard for the diagnosis of *L. infantum* infection in dogs is not available, samples were classified based on results coinciding in both quantitative reference tests. Samples for which discordant results were obtained with the reference tests were not included in the study. Moreover, dogs that had previously been vaccinated with either of the two vaccines available in Spain for the prevention of canine leishmaniosis were also not included in this study.

Of the 244 sera analysed, 232 samples showed concordant results for IFAT and the in-house ELISA. The remaining 12 samples were considered discordant between reference tests, including 2 samples with a low positive result for the in-house ELISA but a negative result (antibody titer < 1:100) for the IFAT, as well as 10 samples with a positive result (antibody titer of 1:100) for the IFAT but negative result for the in-house ELISA. All discordant samples between the two reference tests were classified as seronegative by the rapid test. Among the 232 serum samples characterized, 121 samples were classified as seropositive for *L. infantum* infection and 111 samples were classified as seronegative for *L. infantum* infection (Table 4). Serum samples with different serum

Table 4 Comparison of the *FAST*est LEISH® with IFAT and ELISA results for *Leishmania* spp. antibody detection

Number of samples	IFAT and ELISA combination			IFAT		ELISA		Total
	Concordant results between reference tests		Discordant results between reference tests	Positive	Negative	Positive	Negative	
	Positive	Negative						
*FAST*est LEISH®								
Positive	121	1	0	121	1	121	1	122
Negative	0	110	12	10	112	2	120	122
Total	121	111	12	131	113	123	121	244

anti-*Leishmania* spp. antibody levels were selected for the evaluation (Table 5). All serum samples classified as positive by both reference assays were also positive in the *FAST*est LEISH®. Only one seronegative sample classified by the reference assays was discordant and showed a positive rapid test result. There were no additional freeze–thaw cycle or sample handing differences in testing events between the three tests.

The rapid test achieved sensitivity of 100% (95% confidence intervals (CI) 96.2–100%) and specificity of 99.1% (95% CI 94.4–100%), and the accuracy was 99.6% (95% CI 97.3–100%). The Cohen's kappa coefficient between the rapid test and the combined reference assays for the detection of antibodies was 0.99. The area the receiver operating characteristic curve (AUC-ROC) obtained from the curve was 0.995 (95% CI 0.985–1.000) (Fig. 1). Agreement between the two observers in reading the rapid test was 100% and no invalid results were obtained. The regression line was statistically significant ($P < 0.001$) and Pearson´s correlation coefficient was $r = 0.675$.

Sensitivity and specificity are common parameters for evaluating diagnostic methods [10]. Depending on the purpose of the test, sensitivity or specificity should be prioritized. When a test is used to detect *L. infantum* infection in dogs with suspected clinical leishmaniosis, specificity and sensitivity must be high, but importantly, specificity must not be too low because a confirmatory test should be highly specific to avoid false positives. In contrast, high sensitivity is essential for identifying clinically healthy infected dogs.

When a reliable gold standard is not available, at least two different tests should be performed [11]. In the present study, sample classification was established based on combination of results from ELISA and IFAT, obtaining a better definition of the serological status. When different serological tests are used simultaneously, discrepancies may be observed, and the lack of consistency among tests may depend on their different performances. A possible explanation for these discrepancies may be the inherent differences in the two quantitative assays. Therefore, the differences between the diagnostic performances of the references tests may modify the accuracy of the rapid test analyzed.

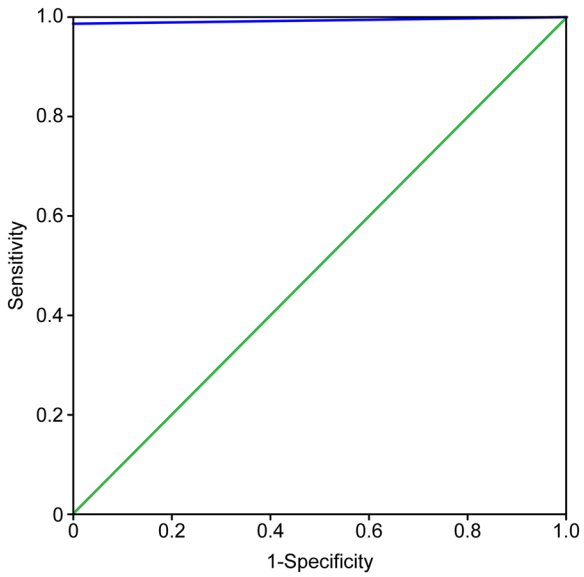

Fig. 1 AUC-ROC curve analysis of the rapid test studied. The Area the receiver operating characteristic curve (AUC-ROC) analysis combines sensitivity and specificity into one measurement, and the result is a single global measure of diagnostic effectiveness. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The blue line represents the AUC-ROC analysis of the *FAST*est LEISH®, and the green line represents the reference line

Numerous rapid tests are available to detect antibodies in dogs. However, these tests show high specificity and variable sensitivity [12]. Differences in diagnostic results obtained between rapid tests are influenced by the serum panel, technology, type of antigen, test threshold and the different laboratory test considered as a reference assay (Table 6). Standardization and comparison of results between the evaluated test and other similar rapid tests from different studies is not possible due to differences in the parameters used to evaluate a test, such as sample size, definition of the clinical status of the dog involved in the study and the selected reference populations. Therefore, examinations of diagnostic tests should follow the STARD guidelines (Standards for the Reporting of Diagnostic Accuracy Studies) to allow an adequate comparison between results.

Table 5 Number of positive samples and antibody levels (IFAT and ELISA)

IFAT	**1:400**	**1:800**	**1:1600**	**1:3200**	**1:6400**	**> 1:6400**
Number of samples	4	8	27	22	14	46
ELISA cutoff: 30 EU	**Low positive (> 30 and < 100 EU)**	**Moderate positive (≥ 100 and < 200 EU)**	**High positive (≥ 200 EU)**			
Number of samples	27	46	48			

Table 6 Concordant results between reference test and results of each reference test for sensitivity and specificity of the *FAST*est LEISH®

	Reference test		
	IFAT and ELISA combination	**IFAT**	**ELISA**
*FAST*est LEISH®			
Sensitivity (95% CI)	100% (96.2–100%)	92.4% (86.1–96.1%)	98.4% (93.7–99.8%)
Specificity (95% CI)	99.1% (94.4–100%)	99.1% (94.5–100%)	99.2% (94.9–100%)
Number of samples included	232	244	244

Sample inclusion can influence assessment measures with any test. Serum samples with high levels of anti-*Leishmania* spp. antibodies are more easily detected by a test and contribute to higher diagnostic values [13]. Samples in the present study had different antibody levels ranging from 42 to 372 EU, according to the in-house ELISA. Conversely, antibody titers detected by IFAT ranged from 1:400 to > 1:6400. The main differences between the ELISA and IFAT techniques are the type of antigen used and the technical method performed to obtain the results. In terms of interpreting the results, IFAT is subjective and depends on the operator´s experience, even when two different experienced observers examine the samples. This situation may explain the discordant results obtained.

A potential limitation of the present study was the lack of samples from dogs serologically reactive to other pathogens, particularly different *Leishmania* spp. or *Trypanosoma cruzi,* which are both protozoa that belong to the Trypanosomatidae family and share various antigens that may produce cross-reactions [14, 15]. All serum samples were collected from dogs from the city of Zaragoza, a region in Spain where *T. cruzi* is not present and *L. infantum* is the parasite responsible for canine leishmaniosis in Europe. Ideally, for a proper clinical sample characterization, the use of techniques of different nature is highly recommended. However, the objective of the study was to evaluated the ability of a rapid test to detect antibodies. For this reason and to characterize the seropositive status of the samples, serological techniques are necessary instead of other methods.

In conclusion, the use of *FAST*est LEISH® as a screening test, may be particularly useful for identifying clinically healthy, seropositive, infected dogs.

Additional file

Additional file 1. Description of the in-house indirect ELISA protocol for detection of anti-*Leishmania infantum* antibodies.

Abbreviations
AUC-ROC: area the receiver operating characteristic curve; CI: confidence intervals; IFAT: immunofluorescence antibody test; ELISA: enzyme-linked immunosorbent assay; EU: ELISA units.

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Prior publication
Data have not been published previously.

Authors' contributions
SVS and VM planned the study and MTV and AL collected the samples. SVS, VM, AF, AL, AB and MTV performed the laboratory analyses and AB, SVS, AF and MTV performed the statistical analyses. SVS and MTV coordinated the study. All authors interpreted the findings. SVS and AF drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study did not require official or institutional ethical approval. The animals were handled according to highly ethical standards and the national legislation. Nevertheless owners were required to sign an informed consent to allow use of samples in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. Pinelli E, Killick-Kendrick R, Wagenaar J, Bernadina W, del Real G, Ruitenberg J. Cellular and humoral immune response in dogs experimentally and naturally infected with *Leishmania infantum*. Infect Immun. 1994;62:229–35.
2. Oliva G, Scalone A, Foglia Manzillo V, Gramiccia M, Pagano A, Di Muccio T, et al. Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. J Clin Microbiol. 2006;44:1318–22.
3. Reis AB, Martins-Filho OA, Teixeria-Carvalho A, Carvalho MG, Mayrink W, França-Silva JC, et al. Parasite density and impaired biochemical/hematological status are associated with severe clinical aspects of canine visceral leishmaniasis. Res Vet Sci. 2006;81:68–75.
4. Paltrinieri S, Solano-Gallego L, Fondati A, Lubas G, Gradoni L, Castagnaro M, et al. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. J Am Vet Med Assoc. 2010. https://doi.org/10.2460/javma.236.11.1184.
5. Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, et al. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors. 2011. https://doi.org/10.1186/1756-3305-4-86.
6. Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet Parasitol. 2008. https://doi.org/10.1016/j.vetpar.2008.07.028.
7. Maia C, Campino L. Biomarkers associated with *Leishmania infantum* exposure, infection, and disease in dogs. Front Cell Infect Microbiol. 2018. https://doi.org/10.3389/fcimb.2018.00302.
8. Wolf D, Failing K, Taubert A, Pantchev N. Serological diagnosis of canine leishmaniosis: comparison of three commercially available tests. Parasitol Res. 2014. https://doi.org/10.1007/s00436-014-3865-1.
9. Riera C, Valladares JE, Gállego M, Aisa MJ, Castillejo S, Fisa R, et al. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. Vet Parasitol. 1999;84:33–47.
10. Adel A, Berkvens D, Abatih E, Soukehal A, Bianchini J, Saegerman C. Evaluation of immunofluorescence antibody test used for the diagnosis of canine leishmaniasis in the Mediterranean basin: a systematic review and meta-analysis. PloS ONE. 2016. https://doi.org/10.1371/journal.pone.0161051.
11. Reitsma JB, Rutjes AW, Khan KS, Coomarasamy A, Bossuyt PM. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. J Clin Epidemiol. 2009. https://doi.org/10.1016/j.jclinepi.2009.02.005.
12. Rodriguez-Cortes A, Ojeda A, Todoli F, Alberola J. Performance of commercially available serological diagnostic tests to detect *Leishmania infantum* infection on experimentally infected dogs. Vet Parasitol. 2013. https://doi.org/10.1016/j.vetpar.2012.09.009.
13. Otranto D, Paradies P, Sasanelli M, Spinelli R, Bradonisio O. Rapid immunochromatographic tests for serodiagnosis of canine leishmaniasis. J Clin Microbiol. 2004;42:2769–70.
14. Baneth G, Nachum-Biala Y, Shabat Simon M, Brenner O, Gaier S, Rojas A, et al. Leishmania major infection in a dog with cutaneous manifestations. Parasite Vector. 2016;9:24. https://doi.org/10.1186/s13071-016-1541-2.
15. Ker HG, Coura-Vital W, Aguiar-Soares RD, Roatt BM, das Dores Moreira N, Carneiro CM, et al. Evaluation of a prototype flow cytometry test for serodiagnosis of canine visceral leishmaniasis. Clin Vaccine Immunol. 2013. https://doi.org/10.1128/cvi.00575-13.

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Original Article

Evaluation of the performance of three serological tests for diagnosis of *Leishmania infantum* infection in dogs using latent class analysis

Avaliação da realização de três testes sorológicos para diagnóstico de infecção por *Leishmania infantum* em cães utilizando análise de classe latente

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Abstract

Canine leishmaniasis (CanL) is a disease caused by *Leishmania infantum*. Serological methods are the most common diagnostic techniques used for the diagnosis of the CanL. The objective of our study was to estimate the sensitivity and specificity of one in-house ELISA kit (ELISA UNIZAR) and three commercially available serological tests (MEGACOR Diagnostik GmbH) including an immunochromatographic rapid test (*FASTest* LEISH®), an immunofluorescent antibody test (MegaFLUO LEISH®) and an enzyme-linked immunosorbent assay (MegaELISA LEISH®), using latent class models in a Bayesian analysis. Two hundred fifteen serum samples were included. The highest sensitivity was achieved for *FAST*est LEISH® (99.38%), ELISA UNIZAR (99.37%), MegaFLUO LEISH® (99.36%) followed by MegaELISA LEISH® (98.49%). The best specificity was obtained by *FAST*est LEISH® (98.43%), followed by ELISA UNIZAR (97.50%), whilst MegaFLUO LEISH® and MegaELISA LEISH® obtained the lower specificity (91.94% and 91.93%, respectively). The results of present study indicate that the immunochromatographic rapid test evaluated *FAST*est LEISH® show similar levels of sensitivity and specificity to the quantitative commercial tests. Among quantitative serological tests, sensitivity and specificity were similar considering ELISA or IFAT techniques.

Keywords: Bayesian analysis, canine leishmaniasis, diagnostic techniques and procedures, gold standard, immunoglobulins.

Resumo

A leishmaniose canina (Lcan) é uma doença causada pela *Leishmania infantum*. Os métodos sorológicos são as técnicas diagnósticas mais utilizadas para o diagnóstico da leishmaniose canina. O objetivo do nosso estudo foi estimar a sensibilidade e a especificidade de um kit ELISA interno (ELISA UNIZAR) e de três testes sorológicos disponíveis comercialmente, feitos pelo mesmo fabricante (MEGACOR Diagnostik GmbH), incluindo um teste rápido imunocromatográfico (*FASTest* LEISH®), um teste de anticorpos imunofluorescentes (Megafluo LEISH®) e um ensaio de imunoabsorção enzimática (Megaelisa LEISH®), utilizando-se modelos de classe latentes numa análise bayesiana. Foram incluídas duzentas e quinze amostras de soro. A maior sensibilidade foi alcançada para Fastest LEISH® (99,38%), ELISA UNIZAR (99,37%), Megafluo LEISH® (99,36%) seguida por Megaelisa LEISH® (98,49%). A melhor especificidade foi obtida por *FASTest* LEISH® (98,43%), seguida por ELISA UNIZAR (97,50%), enquanto Megafluo LEISH® e Megaelisa LEISH® obtiveram a menor especificidade (91,94% e 91,93%, respectivamente). Os resultados do presente estudo indicam que o teste rápido imunocromatográfico, avaliado por *FAST*est LEISH® mostra níveis similares de sensibilidade e especificidade aos testes comerciais quantitativos incluídos. Entre os testes sorológicos quantitativos, a sensibilidade e a especificidade foram semelhantes, considerando-se as técnicas de ELISA ou IFI.

Palavras-chave: Análise bayesiana, leishmaniose canina, técnicas e procedimentos diagnósticos, padrão ouro, imunoglobulinas.

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Introduction

CanL is a vector-borne disease caused by *Leishmania infantum*, which dogs are considered the main domestic reservoir for human infection (Dantas-Torres, 2007). This infection is transmitted by the bite of infected female sand flies from the genera *Phlebotomus* in the Old World or *Lutzomyia* in the New World (Moreno & Alvar, 2002). In the Mediterranean basin, an estimation of 2.5 million dogs are infected by the parasite including subclinically infected dogs and sick dogs exhibiting clinical signs and/or clinicopathological abnormalities (Solano-Gallego et al., 2009). Cases of subclinical infections, defined as a situation in which *Leishmania* infection is confirmed but clinical signs and/or clinicopathological abnormalities are not present have been documented in all areas where CanL is endemic. It is no longer believed that absolutely every infected dog will inevitably develop clinical leishmaniasis (Solano-Gallego et al., 2009). In fact, a small proportion (prevalence of the disease ranges between 3 and 10%) of the dogs infected with *L. infantum* in endemic regions will develop the disease following infection (Baneth et al., 2008). The evolution of this chronic infection depends on the cell-mediated immune response from the host against the parasite, cellular protective immunity is associated with activation of the macrophages by different cytokines, particularly interferon-gamma, tumor necrosis factor-alpha and interleukin-2. By contrast, disease susceptibility, by releasing a mixed T helper (Th1 and Th2) lymphocyte cytokines, tend to promote non-protective antibody formation and correlate with a diminution or absent cell-mediated immunity (Hosein et al., 2017). In this sense, the number of circulating CD4+ cells and the CD4+/CD8+ ratio drop during the disease (Esch et al., 2013). In the case of CD8+, these T cells are responsible for lysis of the macrophages infected with *L. infantum* and their activation increases the synthesis of INF-γ and TNF-α (Barbiéri, 2006).

Different techniques to confirm *L. infantum* infection are available including parasitological methods with the direct identification and observation of the parasite such as cytology, histology, specific immunohistochemistry and the parasite culture; molecular methods to detect parasitic nucleic acids (DNA and RNA) by polymerase chain reaction (PCR) such as conventional PCR, nested PCR and quantitative PCR; and serological methods based on detecting specific antibodies response against *L. infantum* (Maia & Campino, 2018). Serology is the preferred diagnostic method for CanL considering information provided by the World Organisation for Animal Health (OIE, 2016). Among serological methods, enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT), and the immunochromatographic rapid test (ICT) represent the most frequent methods used for the detection of infection in dogs (Maia & Campino, 2008; Bourdeau et al., 2014).

For *L. infantum* infection there is no perfect diagnosis but the diagnosis must be appropriate for each situation. The gold standard test does not necessarily have 100% sensitivity (Se) and 100% specificity (Sp) but it is the most sensitive and specific test for diagnosing that infectious agent. In absence of a reference test, the Latent Class Analysis (LCA) allows the estimation of sensitivity and specificity of two or more tests without assuming the true antibody status of the population under study, avoiding the bias connected to the use of an imperfect test (Branscum et al., 2005). The aim of this study was the statistically evaluation of the Se and Sp of one in-house ELISA test in comparison with three different commercial serologic tests in two groups of canine serum samples, one from non-endemic areas, the other from endemic areas, assuming significant different epidemiological conditions and seroprevalences between the two groups by a latent class models in a Bayesian analysis.

Material and Methods

Study population and serum samples

A total of 215 serum samples from dogs admitted to the Veterinary Teaching Hospital of the University of Zaragoza (Spain) and from three different clinics from United Kingdom were used in this study. Serum samples were collected during the period from January 2017 to December 2018 and conserved at –25 °C until analyzed.

With the aim to evaluate the performances of the tests by means of Bayesian analysis, samples were stratified into two populations with different levels of prevalence: canine sera from a non-endemic area (group 1) and from an endemic area (group 2).

One hundred and ninety-five serum samples (group 2) came from The Ebro´s Valley, a Spanish region where CanL is endemic, and the remaining serum samples (group 1) were residual samples taken from dogs during a routine annual health check-up in the United Kingdom, a non-endemic area. Within group 2, each dog was classified in different subgroups (non-infected dogs, healthy seronegative dogs, infected seropositive dogs, clinically sick dogs

and finally dogs with serological positive result to other pathogens) according to the clinical information sent with the sample to the laboratory for diagnostic purpose. The subgroups were not considered for the statistical evaluation.

Serum samples selection have reflected different clinical settings in veterinary practice from diagnosis of *L. infantum* infection in healthy dogs to clinically sick dogs, based on clinical evaluation, routine red blood cell count, clinical chemistry, urinalysis and serum protein electrophoresis. The serology status was also routinely recorded by means of an in-house ELISA (Solano-Gallego et al., 2009), but this test had the only goal to assure the selection of a heterogeneous population and did not influence the statistical analysis.

In the case of dogs with serological positive result to other pathogens (cross-reaction group), serology was used together with a molecular test to detect *L. infantum* infection performed in a private laboratory. None of the samples used in the study came from dogs previously vaccinated with any of the two vaccines available in Spain to prevent CanL (Solano-Gallego et al., 2017).

Clinical and epidemiological data were considered for the selection and samples were collected for the sole intention of determining a diagnosis. Ethical approval was not needed, but owners were requested to sign an informed consent. The study was reported to the Bioethical Committee of the University of Zaragoza and conducted in accordance with the European (2010/63/UE) and national (RD1201/2005) directives on animal experimentation. This study did not require official or institutional ethical approval.

Non-endemic canine sera (group 1)

Twenty serum samples obtained from The United Kingdom were included. These dogs had never traveled to an endemic area and they had neither clinical signs nor laboratory abnormalities detected by routine red blood cell count, clinical chemistry, urinalysis and a serum protein electrophoresis.

Endemic canine sera (group 2)

A total of 195 sera were collected from dogs living in a leishmaniasis endemic area. To obtain a heterogeneous group of samples, some subgroups were considered: clinically-ill infected dogs, dogs infected by some other pathogens and seemingly healthy dogs that resulted seropositive or seronegative. The heterogeneity was based mainly on clinical evaluation and routine red blood cell count, clinical chemistry, urinalysis and serum protein electrophoresis. The serology status was also recorded, based on ELISA UNIZAR, but this classification did not have any influence on statistical evaluation, based only on the two groups 1 (non-endemic) and 2 (endemic).

Sixty-five serum samples from seronegative healthy dogs were obtained from the Veterinary Teaching Hospital - Zaragoza Veterinary Faculty (University of Zaragoza, Spain). These samples came from dogs that were taken to Hospital for a screening *L. infantum* infection purpose by quantitative serology. Clinical information showed absence of clinical signs during physical examination and no laboratory findings based on routine red blood cell count and clinical chemistry, serum protein electrophoresis and urine analysis.

Eighty-seven sera from naturally infected dogs presenting variable clinical manifestations and/or laboratory alterations were included. Among clinical signs detected compatible with CanL, these were lymphadenomegaly (n=74), skin lesions (n=65), weight loss (n=44), anorexia (n=36), ocular lesions (n=19), pale mucous membranes (n=15), lameness (n=10), fever (n=8), gastrointestinal signs (n=7), epistaxis (n=4) and muscular atrophy (n=2). On the other hand, laboratory abnormalities detected compatible with CanL were non-regenerative anemia (n=50), neutrophilia (n=9), lymphopenia (n=17), lymphocytosis (n=2), thrombocytopenia (n=11), renal azotemia (n=6), hyperproteinemia (n=52), dysproteinemia with hypoalbuminemia and inverted albumin: globulin ratio (n=63), serum protein electrophoresis alteration with hypergammaglobulinemia detected (n=70), proteinuria (n=29) and low urinary specific gravity (n=21). In all 87 sick dogs, *L. infantum* disease was confirmed by a positive serology to the ELISA UNIZAR with moderate (n=44) to high (n=43) concentrations of serum anti-*Leishmania* antibodies.

Twenty-four sera samples from dogs evaluated out of seasonal activity of sand flies with a low positive result to the ELISA UNIZAR were included. Clinical information showed absence of clinical signs during physical examination and no laboratory findings being classified as seropositive asymptomatic infected dogs. All dogs were negative to *L. infantum* infection using a quantitative PCR in blood.

Nineteen samples from dogs with a serological positive result to other pathogens were analyzed to evaluate any possible cross-reaction: *Dirofilaria immitis* (n=3, positive result to heartworm antigen test and modified Knott´s test), *Neospora caninum* (n=1, IFAT antibody titer of 1:100), *Toxoplasma gondii* (n=1, IFAT antibody titer of 1:80),

Rickettsia conorii (n=1, IFAT antibody titer of 1:20), *Ehrlichia canis* (n=1, IFAT antibody titer 1:40), *Anaplasma platys* (n=11, IFAT antibody titers ranging from 1:20 to 1:160) and finally a sample with two co-infections *T. gondii* (IFAT antibody titer of 1:160) and *N. caninum* (IFAT antibody titer of 1:100). All dogs were negative to *L. infantum* infection using a quantitative PCR in blood.

ELISA UNIZAR technique

Prior to performing the in-house ELISA, *L. infantum* antigen (strain MHOM/FR/78/LEM 75 belonging to *L. infantum* zimodeme MON-1) was obtained from parasite culture. For the ELISA UNIZAR, the crude antigen was adjusted to a concentration of 20 µg/mL with sterile phosphate buffered saline (PBS). Each plate was coated lightly with 100 µL/well of the 20 µg/mL antigen solution in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were then frozen and stored at –20 °C. One hundred microliters of dog serum, diluted 1:800 in phosphate buffered saline containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M) were added to each well. The plates were incubated for 1 hour (h) at 37 °C in a moist chamber. After washing the plates three times with PBST for 3 minutes (min) followed by one wash with PBS for 1 min, 100 µL of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted 1:20000 in PBST-M was added to each well. The plates were incubated for 1 h at 37 °C in a moist chamber, followed by washes with PBST and PBS as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were added (100 µL per well) and developed for 20±5 min at room temperature in the dark. The reaction was terminated by adding 100 µL of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm (reference wavelength) in an automatic microELISA reader (ELISA Reader Labsystems Multiskan, Midland, Canada). Each plate included serum samples from a dog infected with *L. infantum* as confirmed by cytological examination as a positive control (calibrator) and serum samples from a healthy, non infected dog from the blood donor program as a negative control. The same calibrator serum sample was used for all assays, and the plates with an interassay variation greater than 10% were tested again. All samples and controls were analyzed in duplicate. The ELISA UNIZAR test was performed by a different researcher who had no knowledge of the rapid test, MegaFLUO LEISH® results and MegaELISA LEISH® results. The results were quantified as ELISA UNIT (EU) compared to a positive control serum sample used as a calibrator that was arbitrarily set to 100 EU. The cutoff value was set to 30 EU (mean+4 standard deviations of values from 70 apparently healthy dogs from a non-endemic area and that were not included in this study). Sera with an EU ≥ 200 were classified as high positive, with an EU ≥ 100 and < 200 as moderate positive, and with an EU > 30 and < 100 as low positive.

MegaFLUO LEISH®

The commercial IFAT test (MegaFLUO LEISH®, MEGACOR Diagnostik GmbH, Hörbranz, Austria) was performed as described in the instructions supplied with the test kit. Slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400× magnification and each well was compared to the fluorescence pattern observed in the positive and negative controls. All samples were examined by two different investigators. If discrepancies arose between results, a third observer participated. They were blinded to the results of the other serological tests.

MegaELISA LEISH®

The MegaELISA LEISH® test (MEGACOR Diagnostik GmbH, Hörbranz, Austria) is a quantitative indirect ELISA for the detection of anti-*Leishmania infantum* antibodies in the canine serum sample following instructions supplied with the test kit. The MegaELISA LEISH® test was performed by a different researcher without knowledge of the *FAST*est LEISH® test, the commercial MegaFLUO LEISH® and the ELISA UNIZAR results.

*FAST*est LEISH®

The ICT *FAST*est LEISH® test (MEGACOR Diagnostik GmbH, Hörbranz, Austria) is a qualitative serological test developed to detect canine antibodies against *L. infantum* based on immunochromatographic technology. All tests were stored at room temperature and were performed as described in the instructions supplied with the test kit. The examiner was blinded to the results of the quantitative serological tests.

Statistical analysis

The Bayesian version (Branscum et al., 2005) of the LCA introduced by Hui & Walter (1980) was adopted for evaluate the accuracy of the four diagnostic tests, given the absence of a gold standard. A four tests - two populations model was built in order to estimate the Se and Sp of each diagnostic test. The model assumptions in the Hui & Walter (1980) version were (1) the different true prevalences of the two populations, (2) the Se and Sp of the tests were constant across subpopulations and (3) the tests were conditionally independent given the true infection status. Accounting for the first assumption, the model was run using endemic versus non-endemic area as subgroups. Additionally, different hypotheses about the dependence among tests were considered in the model building: (Toft et al., 2005) the conditional independence among all the tests, given the infection status (i.e. presence of antibodies against *L. infantum*), MOD 1 in Table 1; the conditional covariance between the ELISA UNIZAR and MegaELISA LEISH® tests and conditional independence among *FAST*est LEISH® and MegaFLUO LEISH® compared to ELISA UNIZAR and MegaELISA LEISH®, given the disease status (MOD 2). For both models, uninformative priors for the test accuracy and prevalences were used. All analyses were carried out using Markov chain Monte Carlo techniques and implemented in WinBUGS software. Posterior inferences were based on 50,000 iterations, after a burn-in of 10,000 iterations. Convergence was assessed by running multiple chains from dispersed starting values, observing autocorrelation among samplings and investigating the Brooks–Gelman–Rubin convergence statistic (Brooks & Gelman, 1998). The Deviance Information Criterion (DIC) was used as measure of the model fitting (smaller value is better). The median of the posterior distributions was used as an estimate for the parameters of interest; the 2.5 and 97.5% points were used as estimates of the 95% credibility intervals (95% Posterior credibility interval, 95% PCI). To test Se and Sp between tests, the Bayesian posterior probabilities (POPR) were calculated and used as hypothesis testing, like in traditional statistical methods.

Table 1. Deviance Information Criterion (DIC), posterior median and 95% Posterior credibility interval (95% PCI) of population specific-prevalence (Prev), sensitivity (Se), specificity (Sp) and conditional covariances (covSe, covSp), assuming conditional independence among the four tests (MOD 1), conditional covariance between ELISA UNIZAR and MegaELISA LEISH® (MOD 2).

DIC	MOD 1		MOD 2	
	50.01	95% PCI	51.6	95% PCI
	median		median	
Prev endemic area	55.85	[48.87;62.70]	55.87	[48.84;62.75]
Prev non-endemic area	3.25	[0.12;16.12]	3.24	[0.12;16.08]
Se ELISA UNIZAR	99.37	[96.75;99.98]	99.31	[96.34;99.97]
Se MegaFLUO LEISH®	99.36	[96.67;99.98]	98.14	[94.50;99.70]
Se *FAST*est LEISH®	99.38	[96.73;99.98]	99.36	[96.67;99.98]
Se MegaELISA LEISH®	98.49	[95.06;99.79]	99.36	[96.69;99.98]
Sp ELISA UNIZAR	97.50	[93.38;99.42]	97.51	[93.33;99.42]
Sp MegaFLUO LEISH®	91.94	[85.79;96.06]	91.33	[85.05;95.67]
Sp *FAST*est LEISH®	98.43	[94.87;99.77]	98.45	[94.92;99.78]
Sp MegaELISA LEISH®	91.93	[85.84;96.06]	91.93	[85.79;96.10]
covSe			0.002	[0.000;0.020]
covSp			0.002	[-0.003;0.022]

Agreement between the results for all serological diagnostic techniques was determined using kappa statistic (measure of agreement between categorical variables), carried out with the SPSS software Version 22 (IBM Inc., Chicago, IL, USA). This parameter was determined as follows: no agreement ($k < 0$), slight agreement ($0 < k < 0.2$), fair agreement ($0.2 < k < 0.4$), moderate agreement ($0.4 < k < 0.6$), substantial agreement ($0.6 < k < 0.8$) and almost perfect agreement ($k > 0.8$).

Results

The cross-tabulated counts of the raw test results are reported in the Table 2 for endemic area and non-endemic area including one of the 16 different test patterns (e.g. combination of results of the four serological tests detected in each group of dogs). 195 samples out of 215 (90.7%) showed concordant results for all tests included.

Table 2. Combination of results of the four serological tests detected in each group of dogs considering endemic and non-endemic areas.

Serological technique				Endemic area (n=195)	Non-endemic (n=20)
ELISA UNIZAR	MegaFLUO LEISH®	*FAST*est LEISH®	MegaELISA LEISH®		
+	+	+	+	108	0
+	+	+	-	1	0
+	+	-	+	0	0
+	-	+	+	0	0
+	+	-	-	0	0
+	-	-	+	0	0
+	-	-	-	2	0
-	+	+	+	0	0
-	-	+	+	0	0
-	+	-	+	0	0
-	+	-	-	8	0
-	+	+	-	0	0
-	-	-	+	8	0
-	-	+	-	1	0
-	-	-	-	67	20

Abbreviations: - negative, + *positive*.

The posterior estimates (median and 95% PCI) are presented in Table 1. Looking at Deviance Information Criterion (DIC) (smaller is better), it seems that the model with conditional independence among all tests (MOD 1) would be the preferable one between the two models under evaluation. However, there were no detectable differences in posterior estimates from the two models. The results highlighted a very low prevalence for non-endemic area (3.25%) compared to the medium/high in the endemic area (55.85%). All tests showed high values of Se, near to 100% for *FAST*est LEISH® (99.38%), ELISA UNIZAR (99.37%) and MegaFLUO LEISH® (99.36%), followed by MegaELISA LEISH® (98.49%). With regard to the Sp, *FAST*est LEISH® (98.43%) and ELISA UNIZAR (97.50%) showed significantly better performances (POPR < 0.05) compared to MegaFLUO LEISH® (91.94%) and MegaELISA LEISH® (91.93%).

Agreement between tests was almost perfect (k>0.80) for the all combination techniques from a maximum k value (k=0.972) obtained between *FAST*est LEISH® and ELISA UNIZAR and the minimum k value (k=0.841) obtained between MegaELISA LEISH® and MegaFLUO LEISH®, being the global results included in Table 3.

Table 3. Agreement between serological techniques included in the study including Kappa statistic, Standard Error and 95% confidence intervals (CI).

Agreement between techniques	Kappa statistic	Standard Error	95% CI	Result
*FAST*est LEISH®-ELISA UNIZAR	0.972	0.016	(0.941-1.000)	Almost perfect agreement
*FAST*est LEISH®- MegaFLUO LEISH®	0.916	0.027	(0.862-0.970)	Almost perfect agreement
MegaFLUO LEISH® – ELISA UNIZAR	0.907	0.029	(0.850-0.963)	Almost perfect agreement
MegaELISA LEISH® - *FAST*est LEISH®	0.907	0.029	(0.850-0.963)	Almost perfect agreement
MegaELISA LEISH® - ELISA UNIZAR	0.897	0.030	(0.838-0.956)	Almost perfect agreement
MegaELISA LEISH® – MegaFLUO LEISH®	0.841	0.037	(0.768-0.913)	Almost perfect agreement

Concerning the ability of each test to detect anti-*Leishmania infantum* antibody (immunoglobulin G) depending on the dog population, some differences were observed among the group of dogs from endemic areas, especially if seemingly healthy (see Materials and Methods). In the case of seronegative healthy dogs, one sample (1/65, 1.5%) was classified as seropositive by *FAST*est LEISH®, 6 samples (6/65, 9.2%) were classified as seropositive by MegaFLUO LEISH® (antibody titer ranging from 1:100 (n=5) to 1:200 (n=1)) and finally MegaELISA LEISH® classified 7 samples (7/65, 10.8%) as seropositive and 4 samples (4/65, 6.2%) as inconclusive result. For samples included in clinically-ill infected dogs, all commercial tests analyzed classified all samples as seropositive: antibody titers ranging from 1:800 to 1:102,400 by MegaFLUO LEISH® and in the case of MegaELISA LEISH® from 12 to 84 Megacor EU. In the case of samples from seropositive asymptomatic infected dogs, two samples (2/24, 8.3%) (90 and 56 EU) were classified as seronegative by the three commercial tests: MegaFLUO LEISH® (antibody titer 1:20), *FAST*est LEISH® and MegaELISA LEISH® (1 and 4 Megacor EU). The MegaELISA LEISH® also classified a third sample (1/24, 4.2%) (90 EU by ELISA UNIZAR) as inconclusive. In non-endemic samples antibodies to *Leishmania* were not detected by any of the three commercial tests. Finally, in the group of dogs with a serological positive result to other pathogens, only *FAST*est LEISH® was totally specific and no positive results were detected. In contrast, MegaFLUO LEISH® test gave a positive result in two samples (2/19, 10.5%) for *A. platys* (antibody titer of 1:20 and 1:40), MegaELISA LEISH® test gave positive result in one sample (1/19, 5.3%) for *D. immitis* and an inconclusive result in other 6 samples (6/19, 31.6%), including 4 samples (4/19, 21.1%) for *A. platys* (antibody titer of 1:20 (n=1), 1:80 (n=2), 1:160 (n=1)), 1 sample for *E. canis* (antibody titer of 1:40) and 1 sample with two co-infections *T. gondii* (IFAT antibody titer of 1:160) and *N. caninum* (IFAT antibody titer of 1:100).

Discussion

The absence of a gold standard technique is a recurrent situation in clinical practice and diagnostic research studies including confirmatory techniques for *L. infantum* infection in dogs (Rodríguez-Cortés et al., 2010), cats (Persichetti et al., 2017), ferrets (Giner et al., 2020) and humans (Galluzzi et al., 2018). Although it is usual, the application of two or more tests combination based on different principles such as molecular, serological or other parasitological techniques when there is no reference standard. Nevertheless, classical validation is based on the use of a reference test such as IFAT technique to evaluate the other index test (OIE, 2016). The estimates of Se and Sp of the index test are biased because the reference test could have high Se and high Sp but not necessarily equal to 100%. This latter bias could be resolved using statistical validation based on latent class models in a Bayesian analysis. Despite the large number of articles focused on confirmatory techniques validation for diagnosis of leishmaniasis, very few studies using a Bayesian statistical approach have been published in dogs (Adel et al., 2016), cats (Persichetti et al., 2017) and other animals such as lagomorphs (De la Cruz et al., 2016).

In the study, Se value was equal for MegaFLUO LEISH®, *FAST*est LEISH® and the ELISA UNIZAR, obtaining a Se approximately equal to 99.40%, whilst MegaELISA LEISH® was the commercial test with less sensitivity in comparison to the others but its sensitivity was high (98.49%). In contrast, the highest Sp was obtained by the *FAST*est LEISH® (98.43%), in comparison to the quantitative tests: ELISA UNIZAR (97.50%), followed by MegaELISA LEISH® and MegaFLUO LEISH® (91.9%).

Various confirmatory methods for evaluating *L. infantum* infection exist, but their performances differ significantly. *In vitro* parasite cultures are laborious, require special facilities, and are limited to research. In contrast, histology, immunohistochemistry, molecular methods and quantitative serological methods are frequently applied in clinical practice, but samples often need to be sent to a specialized laboratory to perform these techniques (Miró et al., 2008).

The group of dogs coming from the endemic area was heterogeneously composed by clinically-ill dogs and apparently healthy dogs, as it is well known that the diagnosis in clinically-ill subjects is easy to obtain with perfect performances by means of any diagnostic test. Among the seropositive asymptomatic infected dogs, some had a previous serological diagnosis of positivity for *Leishmania*, some other did not. In any case, the classification into subgroups did not have any weight in the Bayesian evaluation. The apparently healthy seropositive dogs and clinically-ill infected dogs had different antibody levels ranging from 30 to 372 EU, according to the ELISA UNIZAR and in the case of MegaELISA LEISH® antibody levels ranging from 1 to 84 MEGACOR EU. In both ELISAs the antigen used is sonicated promastigote protein. However, small differences between the same serological ELISA technique were detected between ELISA UNIZAR and MegaELISA LEISH®. Conversely, antibody titers detected by MegaFLUO LEISH® ranged from 1:20 to > 1:12800. The main differences between the ELISA and IFAT techniques are the type of antigen used and the technical method performed to obtain the results. For IFAT, the entire parasite is present

on the slide, and the evaluation is performed with a fluorescence microscope. In contrast, the ELISA technique uses different types of antigens, and the results are obtained by measuring the absorbance with an ELISA plate reader. In terms of interpreting the results, IFAT is subjective and depends on the operator's experience, even when two different experienced observers examine the samples. Intrinsic analytical variability of each quantitative serological technique may explain the differences between results obtained in the present study. In comparison to other studies where ELISA techniques showed better performances compared with in-house IFAT technique, in our case the Se and Sp of the MegaFLUO LEISH®, were similar to the other tests analyzed in the present study.

In-clinic tests, cytological examinations and rapid serological tests are the most rapid and cheap methods for detecting *Leishmania* infection in dogs. Microscopic examinations of samples, including bone marrow, lymph nodes, skin lesions and body fluids, have been used. Bone marrow and lymph nodes are considered the best samples to confirm an infection, but microscopy may not be as sensitive and is also time consuming due to the low numbers and randomly distributed parasites (Saridomichelakis et al., 2005; Moreira et al., 2007).

In Europe, there are a limited number of commercially available ICTs for CanL with diagnostic performance published such as SNAP® Canine *Leishmania* Antibody Test, Speed Leish K®, INGEZIM® LEISHMACROM and WITNESS® *Leishmania* (Rodríguez-Cortés et al., 2010). Diagnostic performance of ICTs shows high specificity and variable sensitivity from low to moderate degree depending on the test evaluated. In our study, the Sp of *FAST*est LEISH® was slightly lower (98.4%) compared to the Sp (100.0%) of other ICTs. On the other hand, the Se of *FAST*est LEISH® was clearly higher in comparison to other tests such as WITNESS® *Leishmania* with a low Se (58.0%) or INGEZIM® LEISHMACROM with a moderate Se (75.0%). Nevertheless, the absence of a common framework makes a correct comparison difficult in relation to the standardization of performance between ICTs.

When a test is available to use in a clinical setting, scientific information that is not obtained from the manufacturer should be collected. A recent study provided additional information about diagnostic measures of other rapid tests that was not included in the technical details (Solano-Gallego et al., 2014). The study (Solano-Gallego et al., 2014) showed important variations in the Se parameter between the technical set up and the rapid test results. A Se of 63.6% was obtained for the Speed Leish K® test, which was not consistent with the higher Se indicated in the manufacturer's instructions. However, the test performance of *FAST*est LEISH® obtained in the present study was similar to previously reported diagnostic performance results supplied with the kit.

Differences in diagnostic performance are described between quantitative serological tests and ICTs evaluated simultaneously with a better performance in favour of quantitative serological tests. In this study, Se and Sp were similar between *FAST*est LEISH® and the ELISA UNIZAR used but this situation was not similar in comparison to the Sp value detected between *FAST*est LEISH® (0.984) and the two quantitative commercial tests (0.919). This *FAST*est LEISH® may be a valid alternative in the absence of a quantitative test as a screening test.

One of the great difficulties in the evaluation of diagnostic tests is the lack of standardization of the clinical classification of the animal with respect to *L. infantum* infection. Different classifications and stages of disease have been proposed in CanL. However, there are notable differences when it comes to how a dog is classified following one or another clinical classification. This added difficulty still complicates the definition of sample selection and the possible bias can be established (Meléndez-Lazo et al., 2018). In the present study the Bayesian method, based on groups selected only on the basis of endemic/not endemic area, avoided this possible bias.

Sample selection should be included representative and different stage of infection status regarding *Leishmania* infection that can be found when serological tests are evaluated for detecting *L. infantum* infection. In our study, all three serological tests discriminate samples provided from clinically-ill infected dogs and samples from non-endemic areas. The main problem from the point of view of the application of serological techniques in a clinical context is the Se of the serology is lowest early in *Leishmania* infection but high with progressive infection, and this situation is not always included in the evaluation of serological techniques making it crucial that this type of samples should be tested. In our study, for a better characterization of this type of dog, an additional confirmatory technique including a quantitative PCR in blood was performed. Although blood is not the best samples selection for detecting parasitic DNA in comparison to bone marrow or lymph node samples, the procedure to obtain these two different samples is painful and difficult to be accepted by the owner when the dog is apparently healthy without clinical signs and laboratory alterations.

A potential limitation of similar studies could be associated with the evaluation of the cross reactivity phenomenon that it is described commonly in serological techniques (Krawczak et al., 2015). We have evaluated an entire gamma of serum samples with a serological positive result to most frequent pathogens present in our geographical area. The inclusion of serum samples positive to other type of *Leishmania* species or *Trypanosoma cruzi* would be desirable

but our region like other regions of the European Mediterranean basin, *L. infantum* is the common parasite and these geographical regions are free of *T. cruzi*. The analysis of serum samples from different regions where other pathogens are present can confirm the potential use of these commercial in other regions aside from Europe. In any case, as above clarified, in this study the Bayesian approach overtakes the meaning of the real status of every single sample, having the only goal to estimate the performances of the tests.

Conclusions

Latent class analysis provides a useable alternative to traditional test evaluations for serological tests for *L. infantum* infection in dogs, overtaking the needing of a gold standard test or samples with a known serological status. Using LCA we have estimated the sensitivity and specificity of the three different serological tests used in the European Mediterranean area to detect anti-*Leishmania* antibodies. The results of present study indicate that the immunochromatographic rapid test evaluated *FAST*est LEISH® shows similar levels of sensitivity and specificity to the quantitative commercial tests included.

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References

Adel A, Berkvens D, Abatih E, Soukehal A, Bianchini J, Saegerman C. Evaluation of immunofluorescence antibody test used for the diagnosis of canine leishmaniasis in the Mediterranean Basin: a systematic review and meta-analysis. *PLoS One* 2016; 11(8): e0161051. http://dx.doi.org/10.1371/journal.pone.0161051. PMid:27537405.

Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis: new concepts and insights on an expanding zoonosis: part one. *Trends Parasitol* 2008; 24(7): 324-330. http://dx.doi.org/10.1016/j.pt.2008.04.001. PMid:18514028.

Barbiéri CL. Immunology of canine leishmaniasis. *Parasite Immunol* 2006; 28(7): 329-337. http://dx.doi.org/10.1111/j.1365-3024.2006.00840.x. PMid:16842269.

Bourdeau P, Saridomichelakis MN, Oliveira A, Oliva G, Kotnik T, Gálvez R, et al. Management of canine leishmaniosis in endemic SW European regions: a questionnaire-based multinational survey. *Parasit Vectors* 2014; 7(1): 110. http://dx.doi.org/10.1186/1756-3305-7-110. PMid:24656172.

Branscum AJ, Gardner IA, Johnson WO. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. *Prev Vet Med* 2005; 68(2-4): 145-163. http://dx.doi.org/10.1016/j.prevetmed.2004.12.005. PMid:15820113.

Brooks SP, Gelman A. General methods for monitoring convergence of iterative simulations. *J Comput Graph Stat* 1998; 7(4): 434-455.

Dantas-Torres F. The role of dogs as reservoirs of *Leishmania* parasites, with emphasis on *Leishmania (Leishmania) infantum* and *Leishmania (Viannia) braziliensis. Vet Parasitol* 2007; 149(3-4): 139-146. http://dx.doi.org/10.1016/j.vetpar.2007.07.007. PMid:17703890.

De la Cruz ML, Pérez A, Domínguez M, Moreno I, García N, Martínez I, et al. Assessment of the sensitivity and specificity of serological (IFAT) and molecular (direct-PCR) techniques for diagnosis of leishmaniasis in lagomorphs using a Bayesian approach. *Vet Med Sci* 2016; 2(3): 211-220. http://dx.doi.org/10.1002/vms3.37. PMid:29067196.

Esch KJ, Juelsgaard R, Martinez PA, Jones DE, Petersen CA. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. *J Immunol* 2013; 191(11): 5542-5550. http://dx.doi.org/10.4049/jimmunol.1301810. PMid:24154626.

Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. Real-time PCR applications for diagnosis of leishmaniasis. *Parasit Vectors* 2018; 11(1): 273. http://dx.doi.org/10.1186/s13071-018-2859-8. PMid:29716641.

Giner J, Basurco A, Alcover MM, Riera C, Fisa R, López RA, et al. First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain. *Vet Parasitol Reg Stud Rep* 2020; 19: 100369. http://dx.doi.org/10.1016/j.vprsr.2020.100369. PMid:32057396.

Hosein S, Blake DP, Solano-Gallego L. Insights on adaptive and innate immunity in canine leishmaniosis. *Parasitology* 2017; 144(1): 95-115. http://dx.doi.org/10.1017/S003118201600055X. PMid:27094260.

Hui SL, Walter SD. Estimating the error rates of diagnostic tests. *Biometrics* 1980; 36(1): 167-171. http://dx.doi.org/10.2307/2530508. PMid:7370371.

Krawczak FS, Reis IA, Silveira JA, Avelar DM, Marcelino AP, Werneck GL, et al. *Leishmania, Babesia* and *Ehrlichia* in urban pet dogs: co-infection or cross-reaction in serological methods? *Rev Soc Bras Med Trop* 2015; 48(1): 64-68. http://dx.doi.org/10.1590/0037-8682-0291-2014. PMid:25860466.

Maia C, Campino L. Biomarkers associated with *Leishmania infantum* exposure, infection, and disease in dogs. *Front Cell Infect Microbiol* 2018; 8: 302. http://dx.doi.org/10.3389/fcimb.2018.00302. PMid:30237985.

Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. *Vet Parasitol* 2008; 158(4): 274-287. http://dx.doi.org/10.1016/j.vetpar.2008.07.028. PMid:18789583.

Meléndez-Lazo A, Ordeix L, Planellas M, Pastor J, Solano-Gallego L. Clinicopathological findings in sick dogs naturally infected with *Leishmania infantum*: comparison of five different clinical classification systems. *Res Vet Sci* 2018; 117: 18-27. http://dx.doi.org/10.1016/j.rvsc.2017.10.011. PMid:29153900.

Miró G, Cardoso L, Pennisi MG, Oliva G, Baneth G. Canine leishmaniosis-new concepts and insights on an expanding zoonosis: part two. *Trends Parasitol* 2008; 24(8): 371-377. http://dx.doi.org/10.1016/j.pt.2008.05.003. PMid:18603476.

Moreira MA, Luvizotto MC, Garcia JF, Corbett CE, Laurenti MD. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. *Vet Parasitol* 2007; 145(3-4): 245-252. http://dx.doi.org/10.1016/j.vetpar.2006.12.012. PMid:17257764.

Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. *Trends Parasitol* 2002; 18(9): 399-405. http://dx.doi.org/10.1016/S1471-4922(02)02347-4. PMid:12377257.

Persichetti MF, Solano-Gallego L, Vullo A, Masucci M, Marty P, Delaunay P, et al. Diagnostic performance of ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum* antibodies in cats using a Bayesian analysis without a gold standard. *Parasit Vectors* 2017; 10(1): 119. http://dx.doi.org/10.1186/s13071-017-2046-3. PMid:28285598.

Rodríguez-Cortés A, López-Fuertes L, Timón M, Alberola J, Ojeda A, Francino O. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard". *Am J Trop Med Hyg* 2010; 82(2): 251-256. http://dx.doi.org/10.4269/ajtmh.2010.09-0366. PMid:20134001.

Saridomichelakis MN, Koutinas AF, Billinis C, Kontos V, Mylonakis M, Leontides LS. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. *Am J Trop Med Hyg* 2005; 73(1): 82-86. http://dx.doi.org/10.4269/ajtmh.2005.73.82. PMid:16014839.

Solano-Gallego L, Cardoso L, Pennisi MG, Petersen C, Bourdeau P, Oliva G, et al. Diagnostic challenges in the era of canine *Leishmania infantum* vaccines. *Trends Parasitol* 2017; 33(9): 706-717. http://dx.doi.org/10.1016/j.pt.2017.06.004. PMid:28689776.

Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, et al. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Vet Parasitol* 2009; 165(1-2): 1-18. http://dx.doi.org/10.1016/j.vetpar.2009.05.022. PMid:19559536.

Solano-Gallego L, Villanueva-Saz S, Carbonell M, Trotta M, Furlanello T, Natale A. Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan®, ID Screen® and *Leishmania* 96®), a rapid test (Speed Leish K®) and an in-house IFAT. *Parasit Vectors* 2014; 7(1): 111. http://dx.doi.org/10.1186/1756-3305-7-111. PMid:24655335.

Toft N, Jørgensen E, Højsgaard S. Diagnosing diagnostic tests: evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. *Prev Vet Med* 2005; 68(1): 19-33. http://dx.doi.org/10.1016/j.prevetmed.2005.01.006. PMid:15795013.

World Organisation for Animal Health – OIE. *Leishmaniosis*. In: World Organisation for Animal Health – OIE, *Manual of diagnostic tests and vaccines for terrestrial animals* [online]. Paris: OIE; 2016. chap. 3.1.11, p. 1-12. Available from: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.11_Leishmaniosis.pdf

ARTÍCULO 3

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Short Communication

First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain

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ABSTRACT

A pet domestic ferret (*Mustela putorius furo*) with a papular lesion involving the right pinna was diagnosed with chronic pyogranulomatous dermatitis by histopathologic examination. Intralesional, intracytoplasmic oval microorganisms compatible with *Leishmania* spp. or *Histoplasma* spp. were observed in macrophages and multinucleate giant cells. *Leishmania infantum* (*L. infantum*) infection was diagnosed by PCR, culture in Novy-MacNeal-Nicolle medium, and immunohistochemistry. Abnormal clinicopathological results included increased alanine transferase, alkaline phosphatase, serum gamma glutamyl transferase and polyclonal gammpathy. Anti-*Leishmania* antibodies were detected by enzyme-linked immunosorbent assay, immunofluorescence antibody test and western blot using *L. infantum* antigen. Immunoreactivity against the 16 kDa specific *L. infantum* antigen fraction was observed by western blot. PCR performed in blood samples obtained from this patient after positive parasite isolation detected *L. infantum* DNA. To the authors' knowledge, this is the first diagnosis and isolation of *L. infantum* in a domestic ferret naturally infected in an endemic region (Spain) where canine and feline leishmaniosis is frequently detected. According to these findings, ferrets should be included as potential reservoir hosts of *L. infantum*. Future investigations should analyze the epidemiological role of ferrets in *L. infantum* infection including the prevalence of infection.

1. Introduction

Leishmaniasis currently ranks second only to malaria in the World Health Organization (WHO) list of protozoan diseases that cause the highest human mortality. It is estimated that approximately 350 million people are under the risk of leishmaniasis infection and 12 million are infected (Alvar et al., 2012). In southern Europe, leishmaniasis is a zoonotic protozoan disease caused by *Leishmania infantum*. This parasite is transmitted by phlebotomine sand flies under natural conditions during feeding. Although various phlebotomine species have been implicated in the transmission of *L. infantum* in Europe, only two species are the most important vector of canine leishmaniosis in the Iberian Peninsula: *Phlebotomus perniciosus* and *Phlebotomus ariasi* (Lucientes et al., 2005). Female *Phlebotomus* feed on a variety of vertebrate hosts, including humans, livestock species, dogs, wild rabbits, hares, rodents and cats.

Dogs are considered the main reservoir host for *L. infantum* but other animals can act as active reservoirs such as cats (Pennisi et al., 2015) and rabbits (García et al., 2014). The identification of other domestic animals that can act as possible reservoir hosts for the parasite could have a significant impact on public health. Equally, from a veterinary point of view, the identification of this disease in a new animal

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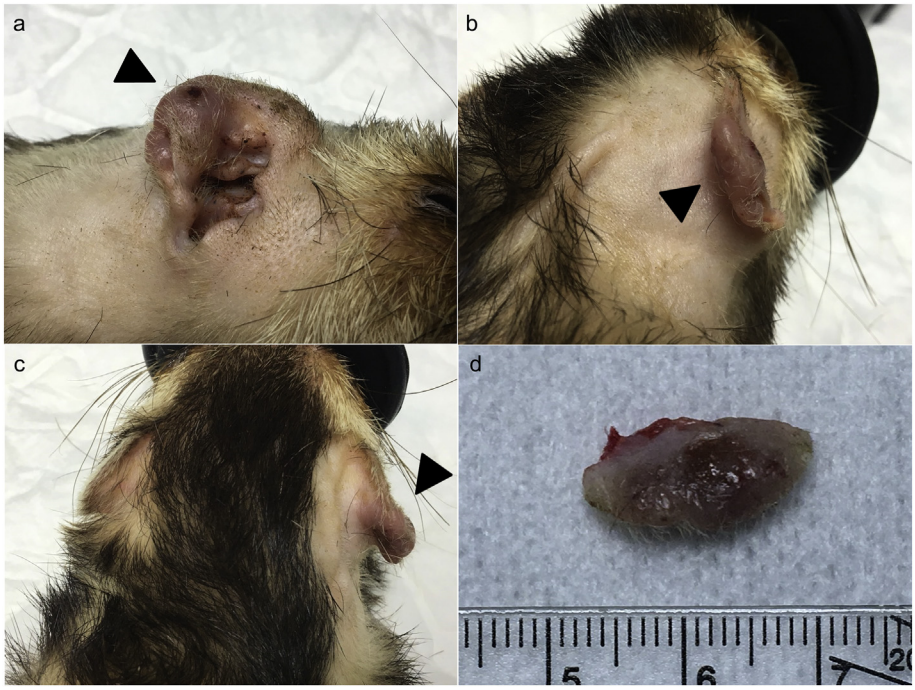

Fig. 1. Different views of dermatologic lesions detected in this ferret (a, b, c) and full thickness biopsy performed from the ear pinna (d).
a, b, c: Erythematous and oedematous papular lesion on the right ear pinna (arrowheads). A superficial ulcer is present on the tip of the ear pinna (a, b).

species is deemed essential.

The potential role of wildlife species as reservoirs of the parasite should be taken into account as well. Among mustelids, *L. infantum* infection has been identified by PCR in liver and/or spleen tissue samples from a polecat (*Mustela putorius*), a European mink (*Mustela lutreola*) and other wild mustelids (*Meles meles, Martes foina* and *Martes martes*) in the northern Iberian Peninsula (Spain) (Del Río et al., 2014). None of the animals showed typical canine leishmaniosis lesions at post-mortem examination.

Although no official ferret population data are available in Europe (d'Ovidio et al., 2014), the domestic ferret (*Mustela putorius furo*) is nowadays a common household pet. This study describes for the first time naturally-occurring *L. infantum* infection in a ferret.

2. Case report

A 4-year-old intact female ferret from Valencia, in the east coast of Spain, was clinically evaluated in February 2019 because of the presence of a non-pruritic dermal lesion in the right pinna (Fig. 1). This ferret was adopted at the age of two years with unknown previous history. At the time of presentation, it lived in an apartment with other ferrets and had access to an outdoor terrace. It was under chronic medical management with prednisolone and cyclosporine A because of inflammatory bowel disease diagnosed one year earlier. This patient was also diagnosed with suppurative cholangitis six months prior to dermatologic presentation.

On physical examination, this ferret was in good condition, active and alert, normothermic and properly hydrated. Cardiac auscultation was within normal limits. Respiratory sounds were also normal and there was no evidence of lymph node enlargement. The fur over the back was coarse. An erythematous, edematous and non-painful papular lesion 5 mm in diameter was observed in the right ear pinna. Cytological evaluation from a sample taken by needle aspiration and stained with Diff-Quick revealed pyogranulomatous dermatitis; no infectious agents were visualized. A full thickness biopsy from the ear pinna was obtained.

2.1. Histopathology and immunohistochemistry specific against L. infantum

The skin biopsy sample was fixed in 10% formalin and embedded in paraffin. Four µm-thick sections were stained with hematoxylin and eosin. Severe chronic diffuse pyogranulomatous dermatitis extending diffusely from the superficial dermis to the subcutis was noted. Inflammation was characterized by dense infiltrates of macrophages, few neutrophils, low numbers of lymphocytes and plasma cells, and rare multinucleate giant cells. The auricular cartilage was unaffected. This lesion was accompanied by areas of necrosis in the dermis and proteinaceous edema and serocellular crusts. Macrophages and multinucleate giant cells contained large numbers of oval organisms with an eccentric nucleus and pale cytoplasm, measuring approximately 3 to 4 µm in diameter, compatible with *Leishmania* spp. amastigotes or *Histoplasma* spp. yeasts were observed (Fig. 2). To determine the presence of *Leishmania* parasites in tissue section, immunohistochemistry was performed using a standard protocol with an Autostainer Link48™ (Dako, Glostrup, Denmark) and an in-house rabbit polyclonal antibody specific for *L. infantum*. Blocking of endogenous peroxidase activity (Dako REAL™ Peroxidase-Blocking Solution, Dako, Glostrup, Denmark) was performed before sections were incubated for 30 min with the primary antiserum at room temperature (RT) (1 in 500 dilution in EnVision Flex™ antibody diluent, Dako). Thereafter, sections were incubated for 30 min at RT with Dako EnVision+ System-HRP,Rb™. The substrate used for detection was 3.3′-diaminobenzidine incubated for 10 min. Sections were then counterstained with hematoxylin for 8 min (EnVision™ FLEX Hematoxylin, Dako) and covered in slides. For the negative control, the primary antibody was replaced with non-immune rabbit serum. A biopsy sample of the lymph node from a dog with clinical leishmaniosis was included as a positive control. The abundant oval microorganisms present within the cytoplasm of dermal macrophages stained positively (brown) (Fig. 3).

2.2. PCR

The presence of *Leishmania* spp. DNA in paraffin embedded skin biopsy was additionally evaluated by amplification of kinetoplast DNA

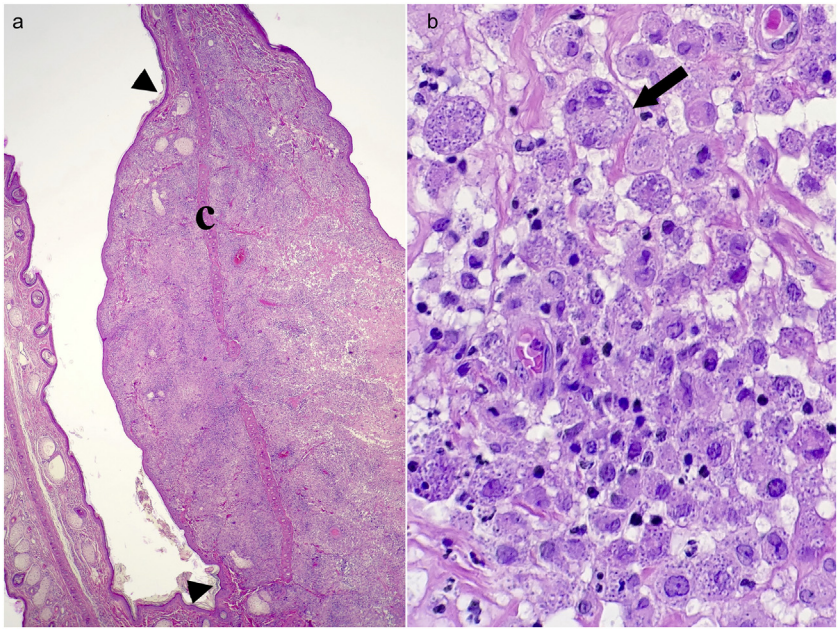

Fig. 2. Histological sections of affected tissues with leishmaniosis in a ferret.
a: Note a papular thickening of the ear (delimited with arrowheads) due to granulomatous inflammation. c = auricular cartilage.
b: A higher magnification of this inflammatory lesion reveals infiltrates of macrophages and low numbers of lymphocytes as a well as a multinucleate giant cell (arrow). The cytoplasm of macrophages and this giant cell are ladden with oval microorganisms with an eccentric nucleus. Hematoxylin and eosin.

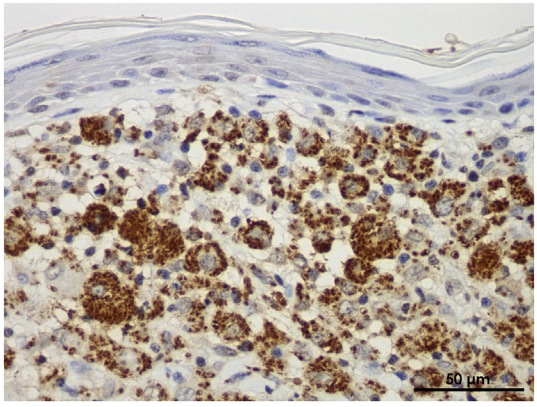

Fig. 3. Immunohistochemical staining labelling of *Leishmania* spp. amastigotes. Note the amastigote forms labelled in brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequence using a quantitative polymerase chain reaction (qPCR). Each amplification was performed in triplicate, in 10 µL reaction, 15 pmol of direct primer (5′-CTT TTC TGG TCC TCC GGG TAG G-3′), 15 pmol of reverse primer (5′-CCA CCC GGC CCT ATT TTA CAC CAA-3′), 50 pmol of the labelled TaqMan probe (FAM-TTT TCG CAG AAC GCC CCT ACC CGC-TAMRA) and 2.5 µL of sample DNA. Amplification and detection were performed in the ABI Prism 7900 system (Applied Biosystems, Foster City, CA, USA.) in a two-step temperature process (94 and 55 °C) for 45 cycles. Positive controls (DNA from *L. infantum* MHOM /ES /04 /BCN-61) and negative controls were included in each RT-PCR analysis (Martín-Ezquerra et al., 2009). A positive result by PCR was obtained from the paraffin block. Additional diagnostic procedures included detection of parasite DNA by PCR from peripheral blood sample and Whatman filter paper number 3 with aspirated material from the perilesional excised area, serology and parasite isolation. Concerning the blood sample and Whatman filter paper results, *Leishmania* spp. DNA was detected in both samples.

2.3. Serology

Anti-*Leishmania* antibodies were detected by western blot (WB) and enzyme-linked immunosorbent assay (ELISA) using sonicated *L. infantum* antigens (MHOM/FR/78/LEM75 zymodeme MON-1). WB was performed as described by Riera et al. (1999), with some modifications. It was done on 0.1% SDS–13% polyacrylamide gel on a Mini-gel Bio RadSystem. Sera diluted at 1/50 were assayed and a protein A peroxidase conjugate (1/1000 dilution; Pierce) was used. A serum positive was considered when immunoreactivity against the 14 and/or16 kDa *Leishmania* antigen fraction was observed. ELISA was performed as described by Riera et al. (1999). Sera were diluted at 1/50 and a protein A peroxidase conjugate (dilution, 1/8000; Pierce) was used. The cutoff was set to 0.137 optical density units (OD) (mean + 4 standard deviations of values from 8 ferrets with a negative result for *Leishmania* real-time PCR in blood). Each test included serum from a *L. infantum* confirmed symptomatic dog from Spain with a *L. infantum* isolation as positive control and serum from a healthy, non-infected dog as negative control because the protein A peroxidase conjugate has been employed in canine samples. Immunoreactivity from the *Leishmania* antigen was detected by WB (band of 16 kDa) and specific antibodies against *L. infantum* were detected by ELISA. Medium levels of antibodies were determined with an OD result of 0.599.

Later, other quantitative serological tests were performed including an in-house immunofluorescence antibody test (IFAT), for detecting *L. infantum* immunoglobulin G antibodies using *L. infantum* (strain MHOM/FR/78/LEM75 zymodeme MON-1) as whole-parasite antigen fixed on multi-spot slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All samples were diluted 1:20 with phosphate-buffered saline (PBS), and the endpoint titer of positive samples was determined by preparing serial twofold dilutions of the serum starting from the cutoff value. Immediately thereafter, 20 µL of every diluted serum sample were applied per well. The slides were incubated for 30 min at 37 °C in a moist chamber. Slides were then washed twice with PBS for 5 min and once with distilled water. Following the washing procedure, 20 µL of fluorescein-conjugated goat anti-ferret immunoglobulin G (Abcam, Cambridge, United Kingdom) diluted 1:16 in Evans blue solution were added to each well. The slides were incubated in a moist chamber at 37 °C for an additional 30 min in the dark. The washing procedure was repeated, and several drops of mounting medium were then added to the cover slips. Slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400 × magnification and each well was compared to the fluorescent pattern observed in the positive

and negative controls. The samples were examined by two different researchers. The cutoff for this technique was set to 1:20 dilution by analyzing 8 serum samples from ferrets with a negative result for *Leishmania* real-time PCR in blood. The same procedure was repeated with the modification of fluorescein-conjugated goat anti-dog immunoglobulin G (Abcam, Cambridge, United Kingdom). Due to the lack of ferret positive reference serum samples, the ferret IFAT was determined by carrying out a parallel canine test run, using positive serum control (a dog from Spain with *L. infantum* confirmed with positive *L. infantum* culture) and negative serum control (dog from United Kingdom, a non-endemic area). Positive and negative controls were included on each slide. The result of the ferret IFAT was positive at 1:160 dilution with fluorescein-conjugated goat anti-ferret immunoglobulin G. However, a negative result of the ferret serum sample was obtained with fluorescein-conjugated goat anti-dog immunoglobulin G.

2.4. In vitro isolation and cultivation

For parasite isolation, Novy–MacNeal–Nicolle (NNN) medium and Schneider medium supplied 100 IU/ mL penicillin and 100 μg/mL streptomycin solution and 10% foetal calf serum was used.

Several drops of the aspirated material from the perilesional skin excised area were introduced into the liquid phase of the NNN and were incubated in medium Schneider at 26 ± 1 °C and cultures were microscopically assessed every day. The culture was positive after three days of incubation and *Leishmania* spp. strain isolated (Clinical Immunology Laboratory, University of Zaragoza) was molecularly type as isolated as *L. infantum* (Laboratory of Parasitology, University of Barcelona) and named MMST/ES/2019/ZGZ103.

2.5. Clinicopathological findings

Complete blood cell count revealed hematological values within normal limits and serum biochemistry profile showed elevated serum alanine transferase (ALT: > 1000 U/L, reference 82–289 U/L), alkaline phosphatase (ALKP: 239 U/L, reference 9–84 U/L), serum gamma glutamyl transferase (GGT: 250 U/L reference 0.2–14 UI/L), alpha-2 globulins (11.1 g/L; reference 4.2–8.5 g/L) and gamma-globulins (22 g/L; reference 2.7–9 g/L (4.8–14.7%)) with an A/G ratio of 0.69 (reference 0.58–1.33). Serum protein electrophoresis revealed polyclonal gammopathy.

3. Discussion

To the authors´ knowledge, this report describes the first clinical case of leishmaniosis in a domestic ferret (*Mustela putorius furo*) and is the first notification of natural *L. infantum* infection detected by parasite culture in mustelids. The diagnosis of clinical leishmaniosis in this ferret was based on the clinical signs, pathologic findings, and confirmatory *L. infantum* techniques, particularly PCR and culture. However, experimental infection has been described in ferrets used as animal models for the study of antileishmanial compounds (White et al., 1989). In dogs, the evolution and pathogenesis of natural infection is highly variable and not easily comparable with experimental infection. There may also be differences in the clinical course as well as in antibody production, among others. This situation therefore could be extrapolable in ferrets.

In the past decade wild animals such as lagomorphs and canids other than dogs have been attributed an important role in the transmission cycle of *L. infantum* (Miró et al., 2017a). Domestic ferrets have probably not played a crucial role as reservoir for *L. infantum* infection; however, studies may be necessary to elucidate the epidemiological role of domestic ferrets as household pets in this transmission cycle.

The ferret from this case report was infected in an urban environment in Valencia, on the East coast of the Iberian Peninsula fronting the Gulf of Valencia on the Mediterranean Sea, a geographical area where *P. perniciosus* is the most prevalent phlebotomine species detected (Aransay et al., 2004). Recent data about seropositivity rate of *L. infantum* in dogs in the East of Spain confirms that detection of anti-*Leishmania* antibodies was higher compared to the rest of the pathogens analyzed including *Anaplasma* spp., *Ehrlichia canis*, *Borrelia burgdorferi* and *Dirofilaria immitis* in this region (Miró et al., 2013). Human leishmaniasis has also been detected in the Valencia region and a recent outbreak of cutaneous leishmaniasis was described (Roth-Damas et al., 2017). In this sense, One Health context confirms the endemic nature and the high prevalence of the infection in the European Mediterranean regions and the potential possibility to detect the presence of *L. infantum* infection in other animals living in the same geographical area.

The presence of parasite in blood samples should be considered an important factor in the risk of iatrogenic transmission of *L. infantum* by blood transfusion as it is reported in other species such as dogs (Tabar et al., 2008). Moreover, transmission by vectors should be taken into account and preventive measures to avoid infection transmission from this and any other ferrets should be applied as is done in dogs (Miró et al., 2017b) and cats (Brianti et al., 2017).

A broad range of immune responses and clinical manifestations have been described in canine leishmaniosis. Infection in dogs may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness. Subclinical infection is not necessarily permanent and factors such as immunosuppression or concomitant diseases could break the equilibrium and lead to the progression of clinical disease in dogs (Solano-Gallego et al., 2009) as in cats (Pennisi et al., 2015) and humans (Alvar et al., 2012). Although ferrets are often relatively resistant to the immunosuppressive effects of prednisolone, this patient was under immune-modulating drugs therapy (prednisolone and cyclosporine) that might have caused immunosuppression and contributed to the clinical manifestation of *Leishmania* infection.

The macroscopic skin lesions of canine and feline leishmaniosis can be diverse, even in the same patient. The main clinical presentations include exfoliative, ulcerative, nodular, sterile pustular and papular dermatitis, nodules at the site of parasite inoculation and onychogryphosis (Manolis et al., 2014; Pennisi et al., 2015). These lesions are the result of granulomatous or pyogranulomatous inflammation that targets different structures of the skin and of the deposition of immune complexes (Manolis et al., 2014). The ferret of this report presented with pyogranulomatous dermatitis compatible with a less common clinicopathological presentation of the disease (papular dermatitis or nodule at the site of parasite inoculation). In dogs, each presentation may reflect a different host–parasite relationship, as in the case of the nodular and papular dermatitis, which are considered markers of high and low susceptibility to canine leishmaniosis, respectively (Manolis et al., 2014).

Clinicopathological findings detected in this ferret included moderately increased serum enzyme activities (ALT, ALKP and GGT) compatible with liver disorders and polyclonal hypergammaglobulinemia. Inflammatory, infectious and toxic hepatic disease. Hepatic lipidosis, and hepatic neoplasia are the most common hepatic diseases encountered in this species (Huynh and Laloi, 2013). Hepatic disease often remains subclinical, which may lead to difficulties in diagnosis. Canine leishmaniosis can occasionally cause hepatitis and elevation of liver enzymes (Rallis et al., 2005). In the ferret of this report, the cause of ALT, ALKP and GGT elevations and if these were related to leishmaniosis cannot be determined because liver biopsies were not obtained.

Hypergammaglobulinemia in ferrets is associated with a variety of infections including Aleutian disease, systemic coronavirus, canine distemper virus, certain neoplasia or systemic mycoses (e. g., blastomycosis and coccidioidomycosis) (Melillo, 2013). In canine leishmaniosis, serum protein electrophoresis reveals an increase of total proteins and globulins, as well as a typically polyclonal gammapathy. In this regard, the serum electrophoretogram detected in the ferret was

very similar to the typical serum protein electrophoresis in dogs (Maia and Campino, 2018) and cats (Pennisi et al., 2013) with clinical leishmaniosis, but not specific for a single infectious disease in ferrets.

The diagnosis of canine and feline leishmaniosis traditionally focuses on the detection of specific antibodies against *L. infantum* in clinical and research settings, with numerous serological techniques developed including IFAT, ELISA and WB techniques. The validation and adaptation of each serological technique to the animal species and reagents employed to perform the technique requires the use of antisera specific to the animal species analyzed. In this regard, serological evaluation of this ferret was performed with two different anti-immunoglobulin G conjugates in the in-house IFAT technique, with a positive result only with specific detection of anti-ferret immunoglobulin G instead of anti-dog immunoglobulin G. Protein A as a conjugated reagent, by contrast, is capable of interacting with the fragment crystallizable region from immunoglobulin G of several animals including ferret, dog (Riera et al., 1999) and cat (Solano-Gallego et al., 2007), being this reagent is very useful to determine total serum immunoglobulin G in animals with clinical leishmaniosis at the time of diagnosis.

The results described are strongly suggestive of a possible role of ferrets as reservoir of *Leishmania* infection. Further epidemiological studies are needed to understand the role of ferrets as a potential reservoir for human infection and how the infection could develop into the disease over time. Equally, therapeutic management for anti-*Leishmania* treatment in pet ferrets should be considered.

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Declaration of Competing Interest

The authors have nothing to disclose.

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References

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7 (5), e35671.

Aransay, A.M., Testa, J.M., Morillas-Marquez, F., Lucientes, J., Ready, P.D., 2004. Distribution of sandfly species in relation to canine leishmaniasis from the Ebro Valley to Valencia, northeastern Spain. Parasitol. Res. 94, 416–420.

Brianti, E., Falsone, L., Napoli, E., Gaglio, G., Giannetto, S., Pennisi, M.G., Priolo, V., Latrofa, M.S., Tarallo, V.D., Solari, B.F., Nazzari, R., Deuster, K., Pollmeier, M., Gulotta, L., Colella, V., Dantas-Torres, F., Capelli, G., Otranto, D., 2017. Prevention of feline leishmaniosis with an imidacloprid 10%/flumethrin 4.5% polymer matrix collar. Parasit. Vectors 10 (334), 14.

Del Río, L., Chitimia, L., Cubas, A., Victoriano, I., De la Rúa, P., Gerrikagoitia, X., Barral, M., Muñoz-García, C.I., Goyena, E., García-Martínez, D., Fisa, R., Riera, C., Murcia, L., Segovia, M., Berriatua, E., 2014. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. Prev. Vet. Med. 113, 430–435.

d'Ovidio, D., Pepe, P., Ianniello, D., Noviello, E., Quinton, J.F., Cringoli, G., Rinaldi, L., 2014. First survey of endoparasites in pet ferrets in Italy. Vet. Parasitol. 203, 227–230.

García, N., Moreno, I., Alvarez, J., de la Cruz, M.L., Navarro, A., Pérez-Sancho, M., García-Seco, T., Rodríguez-Bertos, A., Conty, M.L., Toraño, A., Prieto, A., Domínguez, L., Domínguez, M., 2014. Evidence of *Leishmania infantum* infection in rabbits (*Oryctolagus cuniculus*) in a natural area in Madrid, Spain. Biomed. Res. Int. 2014 (318254).

Huynh, M., Laloi, F., 2013. Diagnosis of liver disease in domestic ferrets (*Mustela putorius*). Vet. Clin. North Am. Exot. Anim. Pract. 16, 121–144.

Lucientes, J., Palmero, J., Guarga, J.L., Gracia, M.J., Peribáñez, M.A., Zárate, J., Castillo, J.A., 2005. Risk of transmission of canine leishmaniosis in eastern Spain. Vet. Rec. 156, 743–744.

Maia, C., Campino, L., 2018. Biomarkers associated with *Leishmania infantum* exposure, infection, and disease in dogs. Front. Cell. Infect. Microbiol. 8, 302.

Manolis, N., Saridomichelakis, M.N., Koutinas, A., 2014. Cutaneous involvement in canine leishmaniosis due to *Leishmania infantum* (syn. *L. chagasi*). Vet. Dermatol. 25, 61–e22.

Martín-Ezquerra, G., Fisa, R., Riera, C., Rocamora, V., Fernández-Casado, A., Barranco, C., Serra, T., Baró, T., Pujol, R.M., 2009. Role of *Leishmania* spp. infestation in non-diagnostic cutaneous granulomatous lesions: report of a series of patients from a Western Mediterranean area. Br. J. Dermatol. 161, 320–325.

Melillo, A., 2013. Applications of serum protein electrophoresis in exotic pet medicine. Vet. Clin. Exot. Anim. 16, 211–225.

Miró, G., Montoya, A., Roura, X., Gálvez, R., Sainz, A., 2013. Seropositivity rates for agents of canine vector-borne diseases in Spain: a multicentre study. Parasit. Vectors 22 (6), 117.

Miró, G., Müller, A., Montoya, A., Checa, R., 2017a. Epidemiological role of dogs since the human leishmaniosis outbreak in Madrid. Parasit. Vectors 26 (10), 209.

Miró, G., Petersen, C., Cardoso, L., Bourdeau, P., Baneth, G., Solano-Gallego, L., Pennisi, M.G., Ferrer, L., Oliva, G., 2017b. Novel areas for prevention and control of canine Leishmaniosis. Trends Parasitol. 33, 718–730.

Pennisi, M.G., Hartmann, K., Lloret, A., Addie, D., Belák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hosie, M.J., Lutz, H., Marsilio, F., Möstl, K., Radford, A.D., Thiry, E., Truyen, U., Horzinek, M.C., 2013. Leishmaniosis in cats: ABCD guidelines on prevention and management. J. Feline Med. Surg. 15, 638–642.

Pennisi, M.G., Cardoso, L., Baneth, G., Bourdeau, P., Koutinas, A., Miró, G., Oliva, G., Solano-Gallego, L., 2015. LeishVet update and recommendations on feline leishmaniosis. Parasit. Vectors 4 (8), 302.

Rallis, T., Day, M.J., Saridomichelakis, M.N., Adamama-Moraitou, K.K., Papazoglou, L., Fytianou, A., Koutinas, A.F., 2005. Chronic hepatitis associated with canine leishmaniosis (*Leishmania infantum*): a clinicopathological study of 26 cases. J. Comp. Pathol. 132, 145–152.

Riera, C., Valladares, J.E., Gállego, M., Aisa, M.J., Castillejo, S., Fisa, R., Ribas, N., Carrió, J., Alberola, J., Arboix, M., 1999. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. Vet. Parasitol. 84, 33–47.

Roth-Damas, P., Sempere-Manuel, M., Mialaret-Lahiguera, A., Fernández-García, C., Gil-Tomás, J.J., Colomina-Rodríguez, J., Palop-Larrea, V., 2017. Community outbreak of cutaneous leishmaniasis in La Ribera region of Valencia, Spain: public health measures. Enferm. Infecc. Microbiol. Clin. 35, 338–343.

Solano-Gallego, L., Rodríguez-Cortés, A., Iniesta, L., Quintana, J., Pastor, J., Espada, Y., Portús, M., Alberola, J., 2007. Cross-sectional serosurvey of feline leishmaniasis in ecoregions around the Northwestern Mediterranean. Am. J. Trop. Med. Hyg. 76, 676–680.

Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Baneth G. Vet. Parasitol. 165, 1–18.

Tabar, M.D., Roura, X., Francino, O., Altet, L., Ruiz de Gopegui, R., 2008. Detection of *Leishmania infantum* by real-time PCR in a canine blood bank. J. Small Anim. Pract. 49, 325–328.

White, M.R., Chapman Jr., W.L., Hanson, W.L., 1989. A comparison of experimental visceral leishmaniasis in the opossum armadillo and ferret. Lab. Anim. Sci. 39, 47–50.

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RESEARCH Open Access

A cross-sectional study of *Leishmania infantum* infection in stray cats in the city of Zaragoza (Spain) using serology and PCR

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Abstract

Background: Feline leishmaniosis is a vector-borne parasitic disease caused by *Leishmania* spp. *Leishmania* infection in dogs is prevalent in the Mediterranean basin, but in other animals, such as cats, it could also play a role in the epidemiology of the disease. Information on the geographical distribution and epidemiological features of *L. infantum* infection in cats is scarce, particularly in urban stray cats living in regions where canine leishmaniosis is endemic. As diagnosis can be challenging, combining different serological and molecular methods is a useful approach. Our aim was to investigate the prevalence of infection of *L. infantum* in apparently healthy stray cats in an endemic region of Spain (Zaragoza city) using serological and molecular methods, and to compare the results of the different techniques.

Methods: The prevalence of *Leishmania* infection was studied in stray cats captured in urban and peri-urban areas of Zaragoza. Blood was collected from each animal for serology and molecular analysis. Three serological methods, namely the immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and western blot (WB), were used to detect *L. infantum* antibodies and a real-time PCR (qPCR) assay was used to detect *L. infantum* DNA. The results were analyzed by Fisher's exact test and Cohen's kappa statistic (κ) to assess the level of agreement between the diagnostic techniques.

Results: Serological analysis of blood samples from 180 stray cats revealed 2.2% (4/179) *Leishmania* infection positivity by IFAT, 2.8% (5/179) by ELISA and 14.5% (26/179) by WB. *Leishmania* DNA was detected by qPCR in 5.6% (10/179) of the cats. Sixteen cats (8.9%) tested positive by only one serological technique and four tested positive by all three serological methods used. The overall rate of infected cats (calculated as the number of cats seropositive and/or qPCR positive) was 15.6%, and only two cats tested positive by all the diagnostic methods. A significant association was found between male cats and a positive qPCR result. Comparison of the techniques revealed a fair agreement in seropositivity between blood qPCR and IFAT ($\kappa = 0.26$), blood qPCR and ELISA ($\kappa = 0.24$), WB and ELISA ($\kappa = 0.37$) and WB and IFAT ($\kappa = 0.40$). The highest agreement between seropositive results was between IFAT and ELISA ($\kappa = 0.89$), and the lowest was between blood qPCR and WB ($\kappa = 0.19$). The prevalence of the feline leukemia virus antigen was 4.49% (8/178 cats) and that of the feline immunodeficiency virus (FIV) antibody was 6.74% (12/178), while co-infection with

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both retroviruses was observed in one female cat (1/178). *Leishmania* ELISA and IFAT seropositivity were statistically associated with FIV status by the chi-square test.

Conclusions: The results obtained in this study, using serological tests and qPCR, indicate the existence of *L. infantum* asymptomatic infection in apparently healthy stray cats in the city of Zaragoza, an endemic area in Spain.

Keywords: FeLV, FIV, *Leishmania infantum*, Serology, PCR, Blood, Cat

Background

Leishmaniosis is a complex vector-borne disease caused by different species of the genus *Leishmania* that is endemic in 88 countries of southern Europe, Africa, Asia and South and Central America. More than 350 million people are estimated to be at risk of the disease [1], with dogs being the main reservoir for *Leishmania infantum* infection. Based on seroprevalence studies and environmental variables, 23.2% of dogs in Italy, Spain, France and Portugal are estimated to be infected [2]. Although *Leishmania* infection in dogs is well documented, there is a need for more information on its prevalence in cats in the same areas.

Various phlebotomine species are implicated in the transmission of *L. infantum* in Europe, of which only two are found in Spain: *Phlebotomus ariasi* and *Phlebotomus perniciosus* [3]. Female *Phlebotomus* spp. feed on a variety of vertebrate reservoirs, including humans, livestock, dogs, wild rabbits, hares, rodents and cats [4], with variable impacts on the epidemiology of leishmaniosis. Colonies of stray cats could play a role in the maintenance of the *Leishmania* life-cycle in an urban environment, where cats are naturally exposed to active vectors.

Free-roaming cats in European cities are a potential source of zoonotic diseases. In the case of *Leishmania*, cats can be infected for several years without exhibiting clinical signs [5]. In dogs, clinical manifestations of infection may range from absent or mild to severe and even fatal disease. This variability is thought to be the result of a cell-mediated immune response from the host, which may be influenced by the genetic background. A similar pattern of humoral and cell-mediated adaptive immune response is detected in cats from endemic areas of *L. infantum* [6]. The most common clinical signs of feline leishmaniosis (FeL) include peripheral lymphadenomegaly, cutaneous and mucocutaneous lesions (e.g. nodular and/or ulcerative dermatitis), generalized weakness, weight loss, anorexia and ocular and oral lesions. Some of the most frequent clinicopathological abnormalities seen in FeL are non-regenerative anemia, hyperproteinemia with hyperglobulinemia and hypoalbuminemia and proteinuria [7].

To carry out the complex diagnosis of FeL, serological and molecular techniques are commonly employed in clinical and epidemiological studies [8]. Serological studies have included the immunofluorescent antibody test (IFAT) [9], the direct agglutination test (DAT) [10], the enzyme-linked immunosorbent assay (ELISA) [11] and western blot (WB) [12]. In contrast with serology techniques, molecular diagnostic techniques are not restricted to bodily fluids, but can also be applied to bone marrow, the lymph node, spleen and skin tissues. Nevertheless, blood is the most commonly used sample for molecular testing in epidemiological surveys.

In an epidemiological survey carried out in the area of Barcelona (Spain), molecular test results indicated that *L. infantum* subclinical infections outnumber clinical infections in cat populations in endemic regions [13]. As serological methods may not be sufficiently sensitive to bring subclinical infections to light, a combination of at least two positive results by molecular and serological techniques is recommended for a more accurate estimation of *Leishmania* infection [14]. Several studies have provided seroprevalence and molecular data in endemic southern European countries, particularly Spain [15], France [16], Portugal, [17] and Italy [18], but considerable gaps in regional data remain to be filled. Given the absence of epidemiological studies on feline infection in the city of Zaragoza, an endemic region of Spain, the aims of the present study were: (1) to investigate the prevalence of *L. infantum* infection in stray cats in Zaragoza using serology and quantitative (qPCR) assays; and (2) to evaluate the screening results of apparently healthy stray cats living in an endemic region by comparing serological and qPCR test data.

Methods

Study areas, cats and sampling

The study was carried out in the city of Zaragoza (41°38′58.8948″N, 0°53′15.7632″W, the Aragon region of Spain) on cats who had lived through at least one transmission season. Based on an expected seroprevalence of 8.5% (the canine seroprevalence in Zaragoza), an accepted 5% deviation from the true prevalence and a confidence level of 95%, the sample size necessary to estimate the seroprevalence was calculated to be 120 cats. The study population comprised 180 stray cats captured in urban and peri-urban areas of Zaragoza (Fig. 1) within the framework of a trap, neuter and release sterilization program run locally to control stray populations.

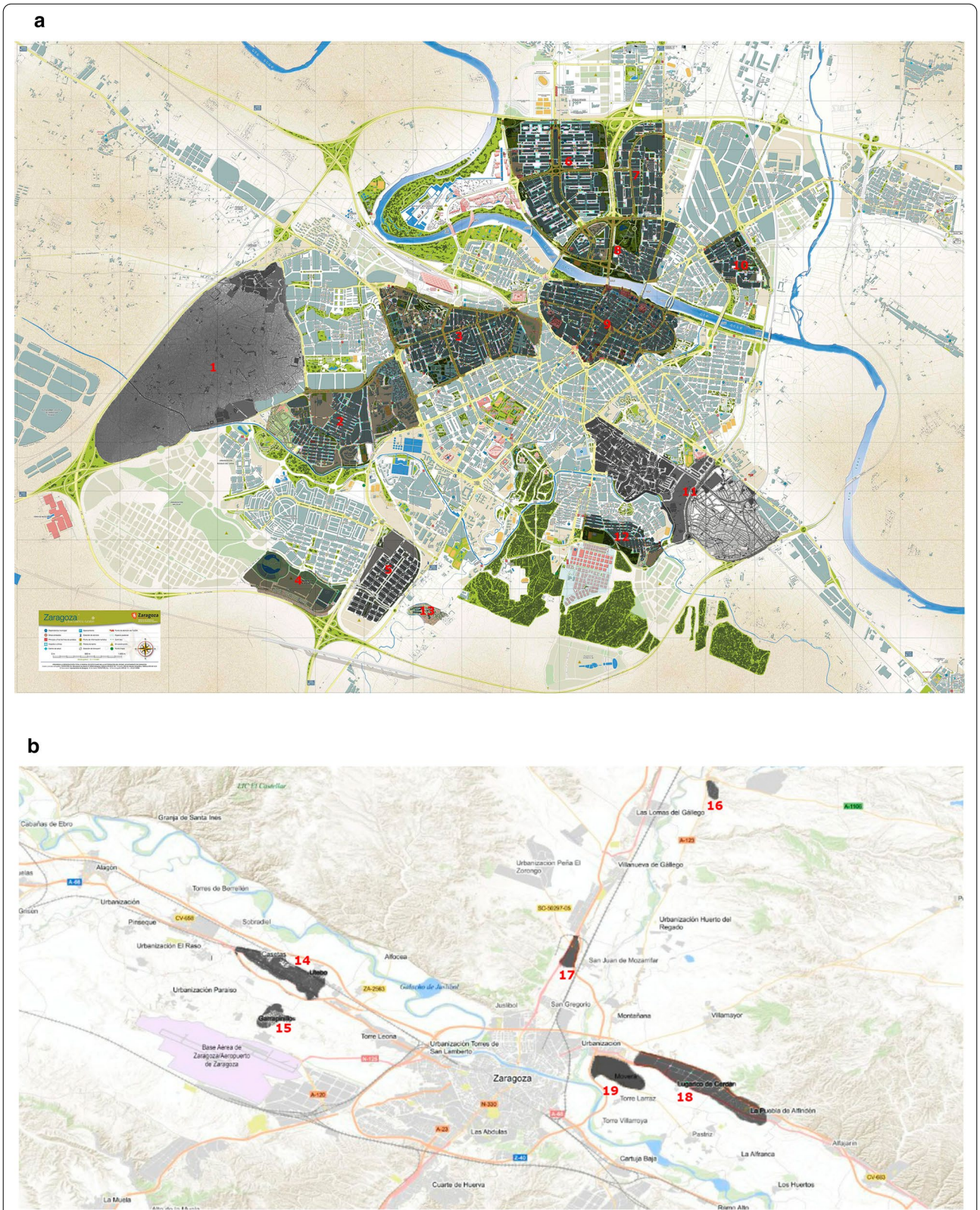

Fig. 1 Location of the stray cat colonies in Zaragoza. **a** Urban colonies: *1* Miralbueno, *2* Valdefierro, *3* Delicias, *4* Valdespartera, *5* Casablanca, *6* Actur, *7* Picarral, *8* Arrabal, *9* Centro, *10* La Jota, *11* San José, *12* Torrero, *13* Urbanización de la Junquera. **b** Peri-urban colonies: *14* Casetas, *15* Garrapinillos, *16* Peñaflor, *17* San Juan de Mozarrifar, *18* Malpica, *19* Movera

Captured stray cats were anesthetized with a combination of dexmedetomidine (Dexdomitor®; 15 µg/kg, subcutaneous injection), ketamine (Anaestamine®; 5 mg/kg, subcutaneous injection) and methadone (Semfortan®; 0.3 mg/kg, subcutaneous injection). Data on the breed, age, gender and colony of origin of each cat were recorded. A complete physical examination was carried out before sampling.

Prior to collecting blood, the fur of the cats was trimmed around the jugular region. Sampling consisted of collecting 2 ml of blood aseptically by jugular venepuncture, with the collected volume divided equally between a sterile blood collection tube (to obtain the serum) and a second tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant (for PCR analysis). Blood and separated serum were stored at − 20 °C until processing. Routine laboratory tests, such as a complete blood count and biochemistry profile, were not performed.

Diagnostic serological tests

Detection of specific antibodies was performed using three in-house serological techniques: the IFAT, ELISA and WB.

Detection of L. infantum antibodies by IFAT

The IFAT was performed according to the standard procedures of the World Organization for Animal Health [19], using promastigote forms of the strain MHOM/MON-1/LEM 75 zymodeme MON-1 as a whole-parasite antigen fixed on multi-spot slides (Thermo Fisher Scientific, Waltham, MA, USA). Sera from the captured cats were assayed in serial twofold dilutions from 1:20 to 1:2560. Briefly, a twofold dilution of each serum was applied per well. The slides were incubated for 30 min at 37 °C in a moist chamber, and then washed twice with phosphate buffered saline (PBS) for 5 min and once more with distilled water. After the washing procedure, 20 µl of goat anti-cat IgG-fluorescein isothiocyanate conjugate (Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:64 in 0.2% Evans blue was added to each well. The slides were incubated in a moist chamber at 37 °C for another 30 min in complete darkness and washed again as described above.

After the second washing procedure, a few drops of mounting medium were placed on the cover slips. The slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400× magnification, and each well was compared to the fluorescence pattern seen in the positive and negative controls. Positive and negative controls were included on each slide. A positive control serum was obtained from a cat from Spain diagnosed with FeL, confirmed by a positive *L. infantum* isolation using a biphasic Novy, McNeal and Nicolle blood agar (NNN) medium, and a negative control serum was obtained from a healthy, non-infected indoor cat. The cut-off value for positive sera was 1:80, in accordance with the literature [8]. Two trained researchers examined every IFAT sample, and a third investigator participated if discrepancies arose between results.

Detection of L. infantum antibodies by a quantitative ELISA

The ELISA was performed on all sera as described previously, with some modifications [20]. Briefly, each plate was coated with 20 µg/ml of crude antigen obtained from *L. infantum* promastigote forms (MHOM/MON-1/LEM 75) in 0.1 M carbonate/bicarbonate buffer (pH 9.6), and incubated overnight at 4 °C. A 100-µl aliquot of cat sera, diluted 1:200 in PBS containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M), was added to each well. The plates were then incubated for 1 h at 37 °C in a moist chamber, following which they were washed and 100 µL of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:20,000 in PBST-M was added. The plates were incubated for 1 h at 37 °C in the moist chamber and were washed again with PBST and PBS as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific) were added to each well and the reaction was allowed to develop for 20 ± 5 min at room temperature in the dark. The reaction was stopped by adding 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (ELISA Reader Labsystems Multiskan, Midland, ON, Canada).

As a positive control (calibrator), each plate included serum from a cat from Spain diagnosed with FeL, confirmed by a positive *L. infantum* isolation using an NNN medium, and as a negative control, serum from a healthy, non-infected cat. The same calibrator serum was used for all assays and plates, with a constant inter-assay variation of < 10%. Plates with an inter-assay variation of > 10% were discarded. All samples and controls were run in duplicate. The results were quantified as ELISA units (EU) compared to the positive control serum used as a calibrator and arbitrarily set at 100 EU. The cut-off was established at 13 EU (mean + 4 standard deviations [SD] of values from 50 indoor cats from northern Spain) and the results above this value were considered to be positive.

Detection of L. infantum antibodies by WB

Anti-*Leishmania* antibodies were detected by WB using a whole antigen of *L. infantum* promastigotes (MHOM/FR/78/LEM75 zymodeme MON-1), as described by

Riera et al. [20] with some modifications. Antigen electrophoresis in 1% sodium dodecyl sulfate/15% polyacrylamide gels together with molecular mass protein standards (Standard Low Range; Bio-Rad, Hercules, CA, USA) was performed on a Mini-Gel AE 6400 Dual Mini Slab Kit (ATTO Corp., Tokyo, Japan). The gels were run at 100 V for 1 h at room temperature.

Polypeptides were transblotted onto nitrocellulose sheets (0.45-mm pore size, HAWP 304 FO; Millipore Corp., Bedford, MA, USA), which were blocked with 20 mM Tris, 0.13 mM NaCl, pH 7.6 (TS) and 5% skimmed milk, overnight at 4 °C. The sheets were washed in TS and introduced into a multiscreen apparatus (Mini Protean II, Multiscreen Apparatus; Bio-Rad). Sera were diluted 1:200 in TS/1% skimmed milk and 0.2% Tween 20. Then 500 µl of each sample was introduced into each channel of the multiscreen apparatus and incubated for 2 h at 37 °C. Bound immunoglobulins were developed by incubation with a 1:1000 dilution of Protein A peroxidase conjugate (Thermo Fisher Scientific) for 1 h. After the sheets were washed three times with TST and a final time with TS, color was developed with 4-chloro-1-naphthol (Thermo Fisher Scientific) and H_2O_2, and the reaction was stopped with tap water after 30 min. The sera were considered to be positive when immunoreactivity from low-molecular-weight polypeptide fractions of 14, 16, 18, 20, 24, 36 and 46 kDa from the *Leishmania* antigen was observed, as previously reported [21, 22].

Detection of L. infantum DNA by qPCR

A qPCR was used in this study. DNA was extracted from 200 µl of blood by the isolation of nucleic acids according to the protocol of the Quick-DNA Miniprep Plus Kit (Zymo Research, Irvine, CA. USA) and eluted in 50 µl of elution buffer, following the manufacturer's instructions.

Leishmania spp. DNA was detected and quantified by amplification of a kinetoplast minicircle DNA sequence by qPCR [23]. Each amplification was performed in triplicate in a 10-µl reaction mixture containing 1× iTaq supermix with Rox (Bio-Rad), 15 pmol of direct primer (5′-CTTTTCTGGTCCTCCGGGTAGG-3′), 15 pmol of reverse primer (5′-CCACCCGGCCCTATTTTACAC CAA-3′), 50 pmol of the labeled TaqMan probe (FAM-TTTTCGCAGAACGCCCCTACCCGCTAMRA) and 2.5 µl of sample DNA. Cycling was performed using the ABI Prism 7900 system (Applied Biosystems [Thermo Fisher Scientific], Foster City, CA, USA) att 94 °C/55 °C for 40 cycles. A non-template control was used in each run as the qPCR negative control. A tenfold dilution series of DNA from promastigotes (MHOM/ES/04/BCN-61, *L. infantum* zymodema MON-1) was used for calibration (serial dilution from 10^5 to 10^{-3} parasites/ml), allowing the plotting of a standard curve. The qPCR was considered to be positive for *Leishmania* when the quantification cycle (Cq) was < 40 and the amplification was detected in all the replicates.

Detection of feline leukemia virus antigens and feline immunodeficiency virus antibodies by an immunochromatographic rapid test

The rapid test (Uranotest FeLV-FIV; URANOVET, Barcelona, Spain) was performed following the instructions of the manufacturer. All tests were stored at room temperature and were performed as described in the instructions supplied with the test kit.

Statistical analysis

The SPSS software package (SPSS Inc. IBM Corp., Chicago, IL, USA) was used for statistical analysis. Associations between *L. infantum* and the recorded variables (gender, location, colony of origin, type of environment [including urban or peri-urban area] and feline immunodeficiency virus/feline leukemia virus [FIV/FeLV] status) were analyzed. The significance of these differences was assessed using Fisher's exact test and a Chi-square test. A P value < 0.05 was considered to be significant. Agreements between diagnostic techniques were evaluated using Cohen's kappa statistic (κ) as follows: no agreement ($\kappa < 0$), slight agreement ($0 < \kappa < 0.2$), fair agreement ($0.2 < \kappa < 0.4$), moderate agreement ($0.4 < \kappa < 0.6$), substantial agreement ($0.6 < \kappa < 0.8$) and almost perfect agreement ($\kappa > 0.8$).

Results

Animals studied

All of the tested cats (97 females, 83 males) were shorthaired breeds, adults (> 1 year old) and assessed as apparently healthy, with no evident systemic or dermatological signs found during the general physical examination. Eighty-two cats came from urban colonies ($n = 10$) and 98 from peri-urban colonies ($n = 9$). Samples of serum for *Leishmania* serological testing and blood for *Leishmania* qPCR were obtained from nearly all of the cats (178/180). A serum sample was not available from one of the cats and blood containing EDTA anticoagulant was not obtained from another. One serum sample was of insufficient volume to perform a serological analysis to detect the presence of FeLV antigens and FIV antibodies but was used for a *Leishmania* serology test.

Serology and qPCR for *L. infantum*

Among the 179 cats for which samples were available for testing, four were seropositive for *L. infantum* by IFAT, with antibody titers ranging from 1:80 (1 cat) to 1:160 (2 cats) and 1:640 (1 cat). Of these four cats, two, with antibody titers of 1:160 and 1:640, respectively, tested

positive for *L. infantum* by all of the diagnostic methods employed; the other two cats tested positive only by ELISA and WB (Table 1).

Ten of the 179 cats tested were seroreactive by IFAT, with antibody titers below the IFAT cut-off: 1:20 in five cats and 1:40 in the other five cats. None of these ten cats tested positive by the other serological techniques or qPCR (Table 1).

Five cats (2.7%) were seropositive to the *L. infantum* antigen (mean ± SD: 39.8 ± 44 EU) by ELISA. One cat (Cat 46) showed a high antibody ELISA titer (118 EU) and tested positive by IFAT, WB and qPCR. The other four ELISA-seropositive cats showed low positivity (range 14–26 EU) (mean ± SD: 20.3 ± 5.7 EU); one of these (Cat 95) was also positive by all four diagnostic methods and a second (Cat 109) was only positive by ELISA. The other 174 cats tested were seronegative by ELISA (mean ± SD: 5.9 ± 1.6 EU). No doubtful results were obtained with this test for any cat (Table 1).

In the WB analysis, none of the polypeptide fractions were recognized in 26 cats (14.52%). The sensitivity to the *L. infantum* antigen is reported in Fig. 2. Of these 26 cats, 16 showed only one molecular mass band, seven showed two molecular mass bands, two showed four molecular mass bands and one showed five molecular mass bands.

The most frequent positive band was at 46 kDa (17/26 cats), followed by a band at 16 kDa (10/26). Nine of these 17 cats with a positive band at 46 kDa tested positive for *Leishmania* spp. DNA by qPCR, of which six were seroreactive to the 46-kDa band, one (Cat 13) to the 24- and 46-kDa bands, one (Cat 95) to the 14-, 16-, 36- and 46-kDa bands and one (Cat 46) to 14-, 16-, 24-, 36- and 46-kDa bands (Table 1). Of these 26 cats that tested positive by WB, four also tested positive by IFAT and ELISA and two by qPCR. In total, seven of the 26 cats were qPCR positive.

The molecular analysis detected *Leishmania* spp. DNA in ten of the 179 animals studied (5.6%), two of which also tested positive by all the serological techniques, one tested positive only by qPCR and the rest were also positive by WB (Table 1).

The overall prevalence of *L. infantum* infection was 15.6%, considering a cat to be infected if it tested positive by at least one of the diagnostic techniques.

Association of *Leishmania* positivity with colonies

Positive results associated with the colony of origin are listed in Table 1. Cats in 11 of the 19 colonies tested positive for *L. infantum* infection, with the majority of these cats located in three colonies: one urban (21.74% of positive cats [5/23], colony 1) and two peri-urban (20.83% of positive cats [5/24], colony 11; and 30.77% of positive cats [4/13], colony 18). In terms of sex, 18.07% of males (15/83) and 7.21% of females (7/97) tested positive by at least one technique.

Two cats, one male (Cat 95) and one female (Cat 46) from different colonies were positive for *L. infantum* infection by all the diagnostic techniques, although physical examination of these two infected cats did not reveal any abnormalities compatible with FeL. Among the 180 cats included in this analysis, two males (Cat 177 and Cat 178) from different colonies yielded a positive result with all of the serological techniques but were negative by qPCR; physical examination of these cats revealed no abnormality.

No significant association was found between positivity for anti-*Leishmania* antibodies and gender, location or type of environment (Table 2). A significant association was found between gender (male) and positivity detected by qPCR ($P = 0.026$).

Agreement between serological and molecular findings

The seroprevalence of *L. infantum* infection, based on a positive result in any serological test, was 15.1% (95% confidence interval [CI] 10.5–21.1%). The seroprevalence rate according to the different tests was 14.5% for WB (95% CI 10.1–20.5%), 2.8% for ELISA (95% CI 1–6.6%) and 2.2% for IFAT (95% CI 0.7–5.8%). The molecular prevalence was 5.6% (95% CI 2.9–5.6%). The overall prevalence of *L. infantum* infection, based on a positive result from at least one of the diagnostic techniques, was 15.6% (95% CI 10.9–21.6%). Kappa agreement was almost perfect between IFAT and ELISA results ($\kappa = 0.89$), and fair between IFAT and qPCR ($\kappa = 0.26$) and ELISA and qPCR ($\kappa = 0.24$). Agreement was higher between IFAT and WB ($\kappa = 0.40$) than between ELISA and WB ($\kappa = 0.37$), and there was a slight agreement between WB and qPCR ($\kappa = 0.19$).

Serological prevalence of FeLV and FIV infections

The seroprevalence of FeLV and FIV infections was 4.49% (8/178 cats) and 6.74% (12/178), respectively. Among the 178 cats, eight cats (5 males and 3 females) were seropositive by the immunochromatographic test for FeLV. The presence of antibodies against FIV was detected in five males and seven females.

No significant association was found between positivity for FeLV infection and positivity for anti-*Leishmania* antibodies, gender, location or type of environment (Table 3). In contrast, a significant association was found between FIV infection and positivity detected by IFAT ($P = 0.023$) and ELISA ($P = 0.037$). No other significant associations were found for the other factors evaluated.

Table 1 Summary of positivity based on different diagnostic tests from all cat colonies

Colony	Location	Gender	ELISA (EU)	IFAT	WB (kDa bands)	PCR blood (Cq)
Colony 1 ($n=23$)	Urban	Male 15/23	0/15	0/15	4/15 Cat 13 (24, 46) Cat 24 (46) Cat 27 (46) Cat 28 (46)	4/14 Cat 13 (Cq 38) Cat 24 (Cq 36) Cat 27 (Cq 38) Cat 28 (Cq 38)
		Female 8/23	0/8	0/8	1/8 Cat 2 (46)	1/8 Cat 2 (Cq 34)
Colony 2 ($n=9$)	Urban	Male 1/9	0/1	0/1	0/1	0/1
		Female 8/9	0/8	0/8	0/8	0/8
Colony 3 ($n=6$)	Urban	Male 3/6	0/3	0/3	2/3 Cat 22 (46) Cat 61 (46)	1/3 Cat 22 (Cq 37)
		Female 3/6	0/3	0/3	0/3	0/3
Colony 4 ($n=8$)	Urban	Male 5/8	0/5	0/5	1/5 Cat 23 (16)	0/5
		Female 3/8	0/3	0/3	0/3	0/3
Colony 5 ($n=2$)	Urban	Female 2/2	0/2	0/2	0/2	0/2
Colony 6 ($n=15$)	Urban	Male 9/15	1/9 Cat 177 (14)	1/9 Cat 177 (1:80)	1/9 Cat 177 (20, 46)	0/9
		Female 6/15	0/6	0/6	1/6 Cat 156 (46)	0/6
Colony 7 ($n=3$)	Urban	Male 1/3	0/1	0/1	0/1	0/1
		Female 2/3	0/2	0/2	0/2	0/2
Colony 8 ($n=7$)	Urban	Male 2/7	0/2	0/2	1/2 Cat 89 (14, 20)	0/2
		Female 5/7	0/5	0/5	0/5	0/5
Colony 9 ($n=5$)	Urban	Male 3/5	0/3	0/3	0/3	0/3
		Female 2/5	0/2	0/2	0/2	0/2
Colony 10 ($n=4$)	Urban	Male 2/4	0/2	0/2	0/2	0/2
		Female 2/4	0/2	0/2	0/2	0/2
Colony 11 ($n=24$)	Peri-urban	Male 12/24	0/12	0/12	2/12 Cat 34 (46) Cat 48 (16)	1/12 Cat 34 (Cq 31)
		Female 12/24	1/12 Cat 46 (118)	1/12 Cat 46 (1:640)	3/12 Cat 30 (18) Cat 38 (16) Cat 46 (14, 16, 24, 36, 46)	1/12 Cat 46 (Cq 31)
Colony 12 ($n=5$)	Peri-urban	Male 2/5	0/2	0/2	0/2	0/2
		Female 3/5	0/3	0/3	0/3	0/3
Colony 13 ($n=10$)	Peri-urban	Male 4/10	0/4	0/4	0/4	0/4
		Female 6/10	0/6	0/6	1/6 Cat 71 (16, 18)	0/6
Colony 14 ($n=4$)	Peri-urban	Male 3/4	1/3 Cat 178 (24)	1/3 Cat 178 (1:160)	1/3 Cat 178 (14, 16, 36, 46)	0/3
		Female 1/4	0/1	0/1	0/1	0/1
Colony 15 ($n=3$)	Peri-urban	Male 1/3	0/1	0/1	0/1	0/1
		Female 2/3	0/2	0/2	0/2	0/2
Colony 16 ($n=3$)	Peri-urban	Female 3/3	0/3	0/3	1/3 Cat 91 (46)	0/3
Colony 17 ($n=21$)	Peri-urban	Male 9/21	0/9	0/9	0/9	0/9
		Female 12/21	0/11	0/11	0/11	0/12

Table 1 (continued)

Colony	Location	Gender	ELISA (EU)	IFAT	WB (kDa bands)	PCR blood (Cq)
Colony 18 ($n=13$)	Peri-urban	Male 7/13	2/7 Cat 95 (17) Cat 109 (26)	1/7 Cat 95 (1:160)	2/7 Cat 95 (14, 16, 36, 46) Cat 176 (16, 46)	2/7 Cat 94 (Cq 38) Cat 95 (Cq 31)
		Female 6/13	0/6	0/6	1/6 Cat 175 (16, 46)	0/6
Colony 19 ($n=15$)	Peri-urban	Male 4/15	0/4	0/4	1/4 Cat 40 (36)	0/4
		Female 11/15	0/11	0/11	3/11 Cat 39 (16, 18) Cat 76 (46) Cat 84 (14)	0/11

ELISA, Enzyme-linked immunosorbent assay; EU, ELISA units; IFAT, immunofluorescent antibody test; WB, western blot; Cq, quantification cycle

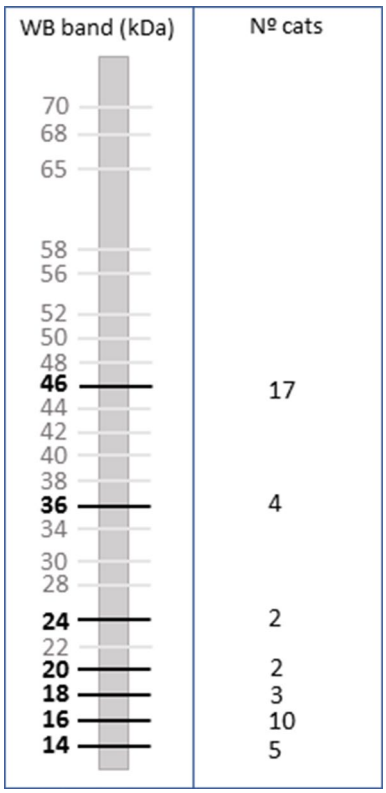

Fig. 2 Antibody recognition of *Leishmania infantum* antigens by western blot (*WB*) in 179 cats from Zaragoza (Spain)

Co-infections

Co-infection with *L. infantum* and FIV was detected in two female cats (Cat 177 and Cat 178), no co-infection with *L. infantum* and FeLV was observed and one female cat was co-infected with both retroviruses.

Discussion

In Spain, the first *Leishmania* infection in cats was described in 1933 [24], and since then the number of natural cases of feline leishmaniosis has increased not only in Spain but also worldwide [25]. In areas endemic for *L. infantum*, dogs and other domestic animals, such as ferrets [26], have been diagnosed with clinical leishmaniosis, but the epidemiological role of the cat is still not completely or clearly understood. In this context, there is an urgent need for epidemiological surveys to ascertain the contribution of infected cats in areas where cases are detected.

Epidemiological studies of cats performed in different regions of Spain report seroprevalences of *L. infantum* ranging from 1.29 to 60% [27, 28]. Surveys using different serological methods have found that the prevalence of *L. infantum* infection in cats in Spain varies from 0.43 to 26% [27, 29] (Table 4).

A study on canine leishmaniosis in Zaragoza found a seroprevalence of 8.5% by IFAT [30]. In the same area, a total of 130 human cases of leishmaniasis were reported from 2000 to 2019 [31]. Nevertheless, the true prevalence of *L. infantum* infection in cats in the Aragon region is unknown; the only published information available is a congress communication on a study of 50 domestic cats, 42% of which were seropositive by the DAT and exhibited immune dysfunction [32].

Epidemiological surveys reporting the presence of anti-*Leishmania* antibodies in feline sera have used a range of techniques, such as IFAT, ELISA, WB and DAT. In general, blood is less suitable for detecting parasitic DNA than bone marrow or the lymph node, but in an epidemiological setting, the procedure to obtain these alternative samples may be impractical and blood is usually the only type of sample available. It should be mentioned that there is a lack of consensus on the ideal biological sample to use in the molecular diagnosis of FeL [33]. In this study, we applied three different serological techniques

Table 2 Characteristics of a population of stray cats in Zaragoza examined for *Leishmania infantum* infection

Factor	*P* value[a]					
	ELISA positive	IFAT positive	WB positive	PCR positive	Positive by any serological method	Positive by any method
Gender	0.126	0.246	0.210	0.026	0.232	0.194
Colony of origin	0.368	0.604	0.587	0.187	0.432	0.297
Environment (urban/peri-urban)	0.240	0.398	0.698	0.335	0.357	0.224

[a] Associations with a *P* value of < 0.05 were considered to be statistically significant

Table 3 Characteristics of a population of stray cats in Zaragoza examined for feline leukemia virus antigens and feline immunodeficiency virus infections

Factor	*P* value[a]	
	FeLV positivity	FIV positivity
Gender	0.476	0.773
Colony of origin	0.327	0.431
Environment (urban/peri-urban)	0.727	0.551
ELISA positive (*L. infantum*)	0.999	0.037
IFAT positive (*L. infantum*)	0.999	0.023
WB positive (*L. infantum*)	0.599	0.656
PCR positive (*L. infantum*)	0.999	0.999

FeLV, Feline leukemia virus antigens; FIV, feline immunodeficiency virus

[a] Associations with a *P* value of < 0.05 were to beconsidered statistically significant

and a molecular test to evaluate the global prevalence of the infection.

Among the serological techniques used in this survey, positivity of 14.5% was detected by WB in feline serum samples, which is similar to the positivity reported in a study performed in Mallorca (15.70%) [29]. ELISA was slightly more effective than IFAT in terms of detecting antibodies against *L. infantum*, in contrast with another study [12]. The main differences between these three serological techniques that would explain the results obtained are the type of antigen used and the technical method performed to obtain the results. WB is less frequently used for the serological detection of infection than IFAT or ELISA and requires the use of a specialized laboratory and reagents to distinguish the molecular weight of the *L. infantum* antigens present in the serum sample.

In the WB analysis, certain bands associated with the diagnosis were detected with more frequency, particularly a band with the molecular weight of 46 kDa and bands with low molecular weight, such as 14, 16, 18 and 20 kDa; all of these bands are considered to be specific for detecting infection. In our study, the presence of bands at 14, 16, 18, 20, 24, 36 and 46 kDa suggested *L. infantum* infection. In contrast, other authors report that bands at 18 kDa or at 16, 28, 34, 54 and 69 kDa do not indicate *L. infantum* infection [12, 34]. Bands at 14, 16, 36 and 46 kDa were detected in two cats that tested positive for *L. infantum* infection by the other three tests. In particular, the 46-kDa band was detected in nine cats with a positive qPCR result. In asymptomatic infection in humans and dogs, the presence of the polypeptide fractions of 14 and/or 16 KDa together with other bands of low molecular weight indicates infection or exposure [35–37]. In our study, we observed low-molecular-weight bands in 16 cats, three of which gave a positive qPCR result, and the 46-KDa band was observed in two of these three cats. In six cats showing reactivity only against the 46-kDa polypeptide fraction, *Leishmani*a DNA in blood was detected by qPCR. However, more serological and molecular studies are needed to ascertain the specificity of this band and whether it can be used to detect prior contact with the parasite or asymptomatic infection in cats [21, 22, 38, 39].

The cats studied by both WB and ELISA showed different seroprevalence results according to the methodology used (8.38 and 2.79%, respectively), probably due to the different sensitivity and specificity of the techniques.

Small differences in the detection of anti-*Leishmania* antibodies between the ELISA and IFAT techniques could be due to the type of antigen used and the technical method performed to obtain the results. Another influential factor is the interpretation of the results; in IFAT, the interpretation of results is subjective and dependent on the operator´s experience. In this study, a cut-off dilution of 1:80 was applied in the IFAT technique, following the LeishVet guidelines [8]. However, some cats were seropositive below the IFAT cut-off, yet seronegative by WB. In a recent publication, a cut-off dilution of 1:10 was established for seropositive cats with titers between 1:20 and 1:40 [40]. In light of our results, it seems reasonable to believe that the 1:80 cut-off point is suitable for

Table 4 Results of epidemiological surveys of feline leishmaniosis in Spain

Year	Region	Type of animal (domestic, mixed, stray)	Number of cats analyzed	Presence of clinical signs and clinicopathological findings	IFAT	ELISA	WB	DAT	PCR (sample)	Direct detection of *L. infantum*	References
2002	Barcelona and Girona	Domestic	117	N.A	N.A	1.7%	N.A	N.A	N.A	N.A	[53]
2002	Aragón	Domestic	50	Immunological alteration	N.A	N.A	N.A	42.00%	N.A	N.A	[32]
2007	Barcelona, Tarragona and Island of Mallorca	Mixed	445	N.A	N.A	5.30–6.30%	N.A	N.A	N.A	N.A	[34]
2007	Southern Spain	Domestic	183	N.A	28.30–60.00%	N.A	N.A	N.A	25.70% (blood)	1.63%	[28]
2008	Barcelona	Domestic	100	Yes; clinical signs or laboratory findings for positive cats inconsistent with leishmaniosis	N.A	N.A	N.A	N.A	3% (blood)	N.A	[13]
2008	Madrid	Domestic	233	Yes; relative lymphocytosis and an increase in alanine aminotransferase value	1.29–4.29%	N.A	N.A	N.A	0.43% (blood)	N.A	[27]
2011	Island of Mallorca	Stray	86	N.A	N.A	N.A	15.70%	N.A	26.00% (blood)	N.A	[29]
2011	Island of Mallorca and Ibiza	Mixed	105	Yes; cutaneous lesions	N.A	13.20%	N.A	N.A	8.70% (blood)	N.A	[11]
2011	Madrid	Domestic	20	N.A. Some cats co-infected with *Tritrichomas foetus*	15.00%	N.A	N.A	N.A	N.A	N.A	[54]
2012	Madrid	Mixed	680	N.A	3.70%	N.A	N.A	N.A	0.60% (blood)	N.A	[55]
2014	Madrid, Toledo and Guadalajara	Stray	346	Yes, 3.2%	3.20%	N.A	N.A	N.A	0.00% (blood)	N.A	[15]
2021	Zaragoza	Stray	180	No	2.23%	2.79%	14.52%	N.A	5.58% (blood)	N.A	This study

N.A., Not available

detecting the presence of anti-*Leishmania* antibodies in cats in endemic areas.

In areas endemic for *Trypanosoma* spp. or other *Leishmania* spp., cross-reactions with *L. infantum* must be taken into account when interpreting serological results, but this is not the case for the geographical area investigated in this study. Cross-reactions can occur in serological tests, especially when a whole-parasite antigen is used; thus, the possibility that new vector-borne pathogens in cats might be able to produce a false positive result for *L. infantum* should not be forgotten. However, in our study, all of the serum samples were collected from cats from the city of Zaragoza [41], a region in Spain where *Trypanosoma* species are not present and new vector-borne parasites have not been reported; moreover, *L. infantum* is the causative parasite of FeL in Europe.

The detection of *L. infantum* DNA by qPCR in different matrices has been evaluated in different studies, including non-invasive procedures to obtain the biological material, such as conjunctival swabs [42], or invasive procedures to obtain blood, lymph node and bone marrow samples [33]. Studies on stray cats in Spain have tested blood by qPCR to detect the parasite and found variable prevalence, depending on the study and the endemic region.

In the present study, qPCR positivity in blood material was slightly higher (5.6%) than in a study performed in Madrid, which involved a higher number of cats and did not detect the parasite DNA in blood samples [15]. The differences between our results and those of other epidemiological studies are likely due to the high rate of infection in cat population. Another possibility might be differences in the performance of the sampling technique between studies. Our findings are in disagreement with the suggestion of a recent study that blood is not a suitable tissue for the molecular diagnosis of *L. infantum* infection in cats [18], as the procedure to obtain blood is easy to perform in epidemiological studies.

In a previous study on of *Leishmania* infection in cats, diagnosis was based on the PCR analysis of blood, skin biopsy, bone marrow and conjunctiva, and positive results were obtained in 13%, 18.2%, 16% and 3.1% of the cats, respectively, the conjunctiva being the least frequently positive matrix [33]. Further research comparing other non-invasive samples (buccal swabs, brush sampling) for molecular diagnosis is needed.

The significant association between gender and *L. infantum* positivity by qPCR ($p = 0.026$) could be related to a risk factor. The higher proportion of positive males than females could be due to differences in behaviour that result in males being more exposed to sand fly bites. In general, male cats occupy a larger area than females and they tend to exist on the periphery of colonies, with large territories that may overlap with several groups of females [43]. One possible explanation for the absence of a significant association between the type of environment and positivity detected by serological techniques and/or qPCR is that *L. infantum* infection is present in both urban and peri-urban cat colonies, and therefore any cat in an urban and peri-urban area endemic for *L. infantum* could be potentially exposed.

FeLV and FIV are immunosuppressive diseases that leave cats susceptible to secondary infections, which may impair their welfare and reduce their lifespan [44]. An association between FIV and *L. infantum* is frequently detected [7], and FeL co-infection with FIV is more prevalent than that with FeLV. In our study, a statistical association was found between FIV and seropositivity detected by IFAT ($P = 0.023$) and ELISA ($P = 0.037$). In contrast, no association was observed between FeLV and *L. infantum* regardless of the technique. These results are in agreement with those reported in the literature [8].

This study, which assessed *L. infantum* infection in 180 asymptomatic stray cats from urban and peri-urban areas in the city of Zaragoza (Aragón, Spain), provides additional confirmation of the value of health surveillance programs for the detection and monitoring of diseases that affect domestic and stray animals. The control and elimination of leishmaniasis requires not only the detection of human and canine infection, but also an effective identification and control of other reservoirs as well as the implementation of vector control. These outdoor animal resevoirs are exposed to vector activity, which could lead to a possible source of infection, as occurred in hares in Fuenlabrada (Madrid, Spain) [45]. One study [46] reported that the density of *Phlebotomus perniciosus*, one of the two vectors of *L. infantum* in Spain, was positively correlated with the presence of cats. The opportunistic feeding behavior of *P. perniciosus*, taking blood meals from a range of animal reservoirs, has been demonstrated in Menorca [47] and other Mediterranean foci [48, 49]. The positive correlation of *P. perniciosus* density with cats is of epidemiological importance, because cats may act as additional reservoirs. None of the animals analyzed here by molecular and serological techniques showed clinical signs compatible with leishmaniosis, which supports the hypothesis that cats are likely reservoirs of *L. infantum* [50]. These findings are similar to results reported in dogs [51] and humans [52], both well known for the occurrence of a chronic subclinical infection. Although the blood of asymptomatic animals is not the most suitable sample to detect leishmaniosis infection, parasitemia was found in the blood of ten cats. Two of the qPCR-positive cats also tested positive in all the serological tests used. Analyzing blood samples for *Leishmania* DNA may help to determine the risk of infection transmission in stray cats.

According to the data provided by the Official College of Veterinarians of Zaragoza, there are 72,811 dogs and 4287 cats registered in the metropolitan area of Zaragoza. The cat population could be significantly higher as their registration is not mandatory in the region of Aragon. There are not any stray dogs, whereas stray cats can be seen around the city. Given the seroprevalence found in this study, the role these animals might play in the leishmaniosis cycle should not be underestimated, especially as the cats live close to humans in an endemic area.

Future studies should focus on determining the urban cycle of *L. infantum*, including the presence of other competent urban reservoirs. The role of the cat as a reservoir should be further explored, as well as the control and prevention of infection transmission in stray cats.

Conclusions

Our results demonstrate the presence of *L. infantum* infection in apparently healthy stray cats in the city of Zaragoza. Parasite DNA was detected in some feline blood samples. The presence of stray cats in urban areas is a factor in the epidemiology of feline infection, given that the evaluated cats were classified as healthy and asymptomatic.

Abbreviations

Cq: Quantification cycle; DAT: Direct agglutination test; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; EU: ELISA units; IFAT: Immunofluorescent antibody test; NNN: Novy, McNeal and Nicolle blood agar; qPCR: Quantitative real-time PCR; WB: Western blot.

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Authors' contributions

SVS, CR, MA and RF designed the survey. CR, MA, RF and SVS supervised the technical work. AB, AF, CR, MA, MV, RF, AY and SVS contributed with data analysis and wrote the manuscript. AG, AM and HR performed the physical examinations and collected samples from cats. AB, AF, MA and SVS performed serological testing and MA and SVS performed the molecular work of this study. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. All analysed data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This survey was included under Project Licence PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol. 2006;22:552–7. https://doi.org/10.1016/j.pt.2006.09.004.
2. Franco AO, Davies CR, Mylne A, Dedet JP, Gállego M, Ballart C, et al. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitology. 2011;138:1878–91. https://doi.org/10.1017/S003118201100148X.
3. Lucientes J, Palmero J, Guarga JL, Gracia MJ, Peribáñez MA, Zárate J, et al. Risk of transmission of canine leishmaniosis in eastern Spain. Vet Rec. 2005;156:743–4. https://doi.org/10.1136/vr.156.23.743.
4. Killick-Kendrick R. The biology and control of phlebotomine sand flies. Clin Dermatol. 1999;17:279–89. https://doi.org/10.1016/s0738-081x(99)00046-2.
5. Spada E, Canzi I, Baggiani L, Perego R, Vitale F, Migliazzo A, Proverbio D. Prevalence of *Leishmania infantum* and co-infections in stray cats in northern Italy. Comp Immunol Microbiol Infect Dis. 2016;45:53–8. https://doi.org/10.1016/j.cimid.2016.03.001.
6. Priolo V, Martínez-Orellana P, Pennisi MG, Masucci M, Prandi D, Ippolito D, et al. *Leishmania infantum*-specific IFN-γ production in stimulated blood from cats living in areas where canine leishmaniosis is endemic. Parasites Vectors. 2019;12:133. https://doi.org/10.1186/s13071-019-3386-y.
7. Pennis MG, Persichetti MF. Feline leishmaniosis: is the cat a small dog? Vet Parasitol. 2018;251:131–7. https://doi.org/10.1016/j.vetpar.2018.01.012.
8. Pennisi MG, Cardoso L, Baneth G, Bourdeau P, Koutinas A, Miró G, et al. LeishVet update and recommendations on feline leishmaniosis. Parasites Vectors. 2015;8:302. https://doi.org/10.1186/s13071-015-0909-z.
9. Spada E, Proverbio D, Migliazzo A, Della Pepa A, Perego R, Bagnagatti G. Serological and molecular evaluation of *Leishmania infantum* infection in stray cats in a non endemic area in Northern Italy. Parasitology. 2013. https://doi.org/10.5402/2013/916376.
10. Sarkari B, Hatam GR, Adnani SJ, Asgari Q. Seroprevalence of feline leishmaniasis in areas of Iran where *Leishmania infantum* is endemic. Ann Trop Med Parasit. 2009;103:275–7. https://doi.org/10.1179/136485909X398276.
11. Sherry K, Miró G, Trotta M, Miranda C, Montoya A, Espinosa C, et al. A serological and molecular study of *Leishmania infantum* infection in cats from the Island of Ibiza (Spain). Vector-Borne Zoonotic. 2011;11:239–45. https://doi.org/10.1089/vbz.2009.0251.
12. Persichetti MF, Solano-Gallego L, Vullo A, Masucci M, Marty P, Delaunay P, et al. Diagnostic performance of ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum* antibodies in cats using a Bayesian analysis without a gold standard. Parasites Vectors. 2017;10:119. https://doi.org/10.1186/s13071-017-2046-3.
13. Tabar MD, Altet L, Francino O, Sánchez A, Ferrer L, Roura X. Vector-borne infections in cats: molecular study in Barcelona area (Spain). Vet Parasitol. 2008;151:332–6. https://doi.org/10.1016/j.vetpar.2007.10.019.
14. Dedola C, Zobba R, Varcasia A, Visco S, Alberti A, Pipia A, et al. Serological and molecular detection of *Leishmania infantum* in cats of Northern

Sardinia, Italy. Vet Parasitol Reg Stud Rep. 2018;13:120–3. https://doi.org/10.1016/j.vprsr.2018.05.003.
15. Miró G, Rupérez C, Checa R, Gálvez R, Hernández L, García M, et al. Current status of *L. infantum* infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis? Parasites Vectors. 2014;7:112. https://doi.org/10.1186/1756-3305-7-112.
16. Ozon C, Marty P, Pratlong F, Breton C, Blein M, Lelièvre A, et al. Disseminated feline leishmaniosis due to *Leishmania infantum* in Southern France. Vet Parasitol. 1998;75:273–7. https://doi.org/10.1016/s0304-4017(97)00174-x.
17. Vilhena H, Martinez-Díaz VL, Cardoso L, Vieira L, Altet L, Francino O, et al. Feline vector-borne pathogens in the north and centre of Portugal. Parasites Vectors. 2013;6:99. https://doi.org/10.1186/1756-3305-6-99.
18. Iatta R, Furlanello T, Colella V, Tarallo VD, Latrofa MS, Brianti E, et al. A nationwide survey of *Leishmania infantum* infection in cats and associated risk factors in Italy. PLOS Negl Trop Dis. 2019;13:e0007594. https://doi.org/10.1371/journal.pntd.0007594.
19. WHO/Technical Report of the TDR Disease Reference Group on Chagas Disease, Human African Trypanosomiasis and Leishmaniasis. Research priorities for Chagas disease, human African trypanosomiasis and leishmaniasis: WHO TRS N°975. 975. 2012. https://www.who.int/neglected_diseases/resources/who_trs_975/en/. Accessed 25 Apr 2020.
20. Riera C, Valladares JE, Gállego M, Aisa MJ, Castillejo S, Fisa R, et al. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. Vet Parasitol. 1999;84:33–47. https://doi.org/10.1016/s0304-4017(99)00084-9.
21. Aisa MJ, Castillejo S, Gallego M, Fisa R, Riera MC, de Colmenares M, et al. Diagnostic potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. Am J Trop Med Hyg. 1998;58:154–9. https://doi.org/10.4269/ajtmh.1998.58.154.
22. Iniesta L, Fernández-Barredo S, Bulle B, Gómez MT, Piarroux R, Gállego M, et al. Diagnostic techniques to detect cryptic leishmaniasis in dogs. Clin Diag Lab Immunol. 2002;9:1137–41. https://doi.org/10.1128/cdli.9.5.1137-1141.2002.
23. Martín-Ezquerra G, Fisa R, Riera C, Rocamora V, Fernández-Casado A, Barranco G, et al. Role of *Leishmania* spp. infestation in nondiagnostic cutaneous granulomatous lesions: report of a series of patients from a Western Mediterranean area. Br J Dermatol. 2009;161:320–5. https://doi.org/10.1111/j.1365-2133.2009.09282.x.
24. Ondovilla AG. Un chat infecté par Leishmania en Espagne. Trabajos. 1993;2:26–7.
25. Fernández-Gallego A, Feo Bernabe L, Dalmau A, Esteban-Saltiveri D, Font A, Leiva M, et al. Feline leishmaniosis: diagnosis, treatment and outcome in 16 cats. J Fel Med Surg. 2020;22(10):993–1007. https://doi.org/10.1177/1098612X20902865 .
26. Giner J, Basurco A, Alcover MM, Riera C, Fisa R, López RA, et al. First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain. Vet Parasitol Reg Stud Rep. 2020;19:100369. https://doi.org/10.1016/j.vprsr.2020.100369.
27. Ayllón T, Tesouro MA, Amusategui I, Villaescusa A, Rodriguez-Franco F, Sainz A. Serologic and molecular evaluation of *Leishmania infantum* in cats from Central Spain. Ann N Y Acad Sci. 2008;1149:361–4. https://doi.org/10.1196/annals.1428.019.
28. Martín-Sánchez J, Acedo C, Muñoz-Pérez M, Pesson B, Marchal O, Morillas-Márquez F. Infection by *Leishmania infantum* in cats: epidemiological study in Spain. Vet Parasitol. 2007;145(2):67–73. https://doi.org/10.1016/j.vetpar.2006.11.005.
29. Millán J, Zanet S, Gomis M, Trisciuoglio A, Negre N, Ferroglio E. An investigation into alternative reservoirs of canine leishmaniasis on the endemic island of Mallorca (Spain). Trans Emerg Dis. 2011;58:352–7. https://doi.org/10.1111/j.1865-1682.2011.01212.x.
30. Castillo Hernández JA, Sánchez Acedo C, Gutiérrez Galindo J, Lucientes Curdi J, Estrada Peña A, et al. Evaluación de diversas pruebas en el diagnóstico de la leishmaniasis canina. In: IV Congreso Nacional de Parasitología, 9–13 July, Tenerife, Spain; 1985. p. 31
31. Boletín Epidemiológico Semanal de Aragón. Semana 32/2020. Zaragoza: Departamento de Sanidad del Gobierno de Aragón. https://www.aragon.es/documents/20127/1650151/BOLETIN+ARAGON+322020.pdf/532edf77-c1c8-94d3-4c22-336e91d98226?t=1597325644731. Accessed 5 May 2020.
32. Zárate-Ramos JJ, Arbea-Sarasa I, Gómez-Ochoa P, Castillo-Hernández JA, García-Salinas, MJ, Morales-Amella MJ. Serological evidence of leishmaniasis in cats in Aragon, Spain. In: Proceedings of the 27th WSAVA Congress. Granada: World Small Animal Veterinary Association; 2002.
33. Chatzis MK, Andreadou M, Leontides L, Kasabalis D, Mylonakis M, Koutinas AF, et al. Cytological and molecular detection of *Leishmania infantum* in different tissues of clinically normal and sick cats. Vet Parasitol. 2014;202:217–25. https://doi.org/10.1016/j.vetpar.2014.02.044.
34. Solano-Gallego L, Rodríguez-Cortés A, Iniesta L, Quintana J, Pastor J, Espada Y, et al. Cross-sectional serosurvey of feline leishmaniasis in ecoregions around the Northwestern Mediterranean. Am J Trop Med Hyg. 2007;76:676–80.
35. Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg. 2004;98:102–10. https://doi.org/10.1016/s0035-9203(03)00015-4.
36. Sobrino R, Ferroglio E, Oleaga A, Romano A, Millan J, Revilla M, et al. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet Parasitol. 2008;2008(155):198–203. https://doi.org/10.1016/j.vetpar.2008.05.003.
37. Alcover MM, Ribas A, Guillén MC, Berenguer D, Tomás-Pérez M, Riera C, et al. Wild mammals as potential silent reservoirs of *Leishmania infantum* in a Mediterranean area. Prev Vet Med. 2020;175:104874.
38. Riera C, Fisa R, López-Chejade P, Serra T, Girona E, Jiménez M, et al. Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain). Transfusion. 2008;48:1383–9. https://doi.org/10.1111/j.1537-2995.2008.01708.x.
39. Guillén MC, Alcover MM, Borruel N, Sulleiro E, Salvador F, Berenguer D, et al. *Leishmania infantum* asymptomatic infection in inflammatory bowel disease patients under anti-TNF therapy. Heliyon. 2020;6:e03940. https://doi.org/10.1016/j.heliyon.2020.e03940.
40. Chatzis MK, Leontides L, Athanasiou LV, Papadopoulos E, Kasabalis D, Mylonakis M, et al. Evaluation of indirect immunofluorescence antibody test and enzyme-linked immunosorbent assay for the diagnosis of infection by *Leishmania infantum* in clinically normal and sick cats. Exp Parasitol. 2014;147:54–9. https://doi.org/10.1016/j.exppara.2014.10.004.
41. Villanueva-Saz S, Basurco A, Martín V, Fernández A, Loste A, Verde MT. Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs. Acta Vet Scand. 2019;61:38. https://doi.org/10.1186/s13028-019-0473-1.
42. Benassi JC, Benvenga GU, Ferreira HL, Pereira VF, Keid LB, Soares R, et al. Detection of *Leishmania infantum* DNA in conjunctival swabs of cats by quantitative real-time PCR. Exp Parasitol. 2017;177:93–7. https://doi.org/10.1016/j.exppara.2017.04.004.
43. Liberg O. Density, spatial organization and reproductive tactics in the domestic cat and other felids. In: Turner D, Bateson P, editors. The domestic cat: the biology of its behaviour. 2nd ed. Cambridge: Cambridge University Press; 2000. p. 120–47.
44. Burling AN, Levy JK, Scott HM, Crandall MM, Tucker SJ, Wood EG, Foster JD, et al. Seroprevalences of feline leukemia virus and feline immunodeficiency virus infection in cats in the United States and Canada and risk factors for seropositivity. J Am Vet Med Assoc. 2017;2017(251):187–94. https://doi.org/10.2460/javma.251.2.187.
45. Carrillo E, Moreno J, Cruz I. What is responsible for a large and unusual outbreak of leishmaniasis in Madrid? Trends Parasitol. 2013;29:579–80. https://doi.org/10.1016/j.pt.2013.10.007.
46. Alcover MM, Ballart C, Serra T, Castells X, Scalone A, Castillejo S, et al. Temporal trends in canine leishmaniosis in the Balearic Islands (Spain): a veterinary questionnaire. Prospective canine leishmaniosis survey and entomological studies conducted on the Island of Minorca, 20 years after first data were obtained. Acta Trop. 2013;128:642–51.
47. De Colmenares M, Portús M, Botet J, Dobaño C, Gállego M, Wolff M, et al. Identification of blood meals of *Phlebotomus perniciosus* (Diptera: Psychodidae) in Spain by a competitive enzyme-linked immunosorbent assay biotin/avidin method. J Med Entomol. 1995;32:229–33. https://doi.org/10.1093/jmedent/32.3.229.
48. Branco S, Alves-Pires C, Maia C, Cortes S, Cristovão JM, Gonçalves L, et al. Entomological and ecological studies in a new potential zoonotic leishmaniasis focus in Torres Novas municipality, Central Region, Portugal.

Acta Trop. 2013;125:339–48. https://doi.org/10.1016/j.actatropica.2012.12.008.
49. Morosetti G, Bongiorno G, Beran B, Scalone A, Moser J, Gramiccia M, et al. Risk assessment for canine leishmaniasis spreading in the north of Italy. Geospat Health. 2009;4:115–27. https://doi.org/10.4081/gh.2009.214.
50. Lima BS, Dantas-Torres F, de Carvalho MR, Marinho-Junior JF, de Almeida EL, Brito ME, et al. Small mammals as hosts of *Leishmania* spp. in a highly endemic area for zoonotic leishmaniasis in North-Eastern Brazil. Trans R Soc Trop Med Hyg. 2013;107:592–7. https://doi.org/10.1093/trstmh/trt062.
51. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis—new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008;24:324–30. https://doi.org/10.1016/j.pt.2008.04.001.
52. Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of *Leishmania infantum* (*L. chagasi*) in human. Acta Trop. 2011;119:69–75. https://doi.org/10.1016/j.actatropica.2011.05.012.
53. Portús M, Gállego M, Riera MC, Aisa MJ, Fisa R, Castillejo S. Wild and domestic mammals in the life cycle of *Leishmania infantum* in Southwest Europe. A literature review and studies performed in Catalonia (Spain). Rev Iber Parasitol. 2002;62:72–6.
54. Miró G, Hernández L, Montoya A, Arranz-Solís D, Dado D, Rojo-Montejo S. First description of naturally acquired *Tritrichomonas foetus* infection in a Persian cattery in Spain. Parasitol Res. 2011;109:1151–4. https://doi.org/10.1007/s00436-011-2359-7.
55. Ayllón T, Diniz PP, Breitschwerdt EB, Villaescusa A, Rodríguez-Franco F, Sainz A. Vector-borne diseases in client-owned and stray cats from Madrid, Spain. Vector-Borne Zoonotic. 2012;12:143–50. https://doi.org/10.1089/vbz.2011.0729.

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4. DISCUSIÓN:

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La investigación recogida en la presente tesis doctoral ha permitido avanzar en el conocimiento de la infección por *Leishmania infantum* en mamíferos de los que hasta ahora no se tenía constancia, como es el caso del hurón doméstico; así como nueva información epidemiológica sobre la infección en gatos de vida libre en la zona de influencia de la ciudad de Zaragoza, llevando a cabo, por primera vez, un estudio epidemiológico en el que se han utilizado hasta cuatro pruebas de confirmación de la infección en el gato incluyendo pruebas de diagnóstico serológico como la técnica ELISA, la técnica de inmunofluorescencia indirecta y la técnica de Western Blot, como una prueba molecular cuantitativa. No hay que olvidar que el principal reservorio peridoméstico de la infección para el hombre es el perro; por ello se ha querido evaluar el rendimiento diagnóstico de una prueba rápida de detección de anticuerpos mediante su comparación frente a dos pruebas cuantitativas consideradas de referencias, como el ELISA y la técnica de inmunofluorescencia indirecta. Adicionalmente, en ausencia de una prueba de referencia o prueba "*Gold Standard*" para la infección por *L. infantum* en el perro, una aproximación de evaluación de cualquier prueba, en igualdad de condiciones, sería la utilización del análisis bayesiano. Dada la amplitud y diversidad de la investigación, sobre todo centrada en los avances sobre el conocimiento del diagnóstico de la infección, se procede a realizar una discusión general dividiendo está en los diferentes aspectos estudiados:

4.1 Diagnóstico serológico de la infección por *L. infantum* en el perro

El conocimiento científico actual sobre la infección de este parásito en perros ha evolucionado notablemente. Inicialmente, la infección por *L. infantum* en perros se consideraba equivalente a enfermedad (Slappendel, 1988). Sin embargo, estudios posteriores revelaron que los perros infectados eran capaces de generar una respuesta inmunitaria celular contra el parásito (Cardoso et al., 1998). Esto demostró que una respuesta inmunitaria adecuada podía permitir que algunos perros infectados por *L. infantum* controlasen la infección sin manifestar síntomas clínicos (Baneth et al., 2008).

La respuesta humoral ha sido analizada en detalle, y se ha encontrado que es posible identificar diferentes tipos de inmunoglobulinas *anti-Leishmania* (Rodríguez-Cortés et al., 2007), siendo la inmunoglobulina tipo G la predominante (Martínez-Moreno et al., 1995). Así, tanto la detección, como la cuantificación de inmunoglobulinas resultan esenciales, ya que pueden indicar tanto la enfermedad (Solano-Gallego et al., 2009) como la exposición (Cavalera et al., 2021). Esto hace que las pruebas de medición de la respuesta humoral sean las más utilizadas, tanto para confirmar sospechas clínicas, como para el cribado rutinario de perros infectados (Maia & Campino, 2008).

La falta de una prueba de referencia es un problema recurrente en la epidemiología de enfermedades infecciosas (Boelaert et al., 1999), incluyendo la infección por *L. infantum* en perros (Rodríguez-Cortés et al., 2010). La prueba de referencia debería permitir discriminar con claridad entre animales infectados y no infectados (Rodríguez-Cortés et al., 2010). Sin embargo, la ausencia de una prueba estándar en animales complica la interpretación de resultados, ya que estos se definen en relación con la enfermedad, y no con la mera presencia de infección (Boelaert et al., 1999). Además, no todos los perros infectados desarrollan síntomas, aunque pueden estar infectados por *L. infantum* (Solano-Gallego et al., 2009). Esto impacta en la eficacia diagnóstica de cualquier prueba, ya que no siempre es posible identificar todos los perros infectados. En general, el diagnóstico de la enfermedad debe basarse en un enfoque integral, que combine la historia clínica, los hallazgos de un examen físico exhaustivo, las pruebas de laboratorio y al menos un resultado positivo en pruebas de confirmación de infección.

Para evaluar y validar pruebas de diagnóstico serológico, se recomienda el uso de muestras comparables, aunque actualmente está estandarización es difícil de lograr. Estas muestras permitirían una mejor comparación entre pruebas diagnósticas. Una forma de evaluar pruebas serológicas es mediante infecciones experimentales en perros (Rodríguez-Cortés et al., 2010). Aunque estos estudios permiten observar la infección en tiempos menores que en infecciones naturales, presentan limitaciones como la variabilidad en el desarrollo de la enfermedad y diferencias en la respuesta clínica. Factores como la vía de administración, dosis de parásitos y presencia o ausencia de componentes de la saliva del vector también influyen en el desarrollo de la infección (Gradoni, 2015). Además, el uso de perros genéticamente homogéneos en estudios experimentales puede reducir la variabilidad clínica observada en condiciones naturales, donde la evolución de la infección es muy variable.

Comparar pruebas serológicas para la infección por *L. infantum* presenta dificultades, ya que la sensibilidad y especificidad dependen del tipo y pureza del antígeno. En técnicas como ELISA, se pueden usar varios antígenos (promastigotes, proteínas recombinantes o purificadas), lo que genera diferencias en los resultados, aun empleando la misma técnica.

Sin embargo, la infección experimental es útil, resulta costosa y es objeto de críticas por razones éticas. Otra estrategia para evaluar pruebas serológicas es usar una prueba contrastada como estándar; por ejemplo, la técnica IFI, recomendada como prueba serológica de referencia por la Organización Mundial de Sanidad Animal (OIE) (Wolf et al., 2014). También es posible combinar dos o más pruebas similares para mejorar la caracterización de las muestras analizadas, aunque esto puede generar discrepancias en los resultados. Una alternativa es considerar tanto los resultados diagnósticos como la información clínicopatológica de los perros de los que se extraen las muestras.

La utilización de diferentes grupos de perros en relación con la infección por *L. infantum* (perros no infectados, infectados clínicamente sanos, enfermos, y con otras enfermedades infecciosas) permitiría una comparación más precisa del rendimiento de las pruebas, evaluando posibles reacciones cruzadas (Villanueva-Saz et al., 2022). Los modelos matemáticos de análisis de clases latentes, en los que se considera la probabilidad de combinación de resultados diagnósticos (Goodman, 1974), han sido útiles en esta investigación como una alternativa a los métodos tradicionales, superando la necesidad de una prueba estándar o de muestras con serología conocida. Así, estimamos la sensibilidad y especificidad de tres pruebas serológicas en el área mediterránea europea para la detección de anticuerpos *anti-Leishmania*.

Por último, el análisis del área bajo la curva ROC (receiver operating characteristic), aunque no es ampliamente usado en estudios de pruebas diagnósticas, ofrece ventajas significativas. Este análisis permite determinar el punto de corte óptimo en pruebas cuantitativas, mejorando su precisión (Gardner & Greiner, 2006). Sin embargo, en pruebas rápidas con resultados dicotómicos, la curva ROC no puede aportar un punto de corte que maximice el rendimiento.

4.2 Primera descripción de la enfermedad en un hurón doméstico y las implicaciones de cara al diagnóstico de la infección y de la enfermedad.

En esta investigación se describe el primer caso clínico de leishmaniosis en un hurón doméstico (*Mustela putorius furo*) naturalmente infectado, y en el que se pudo aislar pudiendo aislar el parásito; convirtiéndose dicha cepa de *L. infantum* en cepa de referencia MMST/ES/2019/ZGZ103. En este caso, el diagnóstico de la infección se basó en la combinación de diferentes pruebas de confirmación de la infección, incluyendo pruebas de diagnóstico serológico adaptadas para dicha especie, el diagnóstico molecular en diferentes muestras biológicas, el examen histopatológico en combinación con la técnica inmunohistoquímica especifica frente a *Leishmania* y, finalmente, el cultivo y aislamiento parasitológico. A pesar de ser un hallazgo de interés y de gran relevancia para los veterinarios clínicos, la primera descripción de la enfermedad en hurón en condiciones de infección experimental utilizando al hurón como modelo animal de investigación, se realizó en 1989 (White et al., 1989).

El hurón naturalmente infectado vivía en un entorno urbano de Valencia, en la costa este de la Península Ibérica frente al Golfo de Valencia en el Mar Mediterráneo, una zona geográfica donde *P. perniciosus* es la especie de flebotomo más prevalente detectada (Aransay et al., 2004; Gálvez et al. 2020) y la seroprevalencia en hurones es de 28,4% (Alcover et al. 2022). Así mismo, la incidencia de leishmaniosis cutánea y leishmaniosis mucocutánea humana en la zona de Valencia ha mostrado un aumento de 3,6 a 13,58 casos/100.000 habitantes en el periodo de estudio (2006-2017) (Garrido-Jareño et al., 2020).

El papel epidemiológico que pueden jugar especies animales silvestres ha sido demostrado en el caso de lagomorfos, felinos y los cánidos que no son perros; lo que puede significar un papel importante en el ciclo de transmisión de *L. infantum* (Pennisi, 2015; Tsakmakidis et al. 2024). El hurón de nuestro caso presentaba un nódulo en el pabellón auricular, que había sido diagnosticado como una dermatitis piogranulomatosa, muy similar a las presentaciones de leishmaniosis en gatos (dermatitis papular o nódulo en el sitio de inoculación del parásito) (Fernandez-Gallego et al. 2020). Las citologías compatibles con la leishmaniosis felina suelen tener una composición celular característica de la inflamación piogranulomatosa, granulomatosa o linfoplasmocítica y patrones similares en estudios histológicos (Di Mattia et al., 2018). El caso se confirmó mediante biopsia excisional seguida de inmunohistoquímica específica a *Leishmania,* evidenciándose la presencia de microorganismos ovales en el citoplasma de los macrófagos dérmicos al igual que han descrito otros autores (Migliazzo et al., 2015).

Así mismo, se obtuvo un resultado positivo mediante qPCR del bloque de parafina, además de la detección de ADN del parásito a partir de muestra de sangre periférica y del material aspirado de la zona extirpada perilesional. La completa caracterización, desde un punto de vista diagnóstico, pudo llevarse a cabo debido a la disponibilidad de diversas técnicas de confirmación de la infección. Es importante recalcar que algunas técnicas como las de tipo parasitológico, como la citología, el estudio histopatológico y las pruebas moleculares pueden ser utilizadas independientemente de la especie animal. Esto es posible debido a que estas pruebas se centran en la visualización morfológica del parásito en preparaciones de tipo citológico, o bien en preparaciones histológicas y su visualización e identificación dependerá en gran medida del propio procesamiento de la muestra y de la pericia y experiencia del observador de las preparaciones. En el caso del diagnóstico molecular cuantitativo, las pruebas se centran en la detección y cuantificación de la carga parasitaria presente en una muestra. Por tanto, al igual que lo comentando para las técnicas parasitológicas, cualquier muestra biológica con independencia de la especie animal de la que haya sido obtenida, potencialmente podremos detectar la presencia del ADN del parásito.

Consideramos que la relevancia de nuestra investigación se centra fundamentalmente en la adaptación de las técnicas serológicas (que tradicionalmente se usan en el perro y el gato), para que sean capaces de detectar la respuesta inmunitaria de tipo humoral que produce el hurón enfermo frente a *L. infantum*. Tal como describimos, muestras de suero de este paciente fueron evaluadas mediante diversas técnicas. Sin embargo, con la técnica inmunofluorescencia indirecta demostramos que, dependiendo del conjugado empleado, puede detectarse marcaje (resultado positivo) o no (resultado negativo). En este trabajo utilizando conjugado específico de perro obtuvimos un resultado negativo en la serología por inmunofluorescencia indirecta; mientras que, al utilizar un conjugado específico de hurón, el resultado fue positivo. Desde un punto de vista de traslado a la práctica clínica, en la mayor parte de las pruebas rápidas de inmunocromatográficas para la detección de anticuerpos *anti-Leishmania*, el conjugado empleado es específico de perro, por lo que no habrá reacción cruzada de anticuerpos y el resultado de la prueba será siempre negativo.

Desde un punto de vista metodológico, no siempre es posible adquirir conjugados específicos de especie, lo que dificultaría la posibilidad de detectar la presencia de anticuerpos *anti-Leishmania* en especies animales para los que no se han desarrollado conjugados específicos. Sin embargo, con el avance del conocimiento de la inmunología, sabemos que existen otros tipos de conjugados, como son la proteína A que se caracteriza por ser capaz de interactuar con la región fragmentaria cristalizable de la inmunoglobulina G de varios animales como hurones, perros (Riera et al., 1999), gatos (Solano-Gallego et al., 2007), y seres humanos (Fisa et al., 1997). Dicho conjugado fue utilizado tanto para la técnica ELISA, como para la técnica del Western Blot, detectando anticuerpos mediante ambas técnicas. En el caso del Western Blot, se observó el mismo patrón de inmunorreactividad que en los perros (Ballart et al., 2013), en los gatos (Persichetti et al., 2017) y en los seres humanos afectados de la enfermedad (Fisa et al., 1997).

4.3 Evaluación de la situación epidemiológica de *L. infantum* en gatos de vida libre en una zona endémica de España

En este estudio se evaluó la infección por *L. infantum* en 180 gatos de vida libre, aparentemente sanos, procedentes del entorno de la ciudad de Zaragoza, incluyendo áreas urbanas como periurbanas. Se utilizaron tanto pruebas de detección de anticuerpos *anti-Leishmania,* como pruebas de detección del ADN del parásito en sangre circulante. Dado que el papel epidemiológico del gato en relación a la infección por *L. infantum*, aún no se conoce totalmente (Pereira & Maia, 2021), la realización de estudios epidemiológicos puede ser útil para dilucidar el potencial que puede jugar esta especie en áreas urbanas y periurbanas.

Diferentes estudios han identificado que la prevalencia de infección por *L. infantum* en gatos en zonas endémicas varía del 0% al 68,5% (Fernández-Gallego et al., 2020). Estudios epidemiológicos realizados en diferentes regiones de España informan de seroprevalencias de *L. infantum* que van desde el 1,29% hasta el 60% (Ayllon et al., 2008; Martín-Sánchez et al., 2007). Cuando se ha utilizado la PCR para la detección del ADN parasitario, la prevalencia de la infección por *L. infantum* en gatos en España varía del 0,43% al 26% (Ayllon et al., 2008, Millán et al., 2011). El único estudio publicado disponible sobre la prevalencia de la infección por *L. infantum* en gatos en la región de Aragón es una comunicación sobre un estudio de 50 gatos domésticos, de los cuales el 42% fueron seropositivos mediante DAT y mostraron disfunción inmunológica (Zárate-Ramos et al., 2002).

Nuestro estudio se centró en el área metropolitana de Zaragoza donde, según los datos proporcionados por el Colegio Oficial de Veterinarios de Zaragoza, hay 72.811 perros y 4.287 gatos censados. La población de gatos podría ser significativamente mayor, ya que su registro no es obligatorio en la región de Aragón. Así mismo, aplicamos tres técnicas serológicas diferentes y una prueba molecular para evaluar la prevalencia global de la infección. Ninguno de los gatos positivos analizados en este estudio, por técnicas moleculares y serológicas, mostró signos clínicos compatibles con leishmaniosis; lo que respalda la hipótesis de que los gatos son probablemente reservorios de *L. infantum* (Pennisi & Persichetti, 2018; Ahuir-Baraja et al., 2021; Chatzis et al., 2014a; Chatzis et al., 2014b; Solano-Gallego et al., 2007). Esto puede ser debido a la existencia de una infección subclínica crónica como sucede en la leishmaniosis canina (Baneth et al., 2008).

Mediante las técnicas serológicas utilizadas en este estudio, se detectó una positividad del 14,5% mediante WB en muestras de suero felino; lo que es similar a la positividad informada en estudios realizados en zonas endémicas de leishmaniosis canina como Mallorca (15,70%) (Millán et al., 2011) o, más recientemente, el Noroeste de Italia (Liguria) (13,20%) (Elmahallawy et al., 2021). La técnica ELISA fue ligeramente más sensible que la IFI en términos de detección de anticuerpos contra *L. infantum*, en contraste con el estudio de Persichetti et al. (2017).

En el análisis de WB, se detectaron bandas asociadas con mayor frecuencia con el diagnóstico de infección por *L. infantum*, particularmente una banda con un peso molecular de 46 kDa y bandas con bajo peso molecular, como 14, 16, 18 y 20 kDa. Esto está en concordancia con lo que otros autores han publicado (Persichetti et al., 2017, Solano-Gallego et al., 2007). Se detectaron bandas en 14, 16, 36 y 46 kDa en dos gatos que dieron positivo para la infección por *L. infantum* por las otras tres pruebas; y la banda de 46 kDa se detectó en nueve gatos con un resultado positivo en qPCR. En seres humanos y perros asintomáticos, la presencia de las fracciones polipeptídicas de 14 y/o 16 kDa, junto con otras bandas de bajo peso molecular, indican infección o exposición (Riera et al., 2004; Sobrino et al., 2008; Alcover et al., 2020).

En nuestro estudio, observamos bandas de bajo peso molecular en 16 gatos; tres de los cuales dieron un resultado positivo en qPCR, y la banda de 46 kDa se observó en dos de estos tres gatos. En seis gatos que mostraron reactividad solo frente a la fracción polipeptídica de 46 kDa, se detectó ADN de *Leishmania* en sangre mediante qPCR. Esto demuestra la necesidad de más estudios serológicos y moleculares para determinar la especificidad de esta banda y si se puede usar para detectar contacto previo con el parásito o infección asintomática en gatos y en seres humanos (Aisa et al., 1998; Iniesta et al., 2002; Riera et al., 2008; Guillén et al., 2020).Tanto el WB, como el ELISA, mostraron resultados de seroprevalencia diferentes (8,38% y 2,79%, respectivamente), probablemente debido a la diferente sensibilidad y especificidad de cada técnica.

Las pequeñas diferencias en la detección de anticuerpos *anti-Leishmania* entre las técnicas de ELISA e IFI podrían deberse al tipo de antígeno utilizado y al método técnico realizado para obtener los resultados. En este estudio, se aplicó una dilución de corte de 1:80 en la técnica de IFI, siguiendo las pautas de LeishVet (Pennisi et al., 2015). Sin embargo, algunos gatos fueron seropositivos por debajo del límite de corte de IFI, pero seronegativos en WB. En una publicación reciente, se estableció una dilución de corte de 1:10 para gatos seropositivos con títulos entre 1:20 y 1:40 (Chatzis et al., 2014b). A la luz de nuestros resultados, parece razonable creer que el punto de corte de 1:80 es adecuado para detectar la presencia de anticuerpos *anti-Leishmania* en gatos en áreas endémicas.

En el presente estudio, la positividad por qPCR en material sanguíneo fue del 5,6%. Este dato contrasta con estudios realizados en Madrid, que involucraron a un mayor número de gatos, donde la seroprevalencia también es menor (2,8-3,7%), y no se detectó el ADN del parásito en las muestras de sangre (Miró et al., 2014; Montoya et al., 2018) ni en biopsias de piel (Montoya et al., 2018). Nuestros hallazgos están en desacuerdo con un estudio que indica que la sangre no es una muestra biológica adecuada para la detección de la presencia del parásito en gatos (Iatta et al., 2019); además de que se trata de una muestra fácil de obtener para realizar estudios epidemiológicos. Aunque la sangre de animales asintomáticos podría no ser la muestra más adecuada para detectar la infección por leishmaniosis, en nuestro estudio se encontró parasitemia en la sangre de diez gatos. Dos de los gatos positivos en qPCR también dieron positivo en todas las pruebas serológicas utilizadas. Un estudio publicado recientemente encontró una asociación significativa entre la positividad de IFI y qPCR en gatos infectados, lo que muestra que los gatos que dan positivo en qPCR tienen una probabilidad 11 veces mayor de ser seropositivos para *L. infantum* IgG en la prueba IFI.

Curiosamente, esta asociación fue significativa sólo para la PCR en sangre. Por lo tanto, los gatos con infección generalizada, es decir, aquellos en los que se encontraron amastigotes en el sistema reticuloendotelial, como en el tejido de los linfonodos, no siempre fueron seropositivos para anticuerpos frente al parásito (Spada et al., 2023). Esto subraya el hecho de que se deben aplicar tantas técnicas como sea posible para identificar a los sujetos infectados (Chatzis et al., 2014a)

Consideramos que analizar muestras de sangre para detectar ADN de *Leishmania*, puede ayudar a determinar el riesgo de transmisión de infección en gatos callejeros. Las diferencias entre nuestros resultados y los de otros estudios epidemiológicos, probablemente, se deban a la alta tasa de infección en la población felina ya que se ha demostrado que la positividad molecular para *L. infantum* es paralela a la aparición de una mayor seroprevalencia en los animales examinados (Sherry et al., 2011).

Dentro de los factores de riesgo investigados, hemos detectado la asociación significativa entre el género y la positividad de *L. infantum* por qPCR, pero no con el resto de las pruebas realizadas. Otros autores también han observado una mayor seroprevalencia en gatos machos (Montoya et al., 2018; Spada et al., 2023), aunque, aparentemente, la infección por *L. infantum* no está relacionada con el sexo en perros o gatos (Montoya et al., 2018). La mayor proporción de positivos en machos que en hembras podría deberse a diferencias en el comportamiento, lo que se traduce en que los machos estén más expuestos a las picaduras de mosquitos (Liberg, 2000). Iatta et al. (2019) también observaron factores de riesgo significativos entre el estado de castración y la edad. Lo justifican basándose en que los gatos no castrados y mayores de 18 meses son más propensos a un estilo de vida libre que los gatos más jóvenes y castrados; además de que poseen instinto depredador más pronunciado, por lo que, estos gatos están más expuestos a las picaduras de flebotomos, lo que con el tiempo genera un mayor riesgo de infección por *L. infantum* (Iatta et al., 2019, Spada et al., 2023). En nuestro caso, todos los animales estaban dentro de un proyecto de esterilización para ser castrados y eran mayores de un año.

Al contrario de lo que observaron Spada et al. (2023), no vimos la asociación significativa entre el tipo de entorno y la positividad detectada por técnicas serológicas y / o qPCR (Spada et al., 2023). Nosotros pensamos que esto se debe a que la infección por *L. infantum* está presente en colonias de gatos urbanas y periurbanas, y por lo tanto cualquier gato en un área urbana y periurbana endémica para *L. infantum* podría estar potencialmente expuesto.

A todos los gatos del estudio se les realizó las pruebas de FeLV y FIV por medio de un test rápido inmunocromatográfico. Se encontró una asociación estadística entre FIV y seropositividad frente a *L. infantum* detectada por IFI y ELISA. En contraste, no se observó ninguna asociación entre FeLV y el parásito, independientemente de la técnica utilizada. Estos resultados están de acuerdo con los reportados en la literatura (Pennisi et al., 2015; Priolo et al., 2022b). La asociación entre FIV y *L. infantum* se detecta con frecuencia, y la coinfección *Leishmania* con FIV es más prevalente que con FeLV, aunque otros estudios no lograron corroborar estos hallazgos (Fernández-Gallego et al., 2020). La leishmaniosis felina se ha asociado con enfermedades inmunosupresoras como las infecciones retrovirales (FIV y FeLV), tratamientos inmunosupresores y enfermedades debilitantes concomitantes como neoplasias malignas o la diabetes mellitus (Fernández-Gallego et al., 2020; García-Torres et al., 2022), que dejan a los gatos susceptibles expuestos a infecciones secundarias, lo que puede afectar a su bienestar y reducir su esperanza de vida (Burling et al., 2017). Sin embargo, otros estudios no lograron corroborar este hallazgo por lo que la relación causa-efecto entre diversos factores etiológicos y patogénicos no siempre es fácil de establecer (Pennisi et al., 2015, Pennisi et al., 2013).

5. CONCLUSIONES:

5. CONCLUSIONES:

De los estudios realizados, que componen esta tesis doctoral, y, teniendo en cuenta las condiciones de trabajo, hemos obtenido las siguientes conclusiones:

- La prueba rápida de inmunocromatografía FASTest LEISH® ha demostrado ser una herramienta útil para la detección de perros seropositivos clínicamente sanos.
- La prueba rápida inmunocromatográfica FASTest LEISH® ha presentado niveles de sensibilidad y especificidad comparables a los de las pruebas cuantitativas de inmunofluorescencia indirecta y del ensayo por inmunoadsorción ligado a enzimas, utilizadas, ambas, en el análisis comparativo.
- El análisis de clases latentes ha demostrado ser una alternativa eficaz a los métodos tradicionales para evaluar el rendimiento diagnóstico de las pruebas serológicas utilizadas en la detección de anticuerpos anti-*Leishmania* en perros.
- La aplicación del análisis de clases latentes ha permitido estimar, con precisión, la sensibilidad y especificidad de tres pruebas serológicas distintas (inmunocromatografía, inmunofluorescencia indirecta y ELISA) utilizadas en la región mediterránea para la detección de anticuerpos anti-*Leishmania*.
- Nuestros resultados han evidenciado la presencia de infección por *Leishmania infantum* en gatos de vida libre, aparentemente sanos, en la ciudad de Zaragoza, detectándose ADN del parásito en muestras de sangre y anticuerpos anti-*Leishmania* en suero, con una tasa global de infección del 15,6%.
- Entre los métodos de confirmación empleados para la detección de la infección en gatos, la técnica de Western Blot fue la que identificó un mayor número de animales positivos, seguida de la reacción en cadena de la polimerasa
- Según nuestros resultados, de las pruebas serológicas y moleculares se puede considerar que los gatos de vida libre podrían desempeñar un papel potencial en la transmisión y mantenimiento de la infección en zonas urbanas endémicas.
- El hallazgo de un hurón con leishmaniosis clínica en una zona endémica, sugiere la consideración esta especie como un posible reservorio para la infección.

6. ANEXO:

Comisión ética asesora para la experimentación animal
Universidad Zaragoza

Fecha: 18 de enero de 2018
Ref. PI62/17
Destinatario/s:
Sergio Villanueva Saz
Farmacología y Fisiología
Facultad de Veterinaria

Asunto: Remitiendo informe emitido por la Comisión Ética Asesora para la Experimentación Animal sobre procedimiento de experimentación.

Adjunto le remito el informe que la Comisión Ética Asesora para la Experimentación Animal de la Universidad de Zaragoza acordó emitir en su reunión de 11 de enero de 2018 respecto al procedimiento "Situación epidemiológica de felinos domésticos que viven en libertad".

El código de identificación asignado al procedimiento es el PI62/17. Este código puede serle requerido en la solicitud de prestación de servicios del Centro donde prevé realizar la experimentación y puede figurar entre los datos identificativos de las jaulas o sistemas de confinamiento de los animales.

En caso de que la Comisión haya considerado que el procedimiento presentado, no cumple en su presente forma con los requisitos exigidos, el investigador responsable podrá corregir los aspectos necesarios, atendiendo a lo indicado, pudiendo ser presentado nuevamente para su evaluación por esta Comisión.

Aprovecho la ocasión para mandarle un cordial saludo.

El Secretario de la Comisión

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7. BIBLIOGRAFÍA:

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Abbate JM, Arfuso F, Napoli E, Gaglio G, Giannetto S, Latrofa MS, Otranto D, Brianti E. *Leishmania infantum* in wild animals in endemic areas of southern Italy. Comp Immunol Microbiol Infect Dis. 2019; 67:101374. doi: 10.1016/j.cimid.2019.101374. .

Abbehusen MMC, Almeida VDA, Solcà MDS, Pereira LDS, Costa DJ, Gil-Santana L, Bozza PT, Fraga DBM, Veras PST, Dos-Santos WLC, Andrade BB, Brodskyn CI. Clinical and immunopathological findings during long term follow-up in *Leishmania infantum* experimentally infected dogs. Sci Rep. 2017; 7:15914. doi: 10.1038/s41598-017-15651-8.

Ahuir-Baraja AE, Ruiz MP, Garijo MM, Llobat L. Feline Leishmaniosis: An Emerging Public Health Problem. Vet Sci. 2021; 8:173. doi: 10.3390/vetsci8090173.

Aisa MJ, Castillejo S, Gallego M, Fisa R, Riera MC, de Colmenares M, Torras S, Roura X, Sentis J, Portus M. Diagnostic potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. Am J Trop Med Hyg. 1998; 58:154-159. doi: 10.4269/ajtmh.1998.58.154.

Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, Sereno D. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. PLoS Negl Trop Dis. 2016; 10:e0004349. doi: 10.1371/journal.pntd.0004349.

Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Cannet A, Ravel C, Marty P, Delaunay P, Kasbari M, Granouillac B, Gradoni L, Sereno D. Leishmania infections: Molecular targets and diagnosis. Mol Aspects Med. 2017; 57:1-29. doi: 10.1016/j.mam.2016.11.012.

Akhtardanesh B, Kheirandish R, Sharifi I, Mohammadi A, Mostafavi A, Mahmoodi T, Ebrahimi M. Low susceptibility of domestic cats to experimental *Leishmania infantum* infection. J Vector Borne Dis. 2018; 55:230-234. doi: 10.4103/0972-9062.249481.

Albuquerque A, Campino L, Cardoso L, Cortes S. Evaluation of four molecular methods to detect *Leishmania infection* in dogs. Parasit Vectors. 2017; 10:57. doi: 10.1186/s13071-017-2002-2.

Alcover MM, Ribas A, Guillén MC, Berenguer D, Tomás-Pérez M, Riera C, Fisa R. Wild mammals as potential silent reservoirs of *Leishmania infantum* in a Mediterranean area. Prev Vet Med. 2020; 175:104874. doi: 10.1016/j.prevetmed.2019.104874.

Alcover MM, Giner J, Rabasedas J, Roca-Geronés X, Verde M, Fernández A, Riera C, Fisa R, Villanueva-Saz S. First epidemiological survey of *Leishmania infantum* in the domestic ferret (*Mustela putorius furo*) in a canine leishmaniosis endemic area using serology and PCR. Parasit Vectors. 2022; 15:372. doi: 10.1186/s13071-022-05517-y.

Alvar J, Cañavate C, Molina R, Moreno J, Nieto J. Canine leishmaniasis. Adv Parasitol. 2004; 57:1-88. doi: 10.1016/S0065-308X(04)57001-X.

Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012; 7:e35671. doi: 10.1371/journal.pone.0035671.

Andrade HM, de Toledo Vde P, Marques MJ, França Silva JC, Tafuri WL, Mayrink W, Genaro O. *Leishmania* (*Leishmania*) *chagasi* is not vertically transmitted in dogs. Vet Parasitol. 2002; 103:71-81. doi: 10.1016/s0304-4017(01)00552-0.

Antoine JC, Prina E, Jouanne C, Bongrand P. Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. Infect Immun. 1990; 58:779-787. doi: 10.1128/iai.58.3.779-787.1990.

Antoniou M, Gramiccia M, Molina R, Dvorak V, Volf P. The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region. Euro Surveill. 2013; 18:20540. doi: 10.2807/1560-7917.es2013.18.30.20540.

Aransay AM, Testa JM, Morillas-Marquez F, Lucientes J, Ready PD. Distribution of sandfly species in relation to canine leishmaniasis from the Ebro Valley to Valencia, northeastern Spain. Parasitol Res. 2004; 94:416-420. doi: 10.1007/s00436-004-1231-4.

Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, de la Fuente S, Martínez AM, Pérez AM, Aránguez E, Iriso A, Sevillano O, Bernal J, Vilas F. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013; 18:20546. doi: 10.2807/1560-7917.es2013.18.30.20546.

Asfaram S, Fakhar M, Teshnizi SH. Is the cat an important reservoir host for visceral leishmaniasis? A systematic review with meta-analysis. J Venom Anim Toxins Incl Trop Dis. 2019; 25:e20190012. doi: 10.1590/1678-9199-JVATITD-2019-0012.

Ashford RW. Leishmaniasis reservoirs and their significance in control. Clin Dermatol. 1996; 14:523-532. doi: 10.1016/0738-081x(96)00041-7.

Ayllon T, Tesouro MA, Amusategui I, Villaescusa A, Rodriguez-Franco F, Sainz A. Serologic and molecular evaluation of *Leishmania infantum* in cats from Central Spain. Ann N Y Acad Sci. 2008; 1149:361-364. doi: 10.1196/annals.1428.019.

Azami-Conesa I, Martínez-Díaz RA, González F, Gómez-Muñoz MT. First detection of *Leishmania infantum* in common urban bats *Pipistrellus pipistrellus* in Europe. Res Vet Sci. 2020; 132:172-176. doi: 10.1016/j.rvsc.2020.06.019.

Ballart C, Alcover MM, Picado A, Nieto J, Castillejo S, Portús M, Gállego M. First survey on canine leishmaniasis in a non classical area of the disease in Spain (Lleida, Catalonia) based on a veterinary questionnaire and a cross-sectional study. Prev Vet Med. 2013; 109:116-127. doi: 10.1016/j.prevetmed.2012.09.003.

Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008; 24:324-330. doi: 10.1016/j.pt.2008.04.001.

Baneth G, Nachum-Biala Y, Zuberi A, Zipori-Barki N, Orshan L, Kleinerman G, Shmueli-Goldin A, Bellaiche M, Leszkowicz-Mazuz M, Salant H, Yasur-Landau D. *Leishmania* infection in cats and dogs housed together in an animal shelter reveals a higher parasite load in infected dogs despite a greater seroprevalence among cats. Parasit Vectors. 2020;13:115. doi: 10.1186/s13071-020-3989-3.

Baneth G, Solano-Gallego L. Leishmaniasis. Vet Clin North Am Small Anim Pract. 2022; 52:1359-1375. doi: 10.1016/j.cvsm.2022.06.012.

Barbiéri CL. Immunology of canine leishmaniasis. Parasite Immunol. 2006; 28:329-337. doi: 10.1111/j.1365-3024.2006.00840.x.

Barbosa de Castro R, Badaró de Moraes JV, Costa Bressan G, de Souza Vasconcellos R, Silva-Júnior A, Lopes Rangel Fietto J. Antigens and their diagnostic performance for Canine Visceral Leishmaniasis: A critical review. Vet Parasitol. 2022; 301:109638. doi: 10.1016/j.vetpar.2021.109638.

Barbosa DS, Belo VS, Bezerra JMT, Figueiredo FB, Werneck GL. Factors associated with *Leishmania infantum* infection in dogs from urban areas endemic for visceral leishmaniasis in Brazil. Res Vet Sci. 2022; 152:651-656. doi: 10.1016/j.rvsc.2022.09.035.

Barrouin-Melo SM, Larangeira DF, Santos SO, Chagas-Júnior AD, Paixão M, Aguiar PH, dos-Santos WL, Pontes-de-Carvalho L. A standardized cytological and immunochemical method for the analysis of fine-needle spleen aspirates: assessment of leukocyte population changes in canine visceral leishmaniosis. Vet Immunol Immunopathol. 2006; 111:251-261. doi: 10.1016/j.vetimm.2006.01.021.

Basset D, Faraut F, Marty P, Dereure J, Rosenthal E, Mary C, Pratlong F, Lachaud L, Bastien P, Dedet JP. Visceral leishmaniasis in organ transplant recipients: 11 new cases and a review of the literature. Microbes Infect. 2005; 7:1370-1375. doi: 10.1016/j.micinf.2005.06.002.

Basurco A, Natale A, Capello K, Fernández A, Verde MT, González A, Yzuel A, Giner J, Villanueva-Saz S. Evaluation of the performance of three serological tests for diagnosis of *Leishmania infantum* infection in dogs using latent class analysis. Rev Bras Parasitol Vet. 2020; 29, e018020. doi:10.1590/S1984-29612020105

Batista JF, Magalhães Neto FDCR, Lopes KSPDP, Sousa CMG, Alcântara DS, Baêta SAF, Alves MMM, Mendonça IL. Clinical, hematological, biochemical, and histopathological evaluations in domestic cats (*Felis catus*) infected by *Leishmania infantum*. Rev Bras Parasitol Vet. 2023; 32:e004723. doi: 10.1590/S1984-29612023037.

Battisti E, Zanet S, Khalili S, Trisciuoglio A, Hertel B, Ferroglio E. Molecular Survey on Vector-Borne Pathogens in Alpine Wild Carnivorans. Front Vet Sci. 2020; 7:1. doi: 10.3389/fvets.2020.00001.

Baxarias M, Viñals J, Álvarez-Fernández A, Alcover MM, Solano-Gallego L. Detection of specific antibodies against *Leishmania infantum* in canine serum and oral transudate using an in-house ELISA. Parasit Vectors. 2022a; 15:164. doi: 10.1186/s13071-022-05246-2.

Baxarias M, Homedes J, Mateu C, Attipa C, Solano-Gallego L. Use of preventive measures and serological screening tools for *Leishmania infantum* infection in dogs from Europe. Parasit Vectors. 2022b;15:134. doi: 10.1186/s13071-022-05251-5.

Belinchón-Lorenzo S, Iniesta V, Parejo JC, Fernández-Cotrina J, Muñoz-Madrid R, Soto M, Alonso C, Gómez Nieto LC. Detection of *Leishmania infantum* kinetoplast minicircle DNA by Real Time PCR in hair of dogs with leishmaniosis. Vet Parasitol. 2013; 192:43-50. doi: 10.1016/j.vetpar.2012.11.007.

Belinchón-Lorenzo S, Parejo JC, Iniesta V, Fernández-Cotrina J, Muñoz-Madrid R, Monroy I, Baz V, Gómez-Luque A, Serrano-Aguilera FJ, Barneto JL, Gómez-Nieto LC. First detection of *Leishmania* kDNA in canine cerumen samples by qPCR. Vet Parasitol. 2016; 228:65-68. doi: 10.1016/j.vetpar.2016.05.021.

Bento MF, Souza GS, Vieira BS, Silva ÂAD, Krawczak FDS, Moura VMBD. Sensitivity of PCR in conjunctival swab samples for the diagnosis of canine visceral leishmaniasis: a systematic review and meta-analysis. Rev Bras Parasitol Vet. 2023; 32:e009423. doi: 10.1590/S1984-29612023063.

Boechat VC, Pereira SA, Júnior AAVM, Dos Santos SA, Miranda LFC, Figueiredo FB, Ferreira LC, de Carvalho Rodrigues FDC, de Oliveira RVC, -de-Freitas RT, Bruno RV, Morgado FN, Menezes RC. Frequency, active infection and load of *Leishmania infantum* and associated histological alterations in the genital tract of male and female dogs. PLoS One. 2020; 15:e0238188. doi: 10.1371/journal.pone.0238188.

Boelaert M, Aoun K, Liinev J, Goetghebeur E, Van der Stuyft P. The potential of latent class analysis in diagnostic test validation for canine *Leishmania infantum* infection. Epidemiol Infect. 1999; 123:499-506. doi: 10.1017/s0950268899003040.

Bogdan C, Röllinghoff M. The immune response to *Leishmania*: mechanisms of parasite control and evasion. Int J Parasitol. 1998; 28:121-134. doi: 10.1016/s0020-7519(97)00169-0.

Bogdan C. Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. Cell Microbiol. 2008; 10:1221-1234. doi: 10.1111/j.1462-5822.2008.01146.x.

Boggiatto PM, Ramer-Tait AE, Metz K, Kramer EE, Gibson-Corley K, Mullin K, Hostetter JM, Gallup JM, Jones DE, Petersen CA. Immunologic indicators of clinical progression during canine *Leishmania infantum* infection. Clin Vaccine Immunol. 2010; 17:267-273. doi: 10.1128/CVI.00456-09.

Boggiatto PM, Gibson-Corley KN, Metz K, Gallup JM, Hostetter JM, Mullin K, Petersen CA. Transplacental transmission of *Leishmania infantum* as a means for continued disease incidence in North America. PLoS Negl Trop Dis. 2011; 5:e1019. doi: 10.1371/journal.pntd.0001019.

Bourdeau P, Saridomichelakis MN, Oliveira A, Oliva G, Kotnik T, Gálvez R, Foglia Manzillo V, Koutinas AF, Pereira da Fonseca I, Miró G. Management of canine leishmaniosis in endemic SW European regions: a questionnaire-based multinational survey. Parasit Vectors. 2014; 7:110. doi: 10.1186/1756-3305-7-110.

Burling AN, Levy JK, Scott HM, Crandall MM, Tucker SJ, Wood EG, Foster JD. Seroprevalences of feline leukemia virus and feline immunodeficiency virus infection in cats in the United States and Canada and risk factors for seropositivity. J Am Vet Med Assoc. 2017; 251:187-194. doi: 10.2460/javma.251.2.187.

Cantos-Barreda A, Escribano D, Bernal LJ, Cerón JJ, Martínez-Subiela S. Quantification of anti-*Leishmania* antibodies in saliva of dogs. Vet Parasitol. 2017; 242:54-58. doi: 10.1016/j.vetpar.2017.05.017.

Cardoso L, Neto F, Sousa JC, Rodrigues M, Cabral M. Use of a leishmanin skin test in the detection of canine *Leishmania*-specific cellular immunity. Vet Parasitol. 1998; 79:213-220. doi: 10.1016/s0304-4017(98)00169-1.

Cardoso L, Schallig H, Persichetti MF, Pennisi MG. New Epidemiological Aspects of Animal Leishmaniosis in Europe: The Role of Vertebrate Hosts Other Than Dogs. Pathogens. 2021; 10:307. doi: 10.3390/pathogens10030307.

Carrillo E, Moreno J. Cytokine profiles in canine visceral leishmaniasis. Vet Immunol Immunopathol. 2009;128:67-70. doi: 10.1016/j.vetimm.2008.10.310.

Carrillo E,Moreno J, Cruz I. What is responsible for a large and unusual outbreak of leishmaniasis in Madrid?. Trends Parasitol. 2013, 29, 579–580. https://doi.org/10.1016/j.pt.2013.10.007

Carstens-Kass J, Paulini K, Lypaczewski P, Matlashewski G. A review of the leishmanin skin test: A neglected test for a neglected disease. PLoS Negl Trop Dis. 2021; 15:e0009531. doi: 10.1371/journal.pntd.0009531.

Castelli G, Bruno F, Reale S, Catanzaro S, Valenza V, Vitale F. Molecular Diagnosis of Leishmaniasis: Quantification of Parasite Load by a Real-Time PCR Assay with High Sensitivity. Pathogens. 2021; 10:865. doi: 10.3390/pathogens10070865.

Cavalera MA, Iatta R, Laricchiuta P, Passantino G, Abramo F, Mendoza-Roldan JA, Otranto D, Zatelli A. Clinical, haematological and biochemical findings in tigers infected by *Leishmania infantum*. BMC Vet Res. 2020; 16:214. doi: 10.1186/s12917-020-02419-y.

Cavalera MA, Iatta R, Panarese R, Mendoza-Roldan JA, Gernone F, Otranto D, Paltrinieri S, Zatelli A. Seasonal variation in canine anti-*Leishmania infantum* antibody titres. Vet J. 2021; 271:105638. doi: 10.1016/j.tvjl.2021.105638.

Chagas ÚMR, de Avelar DM, Marcelino AP, Paz GF, Gontijo CMF. Correlations between tissue parasite load and common clinical signs in dogs naturally infected by *Leishmania infantum*. Vet Parasitol. 2021;291:109368. doi: 10.1016/j.vetpar.2021.109368.

Chatzis MK, Andreadou M, Leontides L, Kasabalis D, Mylonakis M, Koutinas AF, Rallis T, Ikonomopoulos J, Saridomichelakis MN. Cytological and molecular detection of *Leishmania infantum* in different tissues of clinically normal and sick cats. Vet Parasitol. 2014a; 202: 217-225. doi: 10.1016/j.vetpar.2014.02.044.

Chatzis MK, Leontides L, Athanasiou LV, Papadopoulos E, Kasabalis D, Mylonakis M, Rallis T, Koutinas AF, Andreadou M, Ikonomopoulos J, Saridomichelakis MN. Evaluation of indirect immunofluorescence antibody test and enzyme-linked immunosorbent assay for the diagnosis of infection by *Leishmania infantum* in clinically normal and sick cats. Exp Parasitol. 2014b; 147:54-59. doi: 10.1016/j.exppara.2014.10.004.

Chatzis MK, Xenoulis PG, Leontides L, Kasabalis D, Mylonakis ME, Andreadou M, Ikonomopoulos J, Saridomichelakis MN. Evaluation of clinicopathological abnormalities in sick cats naturally infected by *Leishmania infantum*. Heliyon. 2020; 6:e05177. doi: 10.1016/j.heliyon.2020.e05177.

Cirović D, Chochlakis D, Tomanović S, Sukara R, Penezić A, Tselentis Y, Psaroulaki A. Presence of *Leishmania* and *Brucella* species in the golden jackal *Canis aureus* in Serbia. Biomed Res Int. 2014; 2014:728516. doi: 10.1155/2014/728516.

Correia B, Magalhães A, Rocha L, Cardoso I, Ferreira RRF, Mesa-Sanchez I. Prevalence of subclinical infectious agents in a blood donor population tested on every donation. J Small Anim Pract. 2024; 65:176-180. doi: 10.1111/jsap.13698.

Coura-Vital W, Marques MJ, Giunchetti RC, Teixeira-Carvalho A, Moreira ND, Vitoriano-Souza J, Vieira PM, Carneiro CM, Corrêa-Oliveira R, Martins-Filho OA, Carneiro M, Reis AB. Humoral and cellular immune responses in dogs with inapparent natural *Leishmania infantum* infection. Vet J. 2011; 190:e43-e47. doi: 10.1016/j.tvjl.2011.04.005.

Criado-Fornelio A, Gutierrez-Garcia L, Rodriguez-Caabeiro F, Reus-Garcia E, Roldan-Soriano MA, Diaz-Sanchez MA. A parasitological survey of wild red foxes (*Vulpes vulpes*) from the province of Guadalajara, Spain. Vet Parasitol. 2000; 92:245-251. doi: 10.1016/s0304-4017(00)00329-0.

Da Silva AV, de Souza Cândido CD, de Pita Pereira D, Brazil RP, Carreira JC. The first record of American visceral leishmaniasis in domestic cats from Rio de Janeiro, Brazil. Acta Trop. 2008; 105:92-94. doi: 10.1016/j.actatropica.2007.09.001.

Da Silva SM, Rabelo PF, Gontijo Nde F, Ribeiro RR, Melo MN, Ribeiro VM, Michalick MS. First report of infection of *Lutzomyia longipalpis* by *Leishmania* (*Leishmania*) *infantum* from a naturally infected cat of Brazil. Vet Parasitol. 2010; 174:150-154. doi: 10.1016/j.vetpar.2010.08.005.

Daval N, Marchal C, Guillaumot L, Hüe T, Ravel C, Keck N, Kasbari M. First report of autochthonous non-vectorial canine leishmaniasis in New Caledonia, south-western Pacific: implications for new control measures and recommendations on importation of dogs. Parasit Vectors. 2016; 9:108. doi: 10.1186/s13071-016-1388-6.

De Almeida MC, Cardoso SA, Barral-Netto M. *Leishmania* (*Leishmania*) *chagasi* infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage. Int J Parasitol. 2003; 33:153-162. doi: 10.1016/s0020-7519(02)00266-7.

De Araújo VA, Boité MC, Cupolillo E, Jansen AM, Roque AL. Mixed infection in the anteater *Tamandua tetradactyla* (Mammalia: Pilosa) from Pará State, Brazil: *Trypanosoma cruzi*, *T. rangeli* and *Leishmania infantum*. Parasitology. 2013; 140:455-460. doi: 10.1017/S0031182012001886.

De Freitas E, Melo MN, da Costa-Val AP, Michalick MS. Transmission of *Leishmania infantum* via blood transfusion in dogs: potential for infection and importance of clinical factors. Vet Parasitol. 2006; 137:159-167. doi: 10.1016/j.vetpar.2005.12.011.

De Freitas JC, Lopes-Neto BE, de Abreu CR, Coura-Vital W, Braga SL, Reis AB, Nunes-Pinheiro DC. Profile of anti-*Leishmania* antibodies related to clinical picture in canine visceral leishmaniasis. Res Vet Sci. 2012; 93:705-709. doi: 10.1016/j.rvsc.2011.12.009.

De Lima H, Rodríguez N, Barrios MA, Avila A, Cañizales I, Gutiérrez S. Isolation and molecular identification of *Leishmania chagasi* from a bat (*Carollia perspicillata*) in northeastern Venezuela. Mem Inst Oswaldo Cruz. 2008; 103:412-414. doi: 10.1590/s0074-02762008000400018.

De Paiva-Cavalcanti M, de Morais RC, Pessoa-E-Silva R, Trajano-Silva LA, Gonçalves-de-Albuquerque Sda C, Tavares Dde H, Brelaz-de-Castro MC, Silva Rde F, Pereira VR. Leishmaniases diagnosis: an update on the use of immunological and molecular tools. Cell Biosci. 2015; 5:31. doi: 10.1186/s13578-015-0021-2.

De Souza WM, Weaver SC. Effects of climate change and human activities on vector-borne diseases. Nat Rev Microbiol. 2024; 22, 476–491. doi: 10.1038/s41579-024-01026-0

Depaquit J, Grandadam M, Fouque F, Andry PE, Peyrefitte C. Arthropod-borne viruses transmitted by Phlebotomine sandflies in Europe: a review. Euro Surveill. 2010; 15:19507.

Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004; 27:305-318. doi: 10.1016/j.cimid.2004.03.004.

Di Mattia D, Fondevila D, Abramo F, Fondati A. A retrospective histopathological, immunohistochemical and molecular study of the presence of *Leishmania* spp. in the skin of cats with head and neck ulcerative dermatitis. Vet Dermatol. 2018; 29:212-e76. doi: 10.1111/vde.12535.

Diniz SA, Melo MS, Borges AM, Bueno R, Reis BP, Tafuri WL, Nascimento EF, Santos RL. Genital lesions associated with visceral leishmaniasis and shedding of *Leishmania* spp. in the semen of naturally infected dogs. Vet Pathol. 2005; 42:650-658. doi: 10.1354/vp.42-5-650.

Elmahallawy EK, Zanet S, Poggi M, Alsharif KF, Agil A, Trisciuoglio A, Ferroglio E. Feline Leishmaniosis in Northwestern Italy: Current Status and Zoonotic Implications. Vet Sci. 2021; 8:215. doi: 10.3390/vetsci8100215.

Esch KJ, Juelsgaard R, Martinez PA, Jones DE, Petersen CA. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. J Immunol. 2013; 191:5542-5550. doi: 10.4049/jimmunol.1301810.

Esch KJ, Schaut RG, Lamb IM, Clay G, Morais Lima ÁL, do Nascimento PR, Whitley EM, Jeronimo SM, Sutterwala FS, Haynes JS, Petersen CA. Activation of autophagy and nucleotide-binding domain leucine-rich repeat-containing-like receptor family, pyrin domain-containing 3 inflammasome during *Leishmania infantum*-associated glomerulonephritis. Am J Pathol. 2015; 185:2105-2117. doi: 10.1016/j.ajpath.2015.04.017.

Fernández-Bellon H, Solano-Gallego L, Rodríguez A, Rutten VP, Hoek A, Ramis A, Alberola J, Ferrer L. Comparison of three assays for the evaluation of specific cellular immunity to *Leishmania infantum* in dogs. Vet Immunol Immunopathol. 2005; 107:163-169. doi: 10.1016/j.vetimm.2005.04.002.

Fernández-Bellon H, Solano-Gallego L, Bardagí M, Alberola J, Ramis A, Ferrer L. Immune response to *Leishmania infantum* in healthy horses in Spain. Vet Parasitol. 2006; 135:181-185. doi: 10.1016/j.vetpar.2005.09.007.

Fernández-Gallego A, Feo Bernabe L, Dalmau A, Esteban-Saltiveri D, Font A, Leiva M, Ortuñez-Navarro A, Peña MT, Tabar MD, Real-Sampietro L, Saló F, Lloret A, Bardagí M. Feline leishmaniosis: diagnosis, treatment and outcome in 16 cats. J Feline Med Surg. 2020; 22:993-1007. doi: 10.1177/1098612X20902865.

Fernández-Pérez FJ, Gómez-Muñoz MT, Méndez S, Alunda JM. Leishmania-specific lymphoproliferative responses and IgG1/IgG2 immunodetection patterns by Western blot in asymptomatic, symptomatic and treated dogs. Acta Trop. 2003; 86:83-91. doi: 10.1016/s0001-706x(03)00004-4.

Ferreira Sde A, Ituassu LT, de Melo MN, de Andrade AS. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. Vet Parasitol. 2008; 152:257-263. doi: 10.1016/j.vetpar.2007.12.022.

Fisa R, Gállego M, Riera C, Aisa MJ, Valls D, Serra T, de Colmenares M, Castillejo S, Portús M. Serologic diagnosis of canine leishmaniasis by dot-ELISA. J Vet Diagn Invest. 1997; 9:50-55. doi: 10.1177/104063879700900109.

Franceschi A, Merildi V, Guidi G, Mancianti F. Occurrence of *Leishmania* DNA in urines of dogs naturally infected with leishmaniasis. Vet Res Commun. 2007; 31:335-341. doi: 10.1007/s11259-006-3477-z.

Gaglio G, Brianti E, Napoli E, Falsone L, Dantas-Torres F, Tarallo VD, Otranto D, Giannetto S. Effect of night time-intervals, height of traps and lunar phases on sand fly collection in a highly endemic area for canine leishmaniasis. Acta Trop. 2014; 133:73-77. doi: 10.1016/j.actatropica.2014.02.008.

Galán-Puchades MT, Gómez-Samblás M, Suárez-Morán JM, Osuna A, Sanxis-Furió J, Pascual J, Bueno-Marí R, Franco S, Peracho V, Montalvo T, Fuentes MV. Leishmaniasis in Norway Rats in Sewers, Barcelona, Spain. Emerg Infect Dis. 2019;25:1222-1224. doi: 10.3201/eid2506.181027.

Gállego M, Pratlong F, Fisa R, Riera C, Rioux JA, Dedet JP, Portús M. The life-cycle of *Leishmania infantum* MON-77 in the Priorat (Catalonia, Spain) involves humans, dogs and sandflies; also literature review of distribution and hosts of *L. infantum* zymodemes in the Old World. Trans R Soc Trop Med Hyg. 2001; 95:269-271. doi: 10.1016/s0035-9203(01)90231-7.

Gálvez R, Montoya A, Cruz I, Fernández C, Martín O, Checa R, Chicharro C, Migueláñez S, Marino V, Miró G. Latest trends in *Leishmania infantum* infection in dogs in Spain, Part I: mapped seroprevalence and sand fly distributions. Parasit Vectors. 2020; 13:204. doi: 10.1186/s13071-020-04081-7.

García-Castro A, Egui A, Thomas MC, López MC. Humoral and Cellular Immune Response in asymptomatic dogs with visceral leishmaniasis: A review. Vaccines. 2022; 10:947. doi: 10.3390/vaccines10060947.

García-Torres M, López MC, Tasker S, Lappin MR, Blasi-Brugué C, Roura X. Review and statistical analysis of clinical management of feline leishmaniosis caused by *Leishmania infantum*. Parasit Vectors. 2022; 15:253. doi: 10.1186/s13071-022-05369-6.

Gardner IA, Greiner M. Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. Vet Clin Pathol. 2006; 35:8-17. doi: 10.1111/j.1939-165x.2006.tb00082.x.

Garrido-Jareño M, Sahuquillo-Torralba A, Chouman-Arcas R, Castro-Hernández I, Molina-Moreno JM, Llavador-Ros M, Gómez-Ruiz MD, López-Hontangas JL, Botella-Estrada R, Salavert-Lleti M, Pemán-García J. Cutaneous and mucocutaneous leishmaniasis: experience of a Mediterranean hospital. Parasit Vectors. 2020; 13:24. doi: 10.1186/s13071-020-3901-1.

Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol. 2006; 6:895-906. doi: 10.1038/nri1978.

Gazzonis AL, Bertero F, Moretta I, Morganti G, Mortarino M, Villa L, Zanzani SA, Morandi B, Rinnovati R, Vitale F, Manfredi MT, Cardoso L, Veronesi F. Detecting antibodies to *Leishmania infantum* in horses from areas with different epizooticity levels of canine leishmaniosis and a retrospective revision of Italian data. Parasit Vectors. 2020; 13:530. doi: 10.1186/s13071-020-04385-8.

Gibson-Corley KN, Bockenstedt MM, Li H, Boggiatto PM, Phanse Y, Petersen CA, Bellaire BH, Jones DE. An in vitro model of antibody-enhanced killing of the intracellular parasite *Leishmania amazonensis*. PLoS One. 2014; 9:e106426. doi: 10.1371/journal.pone.0106426.

Gomes J, Rocha H, Carvalho C, Bandeira V, Fonseca C, Rosalino LM, Cunha MV. Molecular detection and characterization of *Leishmania infantum* in free-ranging Egyptian mongoose (*Herpestes ichneumon*). Int J Parasitol Parasites Wildl. 2020; 11:158-162. doi: 10.1016/j.ijppaw.2020.02.001.

Gómez-Ochoa P, Castillo JA, Lucientes J, Gascón M, Zarate JJ, Arbea JI, Larraga V, Rodriguez C. Modified direct agglutination test for simplified serologic diagnosis of leishmaniasis. Clin Diagn Lab Immunol. 2003; 10:967-968. doi: 10.1128/cdli.10.5.967-968.2003.

Goncalves R, Silva SO, de Almeida GG, de Souza CC, Tafuri WL, Norma Melo M. Detection of *Leishmania infantum* DNA in the non-parasitized lung of dogs with visceral leishmaniasis. BMC Vet Res. 2018; 14:403. doi: 10.1186/s12917-018-1730-7.

Goodman, L.A. Exploratory latent structure analysis using both identifiable and unidentifiable models, *Biometrika*, Volume 61, Issue 2, August 1974, Pages 215-231, doi.org/10.1093/biomet/61.2.215

Gradoni L. Canine *Leishmania* vaccines: still a long way to go. Vet Parasitol. 2015; 208:94-100. doi: 10.1016/j.vetpar.2015.01.003.

Gramiccia M. Recent advances in leishmaniosis in pet animals: epidemiology, diagnostics and anti-vectorial prophylaxis. Vet Parasitol. 2011; 181:23-30. doi: 10.1016/j.vetpar.2011.04.019.

Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol. 2005; 35:1169-1180. doi: 10.1016/j.ijpara.2005.07.001.

Guillén MC, Alcover MM, Borruel N, Sulleiro E, Salvador F, Berenguer D, Herrera-de Guise C, Rodríguez V, Moure Z, Sánchez-Montalvà A, Molina I, Fisa R, Riera C. *Leishmania infantum* asymptomatic infection in inflammatory bowel disease patients under anti-TNF therapy. Heliyon. 2020; 6:e03940. doi: 10.1016/j.heliyon.2020.e03940.

Hide M, Bañuls AL, Tibayrenc M. Genetic heterogeneity and phylogenetic status of *Leishmania* (*Leishmania*) *infantum* zymodeme MON-1: epidemiological implications. Parasitology. 2001; 123:425-432. doi: 10.1017/s003118200100871x.

Holzmuller P, Hide M, Sereno D, Lemesre JL. *Leishmania infantum* amastigotes resistant to nitric oxide cytotoxicity: Impact on in vitro parasite developmental cycle and metabolic enzyme activities. Infect Genet Evol. 2006;6:187-197. doi: 10.1016/j.meegid.2005.03.003.

Hong A, Zampieri RA, Shaw JJ, Floeter-Winter LM, Laranjeira-Silva MF. One health approach to Leishmaniases: Understanding the disease dynamics through diagnostic tools. Pathogens. 2020;9:809. doi: 10.3390/pathogens9100809.

Hosein S, Blake DP, Solano-Gallego L. Insights on adaptive and innate immunity in canine leishmaniosis. Parasitology. 2017; 144:95-115. doi: 10.1017/S003118201600055X.

Iatta R, Furlanello T, Colella V, Tarallo VD, Latrofa MS, Brianti E, Trerotoli P, Decaro N, Lorusso E, Schunack B, Mirò G, Dantas-Torres F, Otranto D. A nationwide survey of *Leishmania infantum* infection in cats and associated risk factors in Italy. PLoS Negl Trop Dis. 2019; 13:e0007594. doi: 10.1371/journal.pntd.0007594.

Iatta R, Zatelli A, Laricchiuta P, Legrottaglie M, Modry D, Dantas-Torres F, Otranto D. *Leishmania infantum* in Tigers and Sand Flies from a Leishmaniasis-Endemic Area, Southern Italy. Emerg Infect Dis. 2020;26:1311-1314. doi: 10.3201/eid2606.191668.

Iniesta L, Fernández-Barredo S, Bulle B, Gómez MT, Piarroux R, Gállego M, Alunda JM, Portús M. Diagnostic techniques to detect cryptic leishmaniasis in dogs. Clin Diagn Lab Immunol. 2002; 9:1137-1141. doi: 10.1128/cdli.9.5.1137-1141.2002.

Iniesta L, Gállego M, Portús M. Idiotype expression of IgG1 and IgG2 in dogs naturally infected with *Leishmania infantum*. Vet Immunol Immunopathol. 2007; 119:189-197. doi: 10.1016/j.vetimm.2007.05.006.

Jiménez M, González E, Martín-Martín I, Hernández S, Molina R. Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? Vet Parasitol. 2014; 202:296-300. doi: 10.1016/j.vetpar.2014.03.027.

Ker HG, Coura-Vital W, Aguiar-Soares RD, Roatt BM, das Dores Moreira N, Carneiro CM, Machado EM, Teixeira-Carvalho A, Martins-Filho OA, Giunchetti RC, Araújo MS, Coelho EA, da Silveira-Lemos D, Reis AB. Evaluation of a prototype flow cytometry test for serodiagnosis of canine visceral leishmaniasis. Clin Vaccine Immunol. 2013; 20:1792-1798. doi: 10.1128/CVI.00575-13.

Killick-Kendrick R. Phlebotomine vectors of the leishmaniases: a review. Med Vet Entomol. 1990; 4:1-24. doi: 10.1111/j.1365-2915.1990.tb00255.x.

Killick-Kendrick R. The biology and control of phlebotomine sand flies. Clin Dermatol. 1999; 17:279-289. doi: 10.1016/s0738-081x(99)00046-2.

Koutinas AF, Koutinas CK. Pathologic mechanisms underlying the clinical findings in canine leishmaniasis due to *Leishmania infantum/chagasi*. Vet Pathol. 2014; 51:527-538. doi: 10.1177/0300985814521248.

Krawczak Fda S, Reis IA, Silveira JA, Avelar DM, Marcelino AP, Werneck GL, Labruna MB, Paz GF. *Leishmania*, *Babesia* and *Ehrlichia* in urban pet dogs: co-infection or cross-reaction in serological methods? Rev Soc Bras Med Trop. 2015; 48:64-68. doi: 10.1590/0037-8682-0291-2014.

Laurent T, Van der Auwera G, Hide M, Mertens P, Quispe-Tintaya W, Deborggraeve S, De Doncker S, Leclipteux T, Bañuls AL, Büscher P, Dujardin JC. Identification of Old World *Leishmania* spp. by specific polymerase chain reaction amplification of cysteine proteinase B genes and rapid dipstick detection. Diagn Microbiol Infect Dis. 2009; 63:173-181. doi: 10.1016/j.diagmicrobio.2008.10.015.

Leite RS, Souza NA, Barbosa AD, Ferreira AL, de Andrade AS. Evaluation of conjunctival swab as a mass-screening tool for molecular diagnosis of canine visceral leishmaniasis. Parasitol Res. 2015; 114:2255-2262. doi: 10.1007/s00436-015-4418-y.

Liberg O. Density, spatial organization and reproductive tactics in the domestic cat and other felids. In: Turner D, Bateson P, editors. The domestic cat: the biology of its behaviour. 2. Cambridge: Cambridge University Press; 2000. pp. 120–147.

Libert C, Ravel C, Pratlong F, Lami P, Dereure J, Keck N. *Leishmania infantum* infection in two captive barbary lions (*Panthera leo leo*). J Zoo Wildl Med. 2012; 43:685-688. doi: 10.1638/2012-0056.1.

Lobsiger L, Müller N, Schweizer T, Frey CF, Wiederkehr D, Zumkehr B, Gottstein B. An autochthonous case of cutaneous bovine leishmaniasis in Switzerland. Vet Parasitol. 2010; 169:408-414. doi: 10.1016/j.vetpar.2010.01.022.

Lombardo G, Pennisi MG, Lupo T, Migliazzo A, Caprì A, Solano-Gallego L. Detection of *Leishmania infantum* DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. Vet Parasitol. 2012; 184:10-17. doi: 10.1016/j.vetpar.2011.08.010.

Lucientes J, Palmero J, Guarga JL, Gracia MJ, Peribáñez MA, Zárate J, Castillo JA. Risk of transmission of canine leishmaniosis in eastern Spain. Vet Rec. 2005; 156:743-744. doi: 10.1136/vr.156.23.743.

Magalhães-Junior JT, Mota TF, Porfirio-Passos G, Larangeira DF, Franke CR, Barrouin-Melo SM. Xenodiagnosis on dogs with visceral leishmaniasis: Canine and sand fly aspects related to the parasite transmission. Vet Parasitol. 2016; 223:120-126. doi: 10.1016/j.vetpar.2016.04.031.

Maia C, Campino L. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet Parasitol. 2008; 158:274-287. doi: 10.1016/j.vetpar.2008.07.028.

Maia C, Campino L. Biomarkers associated with *Leishmania infantum* exposure, infection, and disease in dogs. Front Cell Infect Microbiol. 2018; 8:302. doi: 10.3389/fcimb.2018.00302.

Maia C, Ramada J, Cristóvão JM, Gonçalves L, Campino L. Diagnosis of canine leishmaniasis: conventional and molecular techniques using different tissues. Vet J. 2009; 179:142-144. doi: 10.1016/j.tvjl.2007.08.009.

Maia C, Cardoso L. Spread of *Leishmania infantum* in Europe with dog travelling. Vet Parasitol. 2015; 213:2-11. doi: 10.1016/j.vetpar.2015.05.003.

Malta MC, Tinoco HP, Xavier MN, Vieira AL, Costa EA, Santos RL. Naturally acquired visceral leishmaniasis in non-human primates in Brazil. Vet Parasitol. 2010; 169:193-719. doi: 10.1016/j.vetpar.2009.12.016.

Mann S, Frasca K, Scherrer S, Henao-Martínez AF, Newman S, Ramanan P, Suarez JA. A Review of Leishmaniasis: Current Knowledge and Future Directions. Curr Trop Med Rep. 2021; 8, 121–132. doi:10.1007/s40475-021-00232-7

Maroli M, Pennisi MG, Di Muccio T, Khoury C, Gradoni L, Gramiccia M. Infection of sandflies by a cat naturally infected with *Leishmania infantum*. Vet Parasitol. 2007; 145:357-360. doi: 10.1016/j.vetpar.2006.11.009.

Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, Gramiccia M, Mortarino M, Pietrobelli M, Gradoni L. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health. 2008; 13:256-264. doi: 10.1111/j.1365-3156.2007.01998.x.

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med Vet Entomol. 2013; 27:123-147. doi: 10.1111/j.1365-2915.2012.01034.x.

Martínez-Moreno A, Moreno T, Martínez-Moreno FJ, Acosta I, Hernández S. Humoral and cell-mediated immunity in natural and experimental canine leishmaniasis. Vet Immunol Immunopathol. 1995; 48:209-220. doi: 10.1016/0165-2427(95)05434-8.

Martín-Sánchez J, Acedo C, Muñoz-Pérez M, Pesson B, Marchal O, Morillas-Márquez F. Infection by *Leishmania infantum* in cats: epidemiological study in Spain. Vet Parasitol. 2007; 145:267-273. doi: 10.1016/j.vetpar.2006.11.005.

Martín-Sánchez J, Torres-Medina N, Corpas-López V, Morillas-Márquez F, Díaz-Sáez V. Vertical transmission may play a greater role in the spread of *Leishmania infantum* in synanthropic *Mus musculus* rodents than previously believed. Transbound Emerg Dis. 2020; 67:1113-1118. doi: 10.1111/tbed.13436.

Maurelli MP, Bosco A, Foglia Manzillo V, Vitale F, Giaquinto D, Ciuca L, Molinaro G, Cringoli G, Oliva G, Rinaldi L, Gizzarelli M. Clinical, molecular and serological

diagnosis of canine leishmaniosis: An integrated approach. Vet Sci. 2020; 7:43. doi: 10.3390/vetsci7020043.

McKenna M, Attipa C, Tasker S, Augusto M. Leishmaniosis in a dog with no travel history outside of the UK. Vet Rec. 2019; 184:441. doi: 10.1136/vr.105157.

Melo GD, Grano FG, Silva JE, Kremer BE, Lima VM, Machado GF. Blood-brain barrier disruption during spontaneous canine visceral leishmaniasis. Parasite Immunol. 2015; 37:635-645. doi: 10.1111/pim.12285.

Merino Goyenechea J, Castilla Gómez de Agüero V, Palacios Alberti J, Balaña Fouce R, Martínez Valladares M. Occurrence of leishmaniasis in Iberian Wolves in Northwestern Spain. Microorganisms. 2023; 11:1179. doi: 10.3390/microorganisms11051179.

Mettler M, Grimm F, Capelli G, Camp H, Deplazes P. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol. 2005; 43:5515-5519. doi: 10.1128/JCM.43.11.5515-5519.2005.

Meyers AC, Edwards EE, Sanders JP, Saunders AB, Hamer SA. Fatal Chagas myocarditis in government working dogs in the southern United States: Cross-reactivity and differential diagnoses in five cases across six months. Vet Parasitol Reg Stud Reports. 2021; 24:100545. doi: 10.1016/j.vprsr.2021.100545.

Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of *Leishmania infantum* (*L. chagasi*) in human. Acta Trop. 2011; 119:69-75. doi: 10.1016/j.actatropica.2011.05.012.

Migliazzo A, Vitale F, Calderone S, Puleio R, Binanti D, Abramo F. Feline leishmaniosis: a case with a high parasitic burden. Vet Dermatol. 2015; 26:69-70. doi: 10.1111/vde.12180.

Millán J, Zanet S, Gomis M, Trisciuoglio A, Negre N, Ferroglio E. An investigation into alternative reservoirs of canine leishmaniasis on the endemic island of Mallorca (Spain). Transbound Emerg Dis. 2011; 58:352-357. doi: 10.1111/j.1865-1682.2011.01212.x.

Millán J, Ferroglio E, Solano-Gallego L. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. Parasitol Res. 2014; 113:2005-2014. doi: 10.1007/s00436-014-3929-2.

Millán J, Chirife AD, Altet L. Serum chemistry reference values for the common genet (*Genetta genetta*): variations associated with *Leishmania infantum* infection. Vet Q. 2015; 35:43-47. doi: 10.1080/01652176.2014.987883.

Millán J. Molecular investigation of vector-borne parasites in wild micromammals, Barcelona (Spain). Parasitol Res. 2018; 117:3015-3018. doi: 10.1007/s00436-018-5971-y.

Miró G, Rupérez C, Checa R, Gálvez R, Hernández L, García M, Canorea I, Marino V, Montoya A. Current status of *L. infantum* infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis? Parasit Vectors. 2014; 7:112. doi: 10.1186/1756-3305-7-112.

Miró G, López-Vélez R. Clinical management of canine leishmaniosis versus human leishmaniasis due to *Leishmania infantum*: Putting "One Health" principles into practice. Vet Parasitol. 2018; 254:151-159. doi: 10.1016/j.vetpar.2018.03.002.

Miró G, Troyano A, Montoya A, Fariñas F, Fermín ML, Flores L, Rojo C, Checa R, Gálvez R, Marino V, Fragío C, Martínez-Nevado E. First report of *Leishmania infantum* infection in the endangered orangutan (*Pongo pygmaeus pygmaeus*) in Madrid, Spain. Parasit Vectors. 2018; 11:185. doi: 10.1186/s13071-018-2772-1.

Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, Melero S, Bernal J. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet Parasitol. 2012; 190:268-271. doi: 10.1016/j.vetpar.2012.05.006.

Montoya A, de Quadros LP, Mateo M, Hernández L, Gálvez R, Alcántara G, Checa R, Jiménez MÁ, Chicharro C, Cruz I, Miró G. Leishmania infantum infection in Bennett´s Wallabies (*Macropus rufogriseus*) in a Spanish Wildlife park. J Zoo Wildl Med. 2016;47:586-593. doi: 10.1638/2014-0216.1.

Montoya A, García M, Gálvez R, Checa R, Marino V, Sarquis J, Barrera JP, Rupérez C, Caballero L, Chicharro C, Cruz I, Miró G. Implications of zoonotic and vector-borne parasites to free-roaming cats in central Spain. Vet Parasitol. 2018; 251:125-130. doi: 10.1016/j.vetpar.2018.01.009.

Moody AH, Chiodini PL. Methods for the detection of blood parasites. Clin Lab Haematol. 2000; 22:189-201. doi: 10.1046/j.1365-2257.2000.00318.x.

Moradin N, Descoteaux A. *Leishmania* promastigotes: building a safe niche within macrophages. Front Cell Infect Microbiol. 2012; 2:121. doi: 10.3389/fcimb.2012.00121.

Morales-Yuste M, Martín-Sánchez J, Corpas-Lopez V. Canine leishmaniasis: Update on epidemiology, diagnosis, treatment, and prevention. Vet Sci. 2022; 9:387. doi: 10.3390/vetsci9080387.

Morales-Yuste M, Morillas-Márquez F, Díaz-Sáez V, Barón-López S, Acedo-Sánchez C, Martín-Sánchez J. Epidemiological implications of the use of various methods for the diagnosis of canine leishmaniasis in dogs with different characteristics and in differing prevalence scenarios. Parasitol Res. 2012; 111:155-164. doi: 10.1007/s00436-011-2812-7.

Morelli S, Colombo M, Dimzas D, Barlaam A, Traversa D, Di Cesare A, Russi I, Spoletini R, Paoletti B, Diakou A. *Leishmania infantum* seroprevalence in cats from touristic areas of Italy and Greece. Front Vet Sci. 2020; 7:616566. doi: 10.3389/fvets.2020.616566.

Moreno J, Alvar J. Canine leishmaniasis: epidemiological risk and the experimental model. Trends Parasitol. 2002; 18:399-405. doi: 10.1016/s1471-4922(02)02347-4.

Nardoni S, Altomonte I, Salari F, Martini M, Mancianti F. Serological and Molecular Findings of *Leishmania* Infection in healthy donkeys (*Equus asinus*) from a canine leishmaniosis endemic focus in Tuscany, Italy: A Preliminary Report. Pathogens. 2019; 8:99. doi: 10.3390/pathogens8030099.

Naucke TJ, Amelung S, Lorentz S. First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. Parasit Vectors. 2016; 9:256. doi: 10.1186/s13071-016-1551-0.

Oleaga A, Vicente J, Ferroglio E, Pegoraro de Macedo MR, Casais R, del Cerro A, Espí A, García EJ, Gortázar C. Concomitance and interactions of pathogens in the Iberian wolf (*Canis lupus*). Res Vet Sci. 2015; 101:22-27. doi: 10.1016/j.rvsc.2015.05.010.

Oleaga A, Zanet S, Espí A, Pegoraro de Macedo MR, Gortázar C, Ferroglio E. *Leishmania* in wolves in northern Spain: A spreading zoonosis evidenced by wildlife sanitary surveillance. Vet Parasitol. 2018; 255:26-31. doi: 10.1016/j.vetpar.2018.03.015.

Olías-Molero AI, Corral MJ, Jiménez-Antón MD, Alunda JM. Early antibody response and clinical outcome in experimental canine leishmaniasis. Sci Rep. 2019; 9:18606. doi: 10.1038/s41598-019-55087-w.

Olías-Molero AI, Moreno I, Corral MJ, Jiménez-Antón MD, Day MJ, Domínguez M, Alunda JM. Infection of dogs by *Leishmania infantum* elicits a general response of IgG subclasses. Sci Rep. 2020; 10:18826. doi: 10.1038/s41598-020-75569-6.

Oliveira TM, Furuta PI, de Carvalho D, Machado RZ. A study of cross-reactivity in serum samples from dogs positive for *Leishmania* sp., *Babesia canis* and *Ehrlichia canis* in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Rev Bras Parasitol Vet. 2008; 17:7-11. doi: 10.1590/s1984-29612008000100002.

Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. Clin Microbiol Rev. 2005; 18:293-305. doi: 10.1128/CMR.18.2.293-305.2005.

Ondovilla A.G. Un chat infecté par *Leishmania* en Espagne. Trabajos. 1933; 2:26-27.

Ordeix L, Montserrat-Sangrà S, Martínez-Orellana P, Solano-Gallego L. Toll-Like Receptors 2, 4, and 7, Interferon-Gamma, Interleukin 10, and Programmed Death Ligand 1 Transcripts in Leishmanin Skin Test-Positive Reactions of Ibizan Hound Dogs. J Immunol Res. 2020; 2020:9602576. doi: 10.1155/2020/9602576.

Ortega-García MV, Salguero FJ, Rodríguez-Bertos A, Moreno I, García N, García-Seco T, Luz Torre G, Domínguez L, Domínguez M. A pathological study of *Leishmania infantum* natural infection in European rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*). Transbound Emerg Dis. 2019; 66:2474-2481. doi: 10.1111/tbed.13305.

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

Oryan A, Akbari M. Worldwide risk factors in leishmaniasis. Asian Pac J Trop Med. 2016; 9:925-932. doi: 10.1016/j.apjtm.2016.06.021.

Otranto D, Paradies P, Sasanelli M, Leone N, de Caprariis D, Chirico J, Spinelli R, Capelli G, Brandonisio O. Recombinant K39 dipstick immunochromatographic test: a new tool for the serodiagnosis of canine leishmaniasis. J Vet Diagn Invest. 2005; 17:32-37. doi: 10.1177/104063870501700107.

Owens SD, Oakley DA, Marryott K, Hatchett W, Walton R, Nolan TJ, Newton A, Steurer F, Schantz P, Giger U. Transmission of visceral leishmaniasis through blood transfusions from infected English foxhounds to anemic dogs. J Am Vet Med Assoc. 2001; 219:1076-1083. doi: 10.2460/javma.2001.219.1076.

Palatnik-de-Sousa CB, Day MJ. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit Vectors. 2011; 4:197. doi: 10.1186/1756-3305-4-197.

Paltrinieri S, Solano-Gallego L, Fondati A, Lubas G, Gradoni L, Castagnaro M, Crotti A, Maroli M, Oliva G, Roura X, Zatelli A, Zini E; Canine Leishmaniasis Working Group, Italian Society of Veterinarians of Companion Animals. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. J Am Vet Med Assoc. 2010; 236:1184-1191. doi: 10.2460/javma.236.11.1184.

Paltrinieri S, Gradoni L, Roura X, Zatelli A, Zini E. Laboratory tests for diagnosing and monitoring canine leishmaniasis. Vet Clin Pathol. 2016; 45:552-578. doi: 10.1111/vcp.12413.

Papadogiannakis EI, Koutinas AF. Cutaneous immune mechanisms in canine leishmaniosis due to *Leishmania infantum*. Vet Immunol Immunopathol. 2015; 163:94-102. doi: 10.1016/j.vetimm.2014.11.011.

Pappa SA, Kontou PI, Bagos PG, Braliou GG. Urine-based molecular diagnostic Tests for Leishmaniasis infection in human and canine populations: A Meta-analysis. Pathogens. 2021; 10:269. doi: 10.3390/pathogens10030269.

Pennisi M.G. An update on feline Leishmaniosis in Europe. EJCAP. 2013; 23(2): 20-27.

Pennisi MG, Hartmann K, Lloret A, Addie D, Belák S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hosie MJ, Lutz H, Marsilio F, Möstl K, Radford AD, Thiry E, Truyen U, Horzinek MC. Leishmaniosis in cats: ABCD guidelines on prevention and management. J Feline Med Surg. 2013; 15:638-642. doi: 10.1177/1098612X13489229.

Pennisi MG. Leishmaniosis of companion animals in Europe: an update. Vet Parasitol. 2015; 208:35-47. doi: 10.1016/j.vetpar.2014.12.023.

Pennisi MG, Cardoso L, Baneth G, Bourdeau P, Koutinas A, Miró G, Oliva G, Solano-Gallego L. LeishVet update and recommendations on feline leishmaniosis. Parasit Vectors. 2015; 8:302. doi: 10.1186/s13071-015-0909-z.

Pennisi MG, Persichetti MF. Feline leishmaniosis: Is the cat a small dog? Vet Parasitol. 2018; 251:131-137. doi: 10.1016/j.vetpar.2018.01.012.

Pereira A, Maia C. *Leishmania* infection in cats and feline leishmaniosis: An updated review with a proposal of a diagnosis algorithm and prevention guidelines. Curr Res Parasitol Vector Borne Dis. 2021; 1:100035. doi: 10.1016/j.crpvbd.2021.100035.

Persichetti MF, Solano-Gallego L, Vullo A, Masucci M, Marty P, Delaunay P, Vitale F, Pennisi MG. Diagnostic performance of ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum* antibodies in cats using a Bayesian analysis without a gold standard. Parasit Vectors. 2017; 10:119. doi: 10.1186/s13071-017-2046-3.

Porrozzi R, Santos da Costa MV, Teva A, Falqueto A, Ferreira AL, dos Santos CD, Fernandes AP, Gazzinelli RT, Campos-Neto A, Grimaldi G Jr. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. Clin Vaccine Immunol. 2007; 14:544-548. doi: 10.1128/CVI.00420-06.

Priolo V, Martínez-Orellana P, Pennisi MG, Masucci M, Prandi D, Ippolito D, Bruno F, Castelli G, Solano-Gallego L. *Leishmania infantum*-specific IFN-γ production in stimulated blood from cats living in areas where canine leishmaniosis is endemic. Parasit Vectors. 2019; 12:133. doi: 10.1186/s13071-019-3386-y.

Priolo V, Martínez-Orellana P, Pennisi MG, Raya-Bermúdez AI, Jurado-Tarifa E, Masucci M, Donato G, Bruno F, Castelli G, Solano-Gallego L. *Leishmania infantum* specific humoral and cellular immune responses in cats and dogs: A comparative cross-sectional study. Vet Sci. 2022a Sep 7;9(9):482. doi: 10.3390/vetsci9090482.

Priolo V, Masucci M, Donato G, Solano-Gallego L, Martínez-Orellana P, Persichetti MF, Raya-Bermúdez A, Vitale F, Pennisi MG. Association between feline immunodeficiency virus and *Leishmania infantum* infections in cats: a retrospective matched case-control study. Parasit Vectors. 2022b; 15:107. doi: 10.1186/s13071-022-05230-w.

Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009; 136:1915-1934. doi: 10.1017/S0031182009991156.

Ramírez GA, Peñafiel-Verdú C, Altimira J, García-González B, Vilafranca M. Naturally acquired visceral leishmaniosis in a captive Bennett's wallaby (*Macropus rufogriseus rufogriseus*). Vet Pathol. 2013; 50:188-190. doi: 10.1177/0300985812446155.

Ready PD. Leishmaniasis emergence in Europe. EuroSurveill. 2010;15, 19505. doi:10.2807/ese.15.10.19505-en

Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis. 2007; 7:581-596. doi: 10.1016/S1473-3099(07)70209-8.

Riera C, Valladares JE, Gállego M, Aisa MJ, Castillejo S, Fisa R, Ribas N, Carrió J, Alberola J, Arboix M. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. Vet Parasitol. 1999; 84:33-47. doi: 10.1016/s0304-4017(99)00084-9.

Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg. 2004; 98:102-110. doi: 10.1016/s0035-9203(03)00015-4. .

Riera C, Fisa R, López-Chejade P, Serra T, Girona E, Jiménez M, Muncunill J, Sedeño M, Mascaró M, Udina M, Gállego M, Carrió J, Forteza A, Portús M. Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain). Transfusion. 2008; 48:1383-1389. doi: 10.1111/j.1537-2995.2008.01708.x.

Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann Parasitol Hum Comp. 1990; 65:111-125. doi: 10.1051/parasite/1990653111.

Risueño J, Ortuño M, Pérez-Cutillas P, Goyena E, Maia C, Cortes S, Campino L, Bernal LJ, Muñoz C, Arcenillas I, Martínez-Rondán FJ, Gonzálvez M, Collantes F, Ortiz J, Martínez-Carrasco C, Berriatua E. Epidemiological and genetic studies suggest a common *Leishmania infantum* transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet Parasitol. 2018; 259:61-67. doi: 10.1016/j.vetpar.2018.05.012.

Ritter U, Frischknecht F, van Zandbergen G. Are neutrophils important host cells for *Leishmania* parasites? Trends Parasitol. 2009; 25:505-510. doi: 10.1016/j.pt.2009.08.003.

Rodríguez-Cortés A, Fernández-Bellón H, Ramis A, Ferrer L, Alberola J, Solano-Gallego L. *Leishmania*-specific isotype levels and their relationship with specific cell-mediated immunity parameters in canine leishmaniasis. Vet Immunol Immunopathol. 2007; 116:190-198. doi: 10.1016/j.vetimm.2007.01.015.

Rodríguez-Cortés A, Ojeda A, Francino O, López-Fuertes L, Timón M, Alberola J. *Leishmania* infection: laboratory diagnosing in the absence of a "gold standard". Am J Trop Med Hyg. 2010; 82:251-256. doi: 10.4269/ajtmh.2010.09-0366.

Rodriguez-Pinto D, Saravia NG, McMahon-Pratt D. CD4 T cell activation by B cells in human *Leishmania* (*Viannia*) infection. BMC Infect Dis. 2014; 14:108. doi: 10.1186/1471-2334-14-108.

Rogers ME, Bates PA. *Leishmania* manipulation of sand fly feeding behavior results in enhanced transmission. PLoS Pathog. 2007; 3:e91. doi: 10.1371/journal.ppat.0030091.

Rombolà P, Barlozzari G, Carvelli A, Scarpulla M, Iacoponi F, Macrì G. Seroprevalence and risk factors associated with exposure to *Leishmania infantum* in dogs, in an endemic Mediterranean region. PLoS One. 2021; 16:e0244923. doi: 10.1371/journal.pone.0244923.

Rosypal AC, Troy GC, Zajac AM, Frank G, Lindsay DS. Transplacental transmission of a North American isolate of *Leishmania infantum* in an experimentally infected beagle. J Parasitol. 2005; 91:970-972. doi: 10.1645/GE-483R.1.

Roth-Damas P, Sempere-Manuel M, Mialaret-Lahiguera A, Fernández-García C, Gil-Tomás JJ, Colomina-Rodríguez J, Palop-Larrea V. Community outbreak of cutaneous leishmaniasis in La Ribera region of Valencia, Spain: Public Health measures. Enferm Infecc Microbiol Clin. 2017; 35:338-343. doi: 10.1016/j.eimc.2016.04.006.

Rotureau B, Ravel C, Couppié P, Pratlong F, Nacher M, Dedet JP, Carme B. Use of PCR-restriction fragment length polymorphism analysis to identify the main new world *Leishmania* species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. J Clin Microbiol. 2006; 44:459-67. doi: 10.1128/JCM.44.2.459-467.2006.

Ruiz-Fons F, Ferroglio E, Gortázar C. *Leishmania infantum* in free-ranging hares, Spain, 2004-2010. Euro Surveill. 2013; 18:20541. doi: 10.2807/1560-7917.es2013.18.30.20541.

Samant M, Sahu U, Pandey SC, Khare P. Role of cytokines in experimental and human visceral leishmaniasis. Front Cell Infect Microbiol. 2021; 11:624009. doi: 10.3389/fcimb.2021.624009.

Santarém N, Silvestre R, Cardoso L, Schallig H, Reed SG, Cordeiro-da-Silva A. Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. J Clin Microbiol. 2010; 48:1866-74. doi: 10.1128/JCM.02402-09.

Scalone A, De Luna R, Oliva G, Baldi L, Satta G, Vesco G, Mignone W, Turilli C, Mondesire RR, Simpson D, Donoghue AR, Frank GR, Gradoni L. Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. Vet Parasitol. 2002; 104:275-285. doi: 10.1016/s0304-4017(01)00643-4.

Schallig HD, Schoone GJ, Beijer EG, Kroon CC, Hommers M, Ozbel Y, Ozensoy S, da Silva ES, Cardoso LM, da Silva ED. Development of a fast agglutination screening test (FAST) for the detection of anti-*Leishmania* antibodies in dogs. Vet Parasitol. 2002; 109:1-8. doi: 10.1016/s0304-4017(02)00268-6.

Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol. 2007; 8:239-245. doi: 10.1038/ni1443.

Sherry K, Miró G, Trotta M, Miranda C, Montoya A, Espinosa C, Ribas F, Furlanello T, Solano-Gallego L. A serological and molecular study of *Leishmania infantum* infection in cats from the Island of Ibiza (Spain). Vector Borne Zoonotic Dis. 2011; 11:239-245. doi: 10.1089/vbz.2009.0251..

Shio MT, Hassani K, Isnard A, Ralph B, Contreras I, Gomez MA, Abu-Dayyeh I, Olivier M. Host cell signalling and *Leishmania* mechanisms of evasion. J Trop Med. 2012; 2012:819512. doi: 10.1155/2012/819512.

Silva DTD, Alves ML, Spada JCP, Leonel JAF, Vioti G, Benassi JC, Carregaro VML, Alves-Martin MF, Starke-Buzetti WA, Oliveira TMFS. Feline leishmaniosis: hematological and biochemical analysis. Rev Bras Parasitol Vet. 2023; 32:e003823. doi: 10.1590/S1984-29612023035.

Singh OP, Hasker E, Boelaert M, Sacks D, Sundar S. Xenodiagnosis to address key questions in visceral leishmaniasis control and elimination. PLoS Negl Trop Dis. 2020; 14:e0008363. doi: 10.1371/journal.pntd.0008363.

Slappendel RJ. Disseminated intravascular coagulation. Vet Clin North Am Small Anim Pract. 1988; 18:169-184. doi: 10.1016/s0195-5616(88)50015-3.

Sobrino R, Ferroglio E, Oleaga A, Romano A, Millan J, Revilla M, Arnal MC, Trisciuoglio A, Gortázar C. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet Parasitol. 2008; 155:198-203. doi: 10.1016/j.vetpar.2008.05.003.

Solano-Gallego L, Cardoso L, Pennisi MG, Petersen C, Bourdeau P, Oliva G, Miró G, Ferrer L, Baneth G. Diagnostic Challenges in the Era of Canine *Leishmania infantum* Vaccines. Trends Parasitol. 2017; 33:706-717. doi: 10.1016/j.pt.2017.06.004.

Solano-Gallego L, Llull J, Ramos G, Riera C, Arboix M, Alberola J, Ferrer L. The Ibizian hound presents a predominantly cellular immune response against natural *Leishmania* infection. Vet Parasitol. 2000; 90:37-45. doi: 10.1016/s0304-4017(00)00223-5.

Solano-Gallego L, Rodríguez-Cortés A, Iniesta L, Quintana J, Pastor J, Espada Y, Portús M, Alberola J. Cross-sectional serosurvey of feline leishmaniasis in ecoregions around the Northwestern Mediterranean. Am J Trop Med Hyg. 2007; 76:676-680.

Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet Parasitol. 2009; 165:1-18. doi: 10.1016/j.vetpar.2009.05.022.

Solano-Gallego L, Miró G, Koutinas A, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G, The LeishVet Group. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit Vectors. 2011; 4:86. doi: 10.1186/1756-3305-4-86.

Solano-Gallego L, Villanueva-Saz S, Carbonell M, Trotta M, Furlanello T, Natale A. Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and *Leishmania* 96), a rapid test (Speed Leish K) and an in-house IFAT. Parasit Vectors. 2014;7:111. doi: 10.1186/1756-3305-7-111.

Solano-Gallego L, Montserrrat-Sangrà S, Ordeix L, Martínez-Orellana P. *Leishmania infantum*-specific production of IFN-γ and IL-10 in stimulated blood from dogs with clinical leishmaniosis. Parasit Vectors. 2016; 9:317. doi: 10.1186/s13071-016-1598-y.

Solcà Mda S, Guedes CE, Nascimento EG, Oliveira GG, dos Santos WL, Fraga DB, Veras PS. Qualitative and quantitative polymerase chain reaction (PCR) for detection of *Leishmania* in spleen samples from naturally infected dogs. Vet Parasitol. 2012; 184:133-140. doi: 10.1016/j.vetpar.2011.08.026.

Solcà MS, Andrade BB, Abbehusen MM, Teixeira CR, Khouri R, Valenzuela JG, Kamhawi S, Bozza PT, Fraga DB, Borges VM, Veras PS, Brodskyn CI. Circulating Biomarkers of Immune Activation, Oxidative Stress and Inflammation Characterize Severe Canine Visceral Leishmaniasis. Sci Rep. 2016; 6:32619. doi: 10.1038/srep32619.

Spada E, Castelli G, Bruno F, Vitale F, La Russa F, Biondi V, Accettulli S, Migliazzo A, Rossi A, Perego R, Baggiani L, Proverbio D. *FeliLeish*: An Update on Feline Leishmaniosis and Factors Associated with Infection in Different Feline Populations from Italy. Pathogens. 2023; 12:1351. doi: 10.3390/pathogens12111351.

Srivastava P, Dayama A, Mehrotra S, Sundar S. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011; 105:1-6. doi: 10.1016/j.trstmh.2010.09.006.

Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasit Vectors. 2016; 9:277. doi: 10.1186/s13071-016-1553-y.

Srividya G, Kulshrestha A, Singh R, Salotra P. Diagnosis of visceral leishmaniasis: developments over the last decade. Parasitol Res. 2012; 110:1065-1078. doi: 10.1007/s00436-011-2680-1.

Strauss-Ayali D, Baneth G, Shor S, Okano F, Jaffe CL. Interleukin-12 augments a Th1-type immune response manifested as lymphocyte proliferation and interferon gamma production in *Leishmania infantum*-infected dogs. Int J Parasitol. 2005; 35:63-73. doi: 10.1016/j.ijpara.2004.10.015.

Tabar MD, Roura X, Francino O, Altet L, Ruiz de Gopegui R. Detection of *Leishmania infantum* by real-time PCR in a canine blood bank. J Small Anim Pract. 2008; 49:325-328. doi: 10.1111/j.1748-5827.2008.00542.x.

Teixeira Neto RG, Giunchetti RC, Carneiro CM, Vitor RW, Coura-Vital W, Quaresma PF, Ker HG, de Melo LA, Gontijo CM, Reis AB. Relationship of *Leishmania*-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis. Vet Parasitol. 2010; 169:248-257. doi: 10.1016/j.vetpar.2010.01.023.

Thakur S, Joshi J, Kaur S. Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. J Parasit Dis. 2020; 44:253-272. doi: 10.1007/s12639-020-01212-w.

Todolí F, Solano-Gallego L, Ojeda A, Quintana J, Lloret A, Roura X, Alberola J, Rodríguez-Cortés A. Anti-*Leishmania* IgA in urine samples from dogs with clinical leishmaniasis. Vet Parasitol. 2009; 159:17-23. doi: 10.1016/j.vetpar.2008.10.010.

Tomassone L, Berriatua E, De Sousa R, Duscher GG, Mihalca AD, Silaghi C, Sprong H, Zintl A. Neglected vector-borne zoonoses in Europe: Into the wild. Vet Parasitol. 2018; 251:17-26. doi: 10.1016/j.vetpar.2017.12.018.

Toplu N, Aydoğan A, Oguzoglu TC. Visceral leishmaniosis and parapoxvirus infection in a Mediterranean monk seal (*Monachus monachus*). J Comp Pathol. 2007; 136:283-287. doi: 10.1016/j.jcpa.2007.02.005.

Toplu N, Aydogan A. An immunohistochemical study in cases with usual and unusual clinicopathological findings of canine visceral leishmaniosis. Parasitol Res. 2011; 109:1051-1057. doi: 10.1007/s00436-011-2345-0.

Travi BL, Cordeiro-da-Silva A, Dantas-Torres F, Miró G. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS Negl Trop Dis. 2018; 12:e0006082. doi: 10.1371/journal.pntd.0006082.

Tsakmakidis I, Lefkaditis M, Zaralis K, Arsenos G. Alternative hosts of *Leishmania infantum*: a neglected parasite in Europe. Trop Anim Health Prod. 2024; 56:128. doi: 10.1007/s11250-024-03978-0.

Tsakmakidis I, Pavlou C, Tamvakis A, Papadopoulos T, Christodoulou V, Angelopoulou K, Dovas CI, Antoniou M, Anastasakis C, Diakou A. *Leishmania* infection in lagomorphs and minks in Greece. Vet Parasitol Reg Stud Reports. 2019; 16:100279. doi: 10.1016/j.vprsr.2019.100279.

Turchetti AP, da Costa LF, Romão Ede L, Fujiwara RT, da Paixão TA, Santos RL. Transcription of innate immunity genes and cytokine secretion by canine macrophages resistant or susceptible to intracellular survival of *Leishmania infantum*. Vet Immunol Immunopathol. 2015; 163:67-76. doi: 10.1016/j.vetimm.2014.11.010.

Velez R, Ballart C, Domenech E, Abras A, Fernández-Arévalo A, Gómez SA, Tebar S, Muñoz C, Cairó J, Gállego M. Seroprevalence of canine *Leishmania infantum* infection in the Mediterranean region and identification of risk factors: The example of North-Eastern and Pyrenean areas of Spain. Prev Vet Med. 2019; 162:67-75. doi: 10.1016/j.prevetmed.2018.10.015.

Verin, R.; Poli, A.; Ariti, G.; Nardoni, S.; Bertuccelli Fanucchi, M.; Mancianti, F. Detection of *Leishmania infantum* DNA in tissues of free-ranging red foxes (*Vulpes vulpes*) in Central Italy. Eur. J. Wild Res. 2010; 56, 689–692. https://doi.org/10.1007/s10344-010-0395-8

Vilas VJ, Maia-Elkhoury AN, Yadon ZE, Cosivi O, Sanchez-Vazquez MJ. Visceral leishmaniasis: a One Health approach. Vet Rec. 2014; 175:42-44. doi: 10.1136/vr.g4378.

Villanueva-Saz S, Basurco A, Martín V, Fernández A, Loste A, Verde MT. Comparison of a qualitative immunochromatographic test with two quantitative serological assays for the detection of antibodies to *Leishmania infantum* in dogs. Acta Vet Scand 2019; 61:38. doi:10.1186/s13028-019-0473-1

Villanueva-Saz S, Lebrero ME, Solsona A, Ramos JJ, de Arcaute MR, Ruíz H, Pérez MD, Bello JM, Verde M, Ortín A, Marteles D, Fernández A, Gómez A, Trotta M, Lacasta D. Presence of anti-*Leishmania infantum* antibodies in sheep (*Ovis aries*) in Spain. Vet Res Commun. 2023; 48, 615–621. doi: 10.1007/s11259-023-10221-y.

Villanueva-Saz S, Martínez M, Ramirez JD, Herrera G, Marteles D, Servián M, Verde M, Giner J, Lacasta D, Ruíz H, Yzuel A, Fernández A. Evaluation of five different rapid immunochromatographic tests for canine leishmaniosis in Spain. Acta Trop. 2022; 229:106371. doi: 10.1016/j.actatropica.2022.106371.

Viol MA, Lima VM, Aquino MC, Gallo G, Alves IP, Generoso D, Perri SH, Lucheis SB, Langoni H, Nunes CM, Bresciani KD. Detection of cross infections by *Leishmania* spp. and *Trypanosoma* spp. in dogs using indirect immunoenzyme assay, indirect fluorescent antibody test and polymerase chain reaction. Parasitol Res. 2012; 111:1607-1613. doi: 10.1007/s00436-012-2999-2.

Vioti G, da Silva MD, Galvis-Ovallos F, Alves ML, da Silva DT, Leonel JAF, Pereira NWB, Benassi JC, Spada JCP, Maia C, Galati EAB, Starke-Buzetti WA, Oliveira TMFS. Xenodiagnosis in four domestic cats naturally infected by *Leishmania infantum*. Transbound Emerg Dis. 2022; 69:2182-2190. doi: 10.1111/tbed.14216.

White MR, Chapman WL Jr, Hanson WL. A comparison of experimental visceral leishmaniasis in the opossum, armadillo and ferret. Lab Anim Sci. 1989; 39:47-50.

WHO (2014a) Leishmaniasis: A Global Brief on Vector Borne Diseases. http://apps.who.int/iris/ bitstream/10665/111008/1/WH O_DCO_ WHD_2014.1_eng.pdf. Accessed July 7, 2023.

WHO (2014b) Strategic framework for leishmaniasis control in the WHO European Region 2014-2020. www. euro.who.int/__data/assets/pdf_file/0017/245330/ Strategic-framework-for-leishmaniasis-control-in-theWHO-European-Region-20142020.pdf. Accessed July 7, 2023

Wolf D, Failing K, Taubert A, Pantchev N. Serological diagnosis of canine leishmaniosis: comparison of three commercially available tests. Parasitol Res. 2014; 113:1997-2002. doi: 10.1007/s00436-014-3865-1.

Zaffaroni E, Rubaudo L, Lanfranchi P, Mignone W. Epidemiological patterns of canine leishmaniasis [correction of leishmaniosis] in Western Liguria (Italy). Vet Parasitol. 1999; 81:11-19. doi: 10.1016/s0304-4017(98)00237-4.

Zafra R, Jaber JR, Pérez-Ecija RA, Barragán A, Martínez-Moreno A, Pérez J. High iNOS expression in macrophages in canine leishmaniasis is associated with low intracellular parasite burden. Vet Immunol Immunopathol. 2008; 123:353-359. doi: 10.1016/j.vetimm.2008.02.022.

Zanette MF, Lima VM, Laurenti MD, Rossi CN, Vides JP, Vieira RF, Biondo AW, Marcondes M. Serological cross-reactivity of *Trypanosoma cruzi*, *Ehrlichia canis*, *Toxoplasma gondii*, *Neospora caninum* and *Babesia canis* to *Leishmania infantum chagasi* tests in dogs. Rev Soc Bras Med Trop. 2014; 47:105-107. doi: 10.1590/0037-8682-1723-2013.

Zárate-Ramos JJ, Arbea-Sarasa I, Gómez-Ochoa P, Castillo-Hernández JA, García-Salinas, MJ, Morales-Amella MJ. Serological evidence of leishmaniasis in cats in Aragon, Spain. In: Proceedings of the 27th WSAVA Congress. Granada: World Small Animal Veterinary Association; 2002.

Zribi L, El-Goulli AF, Ben-Abid M, Gharbi M, Ben-Sghaier I, Boufaden I, Aoun K, Bouratbine A. Use of an Interferon Gamma Release Assay (IGRA) to test T-cell responsiveness to soluble *Leishmania infantum* antigen in whole blood of dogs from endemic areas. Vet Parasitol. 2017; 246:88-92. doi: 10.1016/j.vetpar.2017.08.029.