Universitat Autònoma de Barcelona

Leishmania infantum infection in dogs: from diagnosis to immunotherapy and prevention

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Tesi doctoral

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La Dra. **Laia Maria Solano Gallego**, Catedràtica del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona,

INFORMA:

Que el treball de tesi doctoral titulat

"***Leishmania infantum* infection in dogs: from diagnosis to immunotherapy and prevention**"

Del que és autora la graduada en veterinària

Marta Baxarias Canals

Ha estat realitzat sota la meva direcció i compleix les condicions exigides per optar al títol de Doctora per la Universitat Autònoma de Barcelona.

I per a què així consti, signo el present informe a Bellaterra, Setembre 2022

Dra. Laia Maria Solano Gallego

Directora i tutora

Marta Baxarias Canals

Doctoranda

Agraïments

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List of abbreviations

A/G	Albumin/globulin
A2	Recombinant *Leishmania infantum* and *Leishmania donovani* amastigote-specific antigen
AHCC	Active hexose dietary compound
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Anti-GITR	Anti-glucocorticoid-induced TNF receptor related protein
ASA	Acetyl salicylic acid
AST	Aspartate transaminase
CAN	Only Canileish® vaccine
CAN + LEI	Canileish® vaccine + Leisguard®
CAN + LEI + REP	Canileish® vaccine + Leisguard® + repellent
CAN + REP	Canileish® vaccine + repellent
CanL	Canine leishmaniosis
CBC	Complete blood count
CG	Control group
CI	Confidence interval
CKD	Chronic kidney disease
CL	Cutaneous leishmaniosis
CMI	Cell-mediated immunity
conc	Concentration
CpG	Un-methylated cytosine triphosphate deoxynucleotide followed by phosphodiester link with guanine triphosphate deoxynucleotide
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL10	Chemokine (C-X-C motif) ligand 10
DAT	Direct agglutination test
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
EU	ELISA units
FML	Fucose mannose ligand
GM-CSF	Granulocyte/macrophage colony stimulating factor
HDP	Host defense peptide
HIV	Human immunodeficiency virus
HL	Human leishmaniosis
HMA	Heads of Medicine Agencies
IFAT	Immunofluorescent antibody test
IFI	Indirect immunofluorescence

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobin A
IgG	Immunoglobin G
IgM	Immunoglobin M
IL	Interleukin
IMQ	Imiquimod
IRIS	International Renal Interest Society
LAMP	Loop-mediated isothermal amplification
LBMPL	*L. braziliensis* promastigote protein+ monophosphoryl A
LEI	Only Leisguard®
LEI + REP	Leisguard® + repellent
LeIF	*Leishmania* elongation initiation factor
Leish-111f	Trifusion recombinant protein of TSA, LmSTI1 and LeIF
LmSTI1	*Leishmania major* stress-inducible protein 1
LET	Only Letifend® vaccine
LET + LEI + REP	Letifend® vaccine + Leisguard® + repellent
LET + REP	Letifend® vaccine + repellent
MAb	Monoclonal antibodies
max	Maximum
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCL	Mucocutaneous leishmaniosis
MCP	Monocyte chemoattractant protein
MCV	Mean corpuscular volume
min	Minimum
MISA	Montanide ISA 720
MPL	Monophosphoryl lipid A
n	Number
NBT	Nitro blue tetrazolium
Neg	Negative
NK	Natural killer
NO	Nitric oxide
NON	No preventive measures applied
OD	Optical density
OR	Odds ratio
OT	Oral transudate
Pam3Cys	N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4 hydrochloride
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1

PD-L1	Ligand of programmed cell death protein-1
P-MAPA	Protein aggregate of magnesium-ammoniumphospholinoleate-palmitoleate anhydride
PMN	Polymorphonuclear cell
Pos	Positive
QuilA	Saponin adjuvant produced with *Quilaja saponaria*
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RBC	Red blood cell count
Redox	Reduction-oxidation
REP	Only repellents applied
rLdccys1	Recombinant cysteine proteinase from *L. infantum*
ROS	Reactive oxygen species
rt-PCR	Real-time PCR
SD	Standard deviation
SE	Stable emulsion
SPE	Serum protein electrophoresis
TG	Treated group
TGF	Transforming growth factor
Th	T helper
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alfa
TNF-β	Tumor necrosis factor beta
Treg	Regulatory T cells
TR-IFMA	Time-resolved immunofluorometric assay
TSA	Thiol-specific antioxidant
UPC	Urinary protein/creatinine
USG	Urinary specific gravity
VL	Visceral leishmaniosis
WBC	Leukocytes concentration
WHO	World Health Organization

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Summary

Canine leishmaniosis (CanL) caused by the protozoan *Leishmania infantum* is considered a major zoonosis in Europe and its control and prevention is one of the main objectives of both public health agencies and veterinarians. The development of clinical leishmaniosis is closely influenced by the immune response of the host, so, recently, new methods for the prevention of this disease using immunoprophylaxis have appeared. The use of immunotherapy such as Leisguard® (domperidone) to enhance the specific immune response against *Leishmania* has been explored in dogs with positive results. However, there are still doubts about the use and effectiveness of immunotherapeutics in the clinical field, both in the prevention and treatment of CanL. Furthermore, CanL often requires an integrated approach, including a clinicopathological examination and specific laboratory tests due to its complexity and variability. Several diagnostic techniques are available to detect *L. infantum* infection and these techniques are usually performed using different types of samples such as blood and serum. However, the use of alternative samples such as oral transudate (OT) is promising and could improve the prevention and control of the disease as the collection of OT is non-invasive, cheap and painless and could be performed by untrained personnel.

The first hypothesis of this doctoral thesis was that the current use of immunotherapy (domperidone, Leisguard®) and other preventive measures to prevent *L. infantum* infection in dogs living in high endemic regions has increased in the last decade. The increase in use of these preventive measures should improve the control of *L. infantum* infection in endemic regions and assist on decreasing the prevalence of both infection and clinical disease. Additionally, the second hypothesis was that the use of immunotherapy (Leisguard®) alone could also avoid the development of clinical illness in *Leishmania*-seropositive healthy dogs. The use of immunotherapy in *Leishmania*-seropositive healthy dogs might improve the dogs' immune response and, thus, the dogs would present neither clinical signs nor laboratory findings while treated only with Leisguard®. Finally, early detection of *L. infantum* infection is highly important to control and prevent the disease in endemic countries. Several diagnostic techniques, usually performed with blood, serum, urine and other infected tissues, are available to diagnose *L. infantum* infection. However, the use of alternative samples has also been investigated with promising results. For this reason, it was hypothesized that OT could be a promising sample to diagnose *L. infantum* infection.

Therefore, the general objectives of this doctoral thesis were: 1) to investigate the use of serological screening tools and preventive measures against *L. infantum* infection in dogs from European *Leishmania*-endemic countries, 2) to investigate and validate new diagnostic techniques for the early detection and follow-up of *L. infantum* infection and immune response in dogs in the clinical setting and 3) to evaluate the efficacy and

safety of Leisguard® as an immunotherapeutic for *Leishmania*-seropositive healthy dogs to avoid the development of clinical illness. Additionally, the specific objectives were: 1) to investigate the most used serological screening tools and preventive measures against *L. infantum* infection in dogs from Europe and how their use changed through the years (chapter 3), 2) to determine the seroprevalence of *L. infantum* infection in apparently healthy dogs in Spain (chapter 4), 3) to investigate and validate new diagnostic methods for the detection of *L. infantum* infection and immune response in dogs (chapters 5 and 6), 4) to investigate the signalment, clinicopathological findings and serological status of *Leishmania*-seropositive healthy dogs (chapter 7) and 5) to assess the efficacy and safety of Leisguard® as an immunotherapeutic for *Leishmania*-seropositive healthy dogs (chapter 8).

In this doctoral thesis, the current use of preventive measures in *L. infantum* infection in dogs from Spain is described and confirms that dog owners follow the veterinarian's recommendations as endorsed by the already published guidelines (chapter 3). As expected, repellents were the preferred preventive measure of dog owners followed by the vaccines and Leisguard®. However, there were still dogs that did not use preventive measures in endemic regions (chapter 3). Moreover, chapter 4 described the current seroprevalence rate for *L. infantum* in apparently healthy dogs that live in Spain. Similarly to previous studies performed in Spain, the seroprevalence rates varied from almost no infection in the Northern areas of Spain to being over 10% in the Southeast close to the Mediterranean basin. These results highlight again the imperative need to use preventive measures against *L. infantum* in Spain.

Furthermore, the current use of serological screening tools in *L. infantum* infection in dogs from Spain is also described and a preference for the use of rapid tests in the clinical setting to detect specific *L. infantum* antibodies was found. This reinforces the need to sensitize clinicians about the limitations that qualitative serological techniques can present in the diagnosis of seropositive dogs in endemic areas (chapter 3).

In this doctoral thesis, the development and validation of diagnostic techniques to detect *L. infantum* infection and the development of disease was also investigated. In chapter 5, an in-house ELISA for the detection of specific antibodies against *L. infantum* in OT was developed. This new technique was promising, especially in sick dogs with high antibody levels, as it presented similar results to the routine technique performed with serum. However, further studies should be performed to improve the reliability of the technique. In chapter 6, a study of the effect of storage on nitro blue tetrazolium (NBT) reduction test in dog blood samples was performed with positive results. The study showed that the NBT reduction test could be performed up to 72 h after collection of canine blood if correctly refrigerated at 4ºC.

In chapter 7, *Leishmania*-seropositive healthy dogs, which are a clinically neglected group regarding treatment of *L. infantum*, were investigated. Chapter 7 is a descriptive

study of the signalment and serological status of these *Leishmania*-seropositive healthy dogs. The study demonstrated that apparently healthy dogs by physical examination can present several clinicopathological findings and, therefore, disease could be diagnosed earlier. Most of the dogs that presented clinicopathological findings were classified in LeishVet stage IIa and the most consistent findings were plasma protein alterations including polyclonal hypergammaglobulinemia, hyperproteinemia and decreased A/G ratio, proteinuria and lymphopenia. Furthermore, the majority of healthy dogs without clinicopathological abnormalities presented low antibody levels against *L. infantum* antigen while most apparently healthy dogs with clinicopathological alterations presented medium to high antibody levels. Finally, *Leishmania*-seropositive healthy dogs without clinicopathological abnormalities were further studied in chapter 8 in a blinded, randomized and controlled multicentre clinical trial to assess the efficacy and safety of Leisguard® as an immunotherapeutic treatment. The use of Leisguard® in healthy dogs with low *L. infantum* antibody levels proved to be effective against disease development. Therefore, Leisguard® can be employed in the clinical setting in healthy dogs classified based on physical examination and routine laboratory tests and with low antibody levels. Furthermore, Leisguard® presented a good safety as few dogs presented adverse drug effects which were very mild and self-limiting.

In conclusion, this doctoral thesis demonstrated the importance of immunotherapy in *L. infantum* infection in dogs for both the prevention of the infection and the treatment against disease development in *Leishmania*-seropositive healthy dogs. Furthermore, the significance of preventive measures, screening tools and annual check-ups in *L. infantum*-endemic countries was also highlighted. Finally, the value of new techniques that could be performed routinely in the clinical setting was also underlined.

Resum

La leishmaniosis canina (CanL) causada pel protozoo *Leishmania infantum* es considera una zoonosis important a Europa i el seu control i prevenció és un dels principals objectius tant de les agències de salut pública com dels veterinaris. El desenvolupament de la leishmaniosi clínica està molt influenciat per la resposta immunitària de l'hoste, per la qual cosa, recentment, han aparegut nous mètodes per a la prevenció d'aquesta malaltia mitjançant la immunoprofilaxi. L'ús d'immunoteràpia com Leisguard® (domperidona) per millorar la resposta immunitària específica enfront *Leishmania* s'ha explorat en gossos amb resultats positius. Tanmateix, encara hi ha dubtes sobre l'ús i l'eficàcia de la immunoteràpia en l'àmbit clínic, tant en la prevenció com en el tractament de la CanL. A més, la CanL sovint requereix un enfocament integrat, que inclou un examen clinicopatològic i proves específiques de laboratori degut a la seva complexitat i variabilitat. Es disposa de diverses tècniques de diagnòstic per detectar la infecció per *L. infantum* i aquestes tècniques solen realitzar-se utilitzant diferents tipus de mostres com ara sang i sèrum. No obstant això, l'ús de mostres alternatives com el transsudat oral (OT) és prometedor i podria millorar la prevenció i el control de la malaltia, ja que la recollida d'OT és no invasiva, barata i indolora, i podria ser realitzada per personal no format.

La primera hipòtesi d'aquesta tesi doctoral va ser que l'ús actual de la immunoteràpia (domperidona, Leisguard®) i altres mesures preventives per prevenir la infecció per *L. infantum* en gossos que viuen en regions d'alta endèmia ha augmentat en l'última dècada. L'augment de l'ús d'aquestes mesures preventives hauria de millorar el control de la infecció per *L. infantum* a les regions endèmiques i ajudar a disminuir la prevalença tant de la infecció com de la malaltia clínica. A més, la segona hipòtesi era que l'ús sol de la immunoteràpia (Leisguard®) també podria evitar el desenvolupament de malaltia clínica en gossos sans i *Leishmania* seropositius. L'ús de la immunoteràpia en gossos sans i *Leishmania* seropositius podria millorar la resposta immune dels gossos i, per tant, els gossos no presentarien ni signes clínics ni anomalies de laboratori mentre es tractessin només amb Leisguard®. Finalment, la detecció precoç de la infecció per *L. infantum* és molt important per controlar i prevenir la malaltia als països endèmics. Per diagnosticar la infecció per *L. infantum* es disposa de diverses tècniques de diagnòstic, normalment realitzades amb sang, sèrum, orina i altres teixits infectats. Tanmateix, també s'ha investigat l'ús de mostres alternatives amb resultats prometedors. Per aquest motiu, es va plantejar la hipòtesi que l'OT podria ser una mostra prometedora per diagnosticar la infecció per *L. infantum*.

Per tant, els objectius generals d'aquesta tesi doctoral eren: 1) investigar l'ús d'eines de cribratge serològic i mesures preventives contra la infecció per *L. infantum* en gossos de països europeus amb endèmia de *Leishmania*, 2) investigar i validar noves tècniques de diagnòstic per a la detecció precoç i el seguiment de la infecció per *L.*

infantum i la resposta immunitària en gossos en l'àmbit clínic i 3) avaluar l'eficàcia i la seguretat de Leisguard® com a immunoterapèutic per a gossos sans i *Leishmania* seropositius per evitar el desenvolupament de malaltia clínica. Addicionalment, els objectius específics eren: 1) investigar les eines de cribratge serològic i les mesures preventives més utilitzades contra la infecció per *L. infantum* en gossos d'Europa i com ha canviat el seu ús al llarg dels anys (capítol 3), 2) determinar la seroprevalència de la infecció per *L. infantum* en gossos aparentment sans a Espanya (capítol 4), 3) investigar i validar nous mètodes de diagnòstic per a la detecció de la infecció per *L. infantum* i la resposta immunitària en gossos (capítols 5 i 6), 4) investigar les característiques clinicopatològiques i estat serològic dels gossos sans i *Leishmania* seropositius (capítol 7) i 5) avaluar l'eficàcia i la seguretat de Leisguard® com a immunoterapèutic per a gossos sans i *Leishmania* seropositius (capítol 8).

En aquesta tesi doctoral es descriu l'ús actual de les mesures preventives contra la infecció per *L. infantum* en gossos d'Espanya i es confirma que els propietaris de gossos segueixen les recomanacions dels veterinaris avalades per les directrius ja publicades (capítol 3). Com era d'esperar, els repel·lents eren la mesura preventiva preferida dels propietaris de gossos seguits per les vacunes i Leisguard®. Tanmateix, encara hi havia gossos que no utilitzaven mesures preventives a les regions endèmiques (capítol 3). A més, en el capítol 4 es descriu la taxa de seroprevalència actual de *L. infantum* en gossos aparentment sans que viuen a Espanya. De la mateixa manera que en estudis anteriors realitzats a Espanya, les taxes de seroprevalència van variar des de gairebé cap infecció a les zones del nord d'Espanya fins a superar el 10% al sud-est prop de la conca mediterrània. Aquests resultats tornen a posar de manifest la necessitat imperiosa d'utilitzar mesures preventives contra *L. infantum* a Espanya.

A més, també es descriu l'ús actual d'eines de cribratge serològic en la infecció per *L. infantum* en gossos d'Espanya i es va trobar una preferència per l'ús de proves ràpides en l'àmbit clínic per detectar anticossos específics de *L. infantum*. Això reforça la necessitat de sensibilitzar als clínics sobre les limitacions que les tècniques serològiques qualitatives poden presentar en el diagnòstic de gossos seropositius en zones endèmiques (capítol 3).

En aquesta tesi doctoral també s'ha investigat el desenvolupament i validació de tècniques diagnòstiques per detectar la infecció per *L. infantum* i el desenvolupament de malaltia. Al capítol 5, es va desenvolupar un ELISA in house per a la detecció d'anticossos específics contra *L. infantum* en OT. Aquesta nova tècnica era prometedora, sobretot en gossos malalts amb nivells elevats d'anticossos, ja que presentava resultats similars a la tècnica rutinària realitzada amb sèrum. Tanmateix, s'han de realitzar estudis addicionals per millorar la fiabilitat de la tècnica. En el capítol 6, es va realitzar un estudi de l'efecte de l'emmagatzematge en la prova de reducció de nitroblau de tetrazoli (NBT) en mostres de sang de gos amb resultats positius. L'estudi

va demostrar que la prova de reducció de NBT es podria realitzar fins a 72 h després de la recollida de sang canina si es refrigerava correctament a 4ºC.

En el capítol 7 es van investigar gossos sans i *Leishmania* seropositius, que són un grup clínicament negligit pel que fa al tractament de *L. infantum*. El capítol 7 és un estudi descriptiu de les característiques i l'estat serològic d'aquests gossos sans i *Leishmania* seropositius. L'estudi va demostrar que els gossos aparentment sans poden presentar en l'examen físic diverses anomalies clinicopatològiques i, per tant, la malaltia podria ser diagnosticada abans. La majoria dels gossos que van presentar anomalies clinicopatològiques es van classificar en l'estadi IIa de LeishVet i les troballes més consistents van ser alteracions en proteïnes plasmàtiques, incloent hipergammaglobulinemia policlonal, hiperproteinèmia i disminució de la relació A/G, proteinúria i limfopènia. A més, la majoria de gossos sans sense anomalies clinicopatològiques presentaven nivells baixos d'anticossos contra l'antigen de *L. infantum* mentre que la majoria dels gossos aparentment sans amb alteracions clinicopatològiques presentaven nivells d'anticossos de mitjans a alts. Finalment, els gossos sans i *Leishmania* seropositius sense anomalies clinicopatològiques es van seguir estudiant al capítol 8 en un assaig clínic multicèntric cec, aleatoritzat i controlat per avaluar l'eficàcia i la seguretat de Leisguard® com a tractament immunoterapèutic. L'ús de Leisguard® en gossos sans amb nivells baixos d'anticossos de *L. infantum* va demostrar ser efectiu contra el desenvolupament de malaltia. Per tant, Leisguard® es pot utilitzar en l'àmbit clínic en gossos sans classificats en funció de l'examen físic i les proves de laboratori de rutina i amb nivells baixos d'anticossos. A més, Leisguard® presentava una bona seguretat ja que pocs gossos van presentar efectes adversos del fàrmac que van ser molt lleus i autolimitants.

En conclusió, aquesta tesi doctoral va demostrar la importància de la immunoteràpia en la infecció per *L. infantum* en gossos tant per a la prevenció de la infecció com per al tractament contra el desenvolupament de la malaltia en gossos sans i *Leishmania* seropositius. A més, també es va destacar la importància de les mesures preventives, les eines de cribratge i els controls anuals als països endèmics de *L. infantum*. Finalment, també s'ha subratllat el valor de les noves tècniques que es podrien realitzar rutinàriament en l'àmbit clínic.

Resumen

La leishmaniosis canina (CanL) causada por el protozoo *Leishmania infantum* se considera una zoonosis importante en Europa y su control y prevención es uno de los principales objetivos tanto de las agencias de salud pública como de los veterinarios. El desarrollo de la leishmaniosis clínica está muy influenciado por la respuesta inmunitaria del huésped, por lo que recientemente han aparecido nuevos métodos para la prevención de esta enfermedad mediante inmunoprofilaxis. El uso de inmunoterapia como Leisguard® (domperidona) para potenciar la respuesta inmunitaria específica frente a *Leishmania* se ha explorado en perros con resultados positivos. Sin embargo, aún existen dudas sobre el uso y la eficacia de los inmunoterapéuticos en el ámbito clínico, tanto en la prevención como en el tratamiento de la CanL. Además, la CanL a menudo requiere un enfoque integrado, que incluye un examen clinicopatológico y pruebas de laboratorio específicas debido a su complejidad y variabilidad. Hay varias técnicas de diagnóstico disponibles para detectar la infección por *L. infantum* y estas técnicas generalmente se realizan utilizando diferentes tipos de muestras, como sangre y suero. Sin embargo, el uso de muestras alternativas como el trasudado oral (OT) es prometedor y podría mejorar la prevención y el control de la enfermedad, ya que la recolección de OT no es invasiva, es económica e indolora y podría ser realizada por personal no formado.

La primera hipótesis de esta tesis doctoral fue que el uso actual de inmunoterapia (domperidona, Leisguard®) y otras medidas preventivas para prevenir la infección por *L. infantum* en perros que viven en regiones de alta endemia se ha incrementado en la última década. El aumento en el uso de estas medidas preventivas debería mejorar el control de la infección por *L. infantum* en las regiones endémicas y ayudar a disminuir la prevalencia tanto de la infección como de la enfermedad clínica. Además, la segunda hipótesis era que el uso de inmunoterapia (Leisguard®) por sí sola también podría evitar el desarrollo de la enfermedad clínica en perros sanos y seropositivos para *Leishmania*. El uso de inmunoterapia en perros sanos y seropositivos para *Leishmania* podría mejorar la respuesta inmunitaria de los perros y, por lo tanto, los perros no presentarían signos clínicos ni hallazgos de laboratorio mientras fueran tratados solo con Leisguard®. Finalmente, la detección temprana de la infección por *L. infantum* es muy importante para controlar y prevenir la enfermedad en los países endémicos. Varias técnicas de diagnóstico, generalmente realizadas con sangre, suero, orina y otros tejidos infectados, están disponibles para diagnosticar la infección por *L. infantum*. Sin embargo, también se ha investigado el uso de muestras alternativas con resultados prometedores. Por esta razón, se planteó la hipótesis de que la OT podría ser una muestra prometedora para diagnosticar la infección por *L. infantum*.

Por tanto, los objetivos generales de esta tesis doctoral fueron: 1) investigar el uso de herramientas de cribado serológico y medidas preventivas frente a la infección por *L.*

infantum en perros de países europeos con *Leishmania* endémica, 2) investigar y validar nuevas técnicas de diagnóstico para la detección temprana y el seguimiento de la infección por *L. infantum* y la respuesta inmunitaria en perros en el entorno clínico y 3) evaluar la eficacia y seguridad de Leisguard® como inmunoterapéutico para perros sanos y seropositivos a *Leishmania* para evitar el desarrollo de la enfermedad clínica. Además, los objetivos específicos fueron: 1) investigar las herramientas de detección serológica y las medidas preventivas más utilizadas frente a la infección por *L. infantum* en perros de Europa y cómo cambió su uso a lo largo de los años (capítulo 3), 2) determinar la seroprevalencia de *L. infantum* en perros aparentemente sanos en España (capítulo 4), 3) investigar y validar nuevos métodos de diagnóstico para la detección de la infección por *L. infantum* y la respuesta inmunitaria en perros (capítulos 5 y 6), 4) investigar las características clinicopatológicas y estado serológico de perros sanos y seropositivos a *Leishmania* (capítulo 7) y 5) evaluar la eficacia y seguridad de Leisguard® como inmunoterapéutico para perros sanos y seropositivos a *Leishmania* (capítulo 8).

En esta tesis doctoral se describe el uso actual de las medidas preventivas contra la infección por *L. infantum* en perros de España y se confirma que los dueños de perros siguen las recomendaciones del veterinario tal como lo avalan las guías ya publicadas (capítulo 3). Como era de esperar, los repelentes fueron la medida preventiva preferida de los dueños de perros, seguidos por las vacunas y Leisguard®. Sin embargo, todavía había perros que no usaban medidas preventivas en regiones endémicas (capítulo 3). Además, en el capítulo 4 se describe la tasa de seroprevalencia actual de *L. infantum* en perros aparentemente sanos que viven en España. Al igual que en estudios realizados previamente en España, las tasas de seroprevalencia variaron desde casi ausencia de infección en las zonas del norte de España hasta superar el 10% en el sureste, cerca de la cuenca del Mediterráneo. Estos resultados ponen de manifiesto de nuevo la imperiosa necesidad de utilizar medidas preventivas frente a *L. infantum* en España.

Además, también se describe el uso actual de herramientas de cribado serológico en la infección por *L. infantum* en perros que viven en España y se encuentra una preferencia por el uso de pruebas rápidas en el entorno clínico para detectar anticuerpos específicos frente a *L. infantum*. Esto refuerza la necesidad de sensibilizar a los clínicos sobre las limitaciones que las técnicas serológicas cualitativas pueden presentar en el diagnóstico de perros seropositivos en áreas endémicas (capítulo 3).

En esta tesis doctoral también se investigó el desarrollo y validación de técnicas diagnósticas para detectar la infección por *L. infantum* y el desarrollo de la enfermedad. En el capítulo 5, se describe un ELISA in house para la detección de anticuerpos específicos frente a *L. infantum* en OT. Esta nueva técnica resultó prometedora, especialmente en perros enfermos con niveles elevados de anticuerpos,

ya que presentó resultados similares a la técnica de rutina realizada con suero. Sin embargo, se deben realizar más estudios para mejorar la fiabilidad de la técnica. En el capítulo 6, se detalla un estudio del efecto del almacenamiento en la prueba de reducción de nitro azul de tetrazolio (NBT) en muestras de sangre de perros con resultados positivos. El estudio mostró que la prueba de reducción de NBT se podía realizar hasta 72 h después de la extracción de sangre canina si se refrigeraba correctamente a 4ºC.

En el capítulo 7 se investigaron perros sanos y seropositivos a *Leishmania*, que son un grupo clínicamente desatendido en cuanto al tratamiento de *L. infantum*. El capítulo 7 es un estudio descriptivo de las características y estado serológico de estos perros sanos y seropositivos a *Leishmania*. El estudio demostró que perros aparentemente sanos al examen físico pueden presentar varios hallazgos clinicopatológicos y, por lo tanto, la enfermedad podría diagnosticarse antes. La mayoría de los perros que presentaron hallazgos clinicopatológicos se clasificaron en estadio IIa de LeishVet y los hallazgos más consistentes fueron alteraciones en las proteínas plasmáticas, incluyendo hipergammaglobulinemia policlonal, hiperproteinemia y disminución del cociente A/G, proteinuria y linfopenia. Además, la mayoría de los perros sanos sin anomalías clínicopatológicas presentaron niveles bajos de anticuerpos frente al antígeno de *L. infantum* mientras que la mayoría de los perros aparentemente sanos con alteraciones clinicopatológicas presentaron niveles de anticuerpos medios a altos. Los perros sanos y seropositivos para *Leishmania* sin anomalías clinicopatológicas se estudiaron más a fondo en el capítulo 8 en un ensayo clínico multicéntrico, controlado, aleatorizado y ciego para evaluar la eficacia y la seguridad de Leisguard® como tratamiento inmunoterapéutico. El uso de Leisguard® en perros sanos con niveles bajos de anticuerpos frente a *L. infantum* demostró ser eficaz contra el desarrollo de la enfermedad. Por tanto, Leisguard® se puede emplear en el entorno clínico en perros sanos clasificados según el examen físico y las pruebas de laboratorio de rutina y con niveles bajos de anticuerpos. Además, Leisguard® presentó una buena seguridad ya que pocos perros presentaron efectos adversos del fármaco que fueron muy leves y autolimitados.

En conclusión, esta tesis doctoral demostró la importancia de la inmunoterapia en la infección por *L. infantum* en perros tanto para la prevención de la infección como para el tratamiento contra el desarrollo de la enfermedad en perros sanos y seropositivos a *Leishmania*. Además, también se destacó la importancia de las medidas preventivas, las herramientas de detección y los controles anuales en los países endémicos de *L. infantum*. Finalmente, también se subrayó el valor de las nuevas técnicas que podrían realizarse de forma rutinaria en el entorno clínico.

Justification

Canine leishmaniosis caused by *Leishmania infantum* is a zoonotic disease encountered in more than 80 countries worldwide [1]. Considered a major zoonosis in Europe, its control and prevention is one of the main objectives of both public health agencies and veterinarians [2,3]. The protozoan *L. infantum* is transmitted through the bite of a female phlebotomine sand fly [4,5] and, therefore, one of the most used ways of prevention is repellents, which can be found in different formats such as collars or spot-on [6]. Recently, new methods have appeared for the prevention of this disease, the most important of which are related to immunoprophylaxis (through vaccines) or immunotherapy, which modulates the immune response [6].

Regarding the immune system, a broad range of immune responses and clinical manifestations have been described in canine *L. infantum* infection [7,8]. In fact, the development of clinical leishmaniosis is closely influenced by the host's immune response [9,10]. Therefore, the immune response requires a balance between inflammatory and regulatory responses to control the infection and avoid disease development [9,10]. For example, a dog that displays a protective cell-mediated immune response characterized by interferon gamma (IFN-γ) release should be able to control the infection. In contrast, another dog that displays mainly a non-protective marked humoral immune response combined with absent or diminished cell-mediated immunity will be susceptible to *Leishmania* infection and clinical disease [9].

Once a dog is infected with *L. infantum*, there is no drug that can achieve a complete elimination of the parasite and, therefore, the presence of clinical disease and the following relapses are commonly expected [11]. The treatment of choice for this canine infection includes antimonials, which actively reduce the parasite load, and allopurinol, which keeps the parasite load at low levels [8,11]. Unfortunately, these drugs can present various adverse effects such as nephrotoxicity [12,13], urolithiasis [14] or digestive disorders [15]. In addition, resistance to several of these drugs has also been observed in some *Leishmania* spp [16,17]. Moreover, these treatments are usually long, sometimes without the opportunity to be discontinued, and only aim to reduce the parasitic load so that the immune response of the dog can be effective [8].

For this reason, with the knowledge that the immune response affects strongly the presentation of the disease, the most promising prophylactic and therapeutic approach includes the use of immunotherapy to enhance the specific immune response against this parasite. Leisguard® (domperidone) is a product that has been explored with positive results against *Leishmania* infection in dogs [18–21]. These effects are mainly caused by the reversible increase in blood levels of prolactin [22], which induces an increase in CD4+ lymphocytes, in addition to the release of several interleukins such as interleukin (IL)-2, IL-12, IFN-γ and tumor necrosis factor alfa (TNF-

α), resulting in the activation of both natural killer (NK) and macrophages, followed by a decrease in CD4+ T helper (Th)2 and tumor necrosis factor beta (TNF-β) [23–25]. However, there are still doubts about the use and effectiveness of domperidone in the clinical field, both in the prevention and treatment of CanL.

Regarding the diagnosis of *L. infantum* infection, CanL often requires an integrated approach, including a clinicopathological examination and specific laboratory tests due to its complexity and variability [7,8]. A full clinical history, thorough physical examination and several routine diagnostic tests are necessary to be suspicious of CanL [7,8]. In addition, several diagnostic techniques are available to detect *L. infantum* infection [7,26,27]. These diagnostic techniques must be used with full knowledge of the basis of each test and its limitations, as well as how to correctly interpret the results [7,27]. Interestingly, these diagnostic techniques have been performed using different types of samples such as blood, serum, urine and other infected tissues [28–30]. However, the use of alternative samples such as OT or hair has also been briefly studied with promising results [31–33]. The advantages of using these types of samples instead of serum include a non-invasive, cheap and painless collection which could also be performed by untrained personnel and improve prevention and control of the disease.

The organization of the studies of this thesis was performed chronologically as follows:

- Chapters 1 and 2 describe the introduction, hypotheses and objectives of this thesis. A full review was performed to comprehend the current knowledge of *L. infantum* infection in dogs to be able to develop further experimental studies. Currently, numerous studies have been performed on immunotherapy for preventing and controlling canine *L. infantum* infection.
- Chapter 3 focuses on the current use in Europe of preventive measures and serological screening tools in *L. infantum* infection in dogs. This information was not previously available as most of the questionnaires focused only on the veterinarian recommendations instead of the real use of these products by the dog owners which depends on several factors such as purchasing power and dog owner knowledge about CanL.
- Chapter 4 outlines a serological survey of *L. infantum* infection in apparently healthy dogs that was performed in Spain. This study was performed during the searching for *L. infantum*-seropositive dogs to be enrolled in the blinded, randomized and controlled multicenter clinical trial to assess the efficacy and safety of Leisguard® as an immunotherapeutic treatment (chapter 8). The data collected was very interesting as few serological serosurveys performed in Spain focused specifically in apparently healthy dogs. In addition, a large number of dogs were screened in different areas of Spain.

- Chapters 5 and 6 focus on the development of new diagnostic techniques to detect *L. infantum* infection and the development of disease. These techniques were developed with the idea of being used in the study described in chapter 8. An in-house ELISA for the detection of specific antibodies against *L. infantum* in OT was developed (chapter 5). A study of the effect of storage on NBT reduction test in dog blood samples was performed to confirm if this technique could be used in blood samples collected more than 24 h ago (chapter 6).
- Chapter 7 reports a descriptive study of the signalment and serological status of *Leishmania*-seropositive apparently healthy dogs. This study was performed during the inclusion of dogs in the study described in chapter 8. Few investigations have been published about clinical data of *Leishmania*-seropositive apparently healthy dogs, which are a clinically neglected group regarding treatment of *L. infantum*. Improving the knowledge about these dogs is highly important to control and prevent *L. infantum* infection and disease progression.
- Chapter 8 describes a blinded, randomized and controlled multicenter clinical trial to assess the efficacy and safety of Leisguard® as an immunotherapeutic treatment for healthy dogs infected with *L. infantum*. Few studies have been published about the use of immunotherapy, specifically Leisguard®, in healthy dogs infected with *L. infantum* which are one of the most clinically neglected dogs. The use of immunotherapy could open new horizons on *L. infantum* treatment in dogs.
- Finally, chapter 9 includes a discussion of all the data collected in the previous chapters and highlights the importance of these findings and future studies to be developed.

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Chapter 1

Introduction

Leishmania, vectors and reservoirs

Leishmanioses are important neglected tropical diseases caused by over 20 different species of the protozoan genus *Leishmania* [1–3]. Furthermore, over 90 sand fly species are known to transmit *Leishmania* parasites [1–3]. Nevertheless, only four *Leishmania* spp. are endemic in Europe. The first one is *Leishmania infantum*, which causes a zoonotic disease with the dog (*Canis lupus familiaris*) as the main domestic reservoir [3–5], and is encountered most frequently in the Mediterranean basin [4,5] (Table 1.1). The second and third are *Leishmania tropica* and *Leishmania donovani*, which mainly cause clinical manifestations in humans and have been reported in Greece [6–9], particularly in Crete [10,11] and Cyprus [9,11] (Table 1.1). Finally, the fourth species endemic in Europe is *Leishmania tarentolae*, which has been documented in humans, dogs, cats and reptiles from Spain [12,13] and Italy [14–17]. However, this *Leishmania* species appears to be non-pathogenic to mammals [14,16,17]. Other *Leishmania* species that cause severe disease manifestations are distributed in America and include *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania mexicana* with rodents frequently serving as main reservoirs. In addition, *Leishmania major* and *L. tropica* are mostly found in Africa and the Middle East [1,3]. Finally, *L. donovani* which mainly affects humans is present in India, Bangladesh and East Africa [1,3,18] (Table 1.1).

Leishmanioses are mainly transmitted through the bite of a female sand fly [19,20], belonging to the genera *Phlebotomus* and *Lutzomyia* depending on the geographical distribution [1] (Table 1.1). The sand fly is a noiseless 2-3 mm long arthropod that is usually active from dusk till dawn although there are some species which can be also active in the daylight [1]. There are also other potential routes of transmission such as venereal [21,22], transplacental [23–25] and through blood transfusion [26,27], that may play a marginal role when compared to the vector transmission [28].

The primary hosts of *Leishmania* are usually sylvatic mammals such as rodents, marsupials and canids, among others. Dogs are considered the main domestic and peridomestic reservoir for *L. infantum* infection in the Mediterranean basin [4,28,29] while other mammals such as wild canids [30], rodents [31] and lagomorphs [32] may be able to maintain a wild cycle.

Life cycle

The biological cycle of *Leishmania* is alternated between two different phases (Fig 1.1): (a) a flagellated promastigote phase which is an extracellular and motile form that colonizes the digestive tract of the sand fly vector, and (b) a non-flagellated amastigote phase which is an intracellular and non-motile form that colonizes the monocyte-macrophage lineage of the vertebrate host [1,34]. Amastigotes develop and multiply within the phagocyte until they are released by cell lysis in order to infect more macrophages [1]. Furthermore, amastigotes transform into promastigotes after being

ingested by the female sand fly through a blood meal [34]. The change in conditions such as decrease in temperature and increase in pH triggers morphological transformation and development of the parasite in the vector [34].

Table 1.1 Sand fly vector species, main affected regions and reservoirs. Adapted from Ready et al. 2013 [33].

Leishmania species	Sand fly vector species	Main affected regions	Reservoir
L. aethiopica	*Phlebotomus longipes* *P. pedifer*	Ethiopia, Kenya	Hyraxes
L. amazonensis	*Lutzomyia flaviscutellata*	East Andes	Rodents
L. braziliensis	*L. ovallesi* *L. wellcomei* *L. neivai* *L. whitmani*	East and West Andes	Rodents, marsupials, dog
L. donovani	*P. argentipes* *P. martini* *P. orientalis* *P. alexandri*	Northeast India, Nepal, Bangladesh, Bhutan Sudan, Ethiopia Greece	Human
L. guyanensis	*L. umbratilis*	East Andes	Arboreal edentate mammals
L. infantum	*P. ariasi* *P. perniciosus* *P. longicupis* *P. langeroni* *P. perfiliewi* *P. galilaeus* *P. syriacus* *P. tobbi* *P. halepensis* *L. longipalpis* *L. migonei*	Mediterranean region Latin America	Dog
L. major	*P. duboscqi* *P. salehi* *P. papatasi* *P. caucasicus*	Sub-Saharan Africa, Yemen North Africa, Middle East, Iran, Pakistan, India	Rodents Gerbils, rodents
L. mexicana	*L. olmeca olmeca*	West Andes	Rodents, marsupials
L. panamensis	None proven	West Andes	Arboreal edentate mammals
L. peruviana	None proven	Peru	Rodents, marsupials, dog
L. tarentolae	*Sergentomyia minuta*	Algeria, Spain, Italy	Reptiles
L. tropica	*P. sergenti* *P. similis* *P. arabicus* *P. guggisbergi*	North Africa, Middle East, Iran, Afghanistan Greece North and sub-Saharan Africa, North Africa	Human Hyraxes

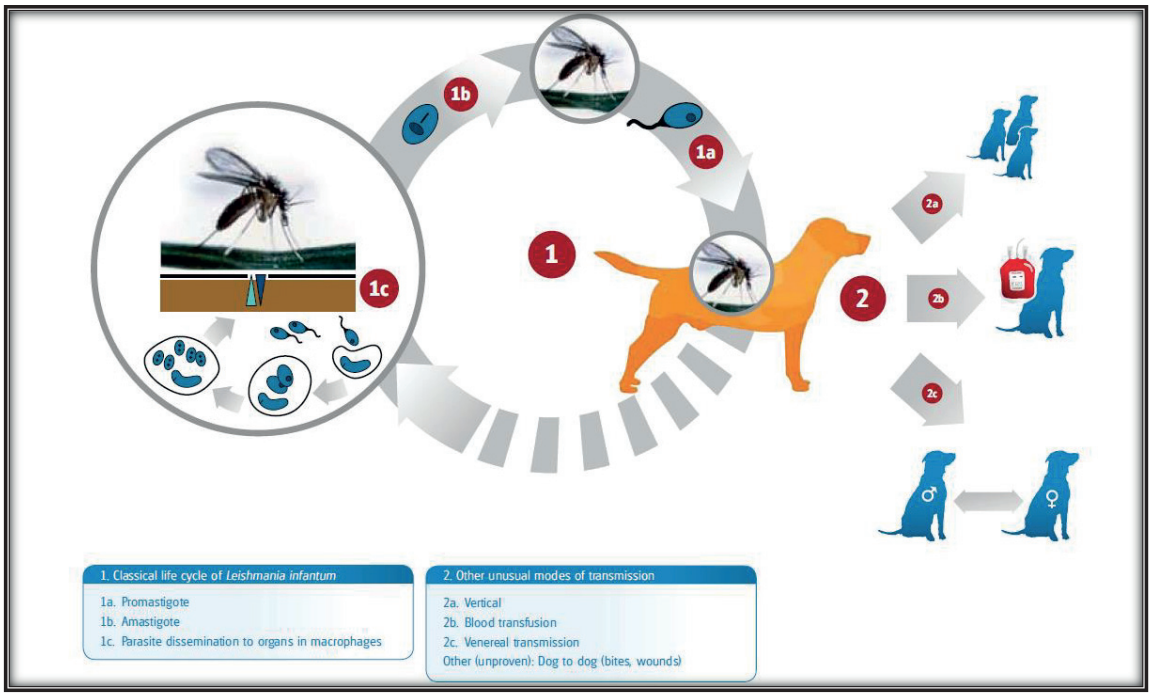

Fig 1.1 The life cycle of *L. infantum* with indication of proven and unproven non-sand fly routes of transmission to dogs. From Solano-Gallego et al. 2011 [28].

Epidemiology

Human leishmaniosis (HL) affects some of the poorest people and is associated with malnutrition, population displacement, poor housing, a weak immune system and lack of financial resources, and is also linked to environmental changes such as deforestation, building of dams, irrigation schemes and urbanization [2]. An estimated 700,000 to 1 million new cases occur annually worldwide, most of them located in Brazil, India, East Africa, the Middle East and Central Asia [2]. However, asymptomatic infections are also common in humans and only a small fraction of infected people will eventually develop the disease [2,35]. Regarding HL in Europe, 199 cases imported mainly from Africa and America were reported in 2020 [2]. In Spain, the incidence rate was of 0.62 cases per 100,000 inhabitants between 2005 and 2017, with cases mainly distributed in the Mediterranean region [36].

The overall seroprevalence of *L. infantum*-infected healthy dogs in western Europe was 23% between 1971 and 2006, although the median was 10% [37]. The country with the highest seroprevalence rate was Italy with a median of nearly 20% while other countries (Spain, Portugal and France) presented median seroprevalence rates between 6 and 8% [37]. In Spain, the seroprevalence rate in dogs between 2011 and 2020 has been detected to be around 10%, although it can range from 0 to 57% depending on the region [38–40]. For example, the North of Spain (such as Asturias and Basque country) has always presented one of the lowest seroprevalence rates [38–40],

usually lower than 5%, while the rates in the Southeast (such as the region of Murcia and the Valencian community) are usually higher than 15% [38]. Moreover, the prevalence of dogs that develop the clinical disease is usually lower than 10% in infected dogs [41,42]. Interestingly, the risk of being seropositive to *L. infantum* has been associated with several factors such as age, breed and the dog's environment, among others. It has been reported that the risk of seropositivity increases with the dog age [43–46] which seems to be related to the repeated exposure to *Leishmania*, although a bimodal age distribution with one peak in young dogs (under 2 years old) and a second peak in older dogs (over 8 years old) has also been commonly reported [47]. Male dogs have also been occasionally reported to have a higher risk of exposure to *Leishmania* infection than female dogs [43,46], although other studies did not detect differences between sexes [45,48]. Furthermore, different dog breeds have also been associated to either an increase or resistance to CanL. Generally, purebred dogs seem to be more likely to present clinical illness [43,45]; however, certain dog breeds such as Ibizan hounds rarely develop CanL due to their predominant immune cellular response [49–51]. Even so, environmental factors such as living outdoors or indoors are also important. Owned dogs have usually a lower risk of infection than dogs living in kennels or hunting dogs [43], which could be associated to being more frequently tested in the clinical setting and also the more likely use of preventive measures against *L. infantum* infection, although recent studies have detected the opposite [46]. However, living outdoors has largely being accepted as a huge risk factor for *L. infantum* infection [43,45,47,48].

Infection *versus* disease

As stated previously, *Leishmania* infection in both humans and dogs is a complex infection and only a small fraction of infected hosts will develop the disease [2,35,41,42]. The wide range of clinical manifestations found in *Leishmania* infection can vary from a total absence of clinical signs and laboratory findings to a severe fatal clinical disease depending on the infecting species and the host's immune response [1,28,52,53]. Furthermore, immunosuppressive conditions such as human immunodeficiency virus (HIV) and concomitant diseases may also influence and affect the outcome and expression of *Leishmania* infection and disease [1,54,55].

In humans, HL is classically classified into three different forms:

- Visceral leishmaniosis (VL) which is commonly characterized by fever, anorexia, weight loss and weakness [1].
- Cutaneous leishmaniosis (CL) which can present different types of skin lesions depending on the infecting *Leishmania* species and geographical location [1].

- Mucocutaneous leishmaniosis (MCL) which occurs when cutaneous lesions expand into the nasal mucosa, oropharynx, oral palate, lips, tongue, larynx, the trachea and upper respiratory tree [1,56].

In dogs, the most common clinical signs of CanL due to *L. infantum* are skin lesions, weight loss and generalized lymphadenomegaly, among a large variety of other clinical conditions [28,57] (Table 1.2). Furthermore, LeishVet, a non-profit scientific association focused on research and clinical activity on leishmaniosis in veterinary medicine, has previously published several guidelines with recommendations for the management of CanL which includes diagnosis, treatment and prognosis [28] (Table 1.3). LeishVet also proposed a system of four stages of CanL based on clinical signs, clinicopathological abnormalities and serological status, and different treatment protocols and prognoses are suggested for each clinical stage from stage I (mild disease) to stage IV (very severe disease) [28] (Table 1.3). The most common treatment for CanL includes the use of antimonials or miltefosine that reduces the parasitic load, and allopurinol that maintains the parasitic load at low levels [28,58]. However, conventional anti-*Leishmania* drugs used in dogs can induce side effects such as nephrotoxicity, urolithiasis and digestive disorders [59–61], and drug resistance has been already described in dogs [62–65].

As stated previously, the manifestations of leishmaniosis are closely influenced by the host's immune response which is very complex, still fairly unknown and largely determined by genetics as well as acquired factors [1,54,66]. The immune response requires a balance between inflammatory and regulatory responses to control *L. infantum* infection [1,54,66]. In brief, a host that displays a protective lymphocyte T helper (Th)1-cell mediated immune response with interferon gamma (IFN-γ) release that stimulates activated macrophages to produce nitric oxide (NO) and reactive oxygen species (ROS) and intracellular killing of amastigotes should be able to control *Leishmania* infection while another host that displays mainly a non-protective marked humoral immune response combined with absent or diminished cell-mediated immunity (CMI) will be susceptible to *Leishmania* infection, present a high parasite burden and finally clinical disease [1,54]. For example, neutrophils and macrophages have very important and distinctive roles in the dog's initial immune ability to control the infection or develop progression towards disease [66]. Both neutrophils and macrophages phagocytise the parasite and can lead to its elimination by ROS or its survival leading to parasite persistence and dissemination [66]. Moreover, T lymphocytes also play an integral role in preventing parasite growth and disease development as these T cells produce IFN-γ among other cytokines such as tumor necrosis factor alfa (TNF-α), interleukin (IL)-3 or chemokine (C-X-C motif) ligand 2 (CXCL2) which results in the differentiation, recruitment and activation of macrophages [66]. However, as the infection progresses towards disease, there is a decrease of T cell proliferation, IFN-γ production and a lack of macrophage activation resulting in a reduction of parasite elimination [66]. The expression of programmed cell death protein-1 (PD-1) and its

ligands on T cells and macrophages has also been investigated in *Leishmania* infection with interesting results [67–69]. PD-1 can induce T cell anergy, T cell apoptosis and exhaustion, diversion of T cells toward Th2 and regulatory T cells (Treg), but also can inhibit macrophage activities by suppression of NO and ROS production, and could be associated to disease severity [67,69,70].

Table 1.2 Clinical manifestations and laboratory abnormalities found in CanL due to *L. infantum*. Adapted from Solano-Gallego et al. 2011 [28].

Clinical manifestations	Laboratory abnormalities
General • Generalized lymphadenomegaly • Loss of body weight • Decreased or increased appetite • Lethargy • Mucous membranes pallor • Splenomegaly • Polyuria and polydipsia • Fever • Vomiting • Diarrhea	**CBC/Hemostasis** • Mild to moderate non-regenerative anemia • Leukocytosis or leukopenia (lymphopenia, neutrophilia, neutropenia) • Thrombocytopathy • Thrombocytopenia • Impaired secondary hemostasis and fibrinolysis
Cutaneous • Non-pruritic exfoliative dermatitis with or without alopecia • Erosive-ulcerative dermatitis • Nodular dermatitis • Papular dermatitis • Pustular dermatitis • Onychogryphosis	**Biochemical profile/Urinalysis** • Hyperproteinemia • Hyperglobulinemia (polyclonal beta and/or gammaglobulinemia) • Hypoalbuminemia • Decreased A/G ratio • Renal azotemia • Elevated liver enzyme activities • Proteinuria
Ocular • Blepharitis (exfoliative, ulcerative, or nodular) and conjunctivitis (nodular) • Keratoconjunctivitis, either common or sicca • Anterior uveitis • Endophtalmitis	
Other • Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital and nasal) • Epistaxis • Lameness (erosive or non-erosive polyarthritis, osteomyelitis, polymyositis) • Atrophic masticatory myositis • Vascular disorders (systemic vasculitis, arterial thromboembolism) • Neurological disorders	

Abbreviations: A/G: albumin/globulin.

Table 1.3 Clinical staging of CanL based on serological status, clinical signs, laboratory findings, and type of therapy and prognosis for each stage. Adapted from Solano-Gallego et al. 2017 [53].

Clinical stages	Clinical signs	Laboratory findings	Quantitative serology	Therapy	Prognosis
Stage I Mild disease	Mild clinical signs (e.g.peripheral lymphadenomegaly or papular dermatitis)	No clinicopathological abnormalities observed Normal renal profile: creatinine<1.4 mg/dl; non-proteinuric: UPC<0.5	Negative to low positive antibody levels	Scientific neglect Limited information, treatment options remain to be defined Monitoring of disease progression	Good
Stage II Moderate disease	Clinical signs of stage I and diffuse or symmetrical cutaneous lesions such as exfoliative dermaitits, onychogryphosis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), generalized lymphadenomegaly, anorexia, weight loss, fever and epistaxis	Clinicopathological abnormalities such as mild non-regenerative anemia, hyperglobulinemia and hypoalbuminemia Substages a)Normal renal profile: creatinine<1.4 mg/dl; non-proteinuric: UPC<0.5 b)Creatinine<1.4 mg/dl; UPC=0.5-1	Low to high antibody levels	Allopurinol + meglumine antimoniate or miltefosine Substage b: follow IRIS guidelines for CKD	Good to guarded
Stage III Severe disease	Clinical signs of stages I and II, and signs originating from immune-complex lesions such as vasculitis, arthritis, uveitis and glomerulonephritis	Clinicopathological abnormalities of stage II and CKD IRIS stage I with UPC>1 or stage II (creatinine 1.4-2.8 mg/dl)	Medium to high antibody levels	Allopurinol + meglumine antimoniate or miltefosine Follow IRIS guidelines for CKD	Guarded to poor
Stage IV Very severe disease	Clinical signs of stages I, II and III, and pulmonary thromboembolism or nephrotic syndrome and end stage renal disease	Clinicopathological abnormalities of stages II and III, and CKD IRIS stage III (creatinine 2.9-5 mg/dl) and stage IV (creatinine>5 mg/dl) or nephrotic syndrome or marked proteinuria UPC>5	Medium to high antibody levels	Specific treatment should be instituted individually Follow IRIS guidelines for CKD	Poor

Abbreviations: CKD: chronic kidney disease; IRIS: International Renal Interest Society; UPC: urinary protein/creatinine.

Diagnosis

The diagnosis of CanL often requires an integrated approach often including both a clinicopathological examination and specific laboratory tests [28,52,53]. To be suspicious of CanL, a clinicopathological examination must include a physical examination and several routine laboratory tests such a complete blood count (CBC), biochemical profile, serum electrophoresis and urinalysis [28,52]. In addition, several diagnostic techniques are available to make a definitive diagnosis of *L. infantum* infection such as:

- Parasitological diagnosis: consists of microscopic examination of different samples of the infected host (lymph nodes, bone marrow, cutaneous lesions, etc.) and can detect the presence of the parasite by direct observation of *Leishmania*. The direct observation of the parasite can also be performed after parasite culture and xenodiagnosis, but these techniques are not used in the clinical setting [52,71]. The parasite can be directly observed by cytology, histology and immunohistochemistry [52]. Cytology can be used to observe the presence of *Leishmania* amastigotes within macrophages or in the background after cell lysis [52]. Histology can be used to demonstrate the presence of *Leishmania* in routinely hematoxylin- and eosin-stained sections, although the identification of amastigotes is more difficult than in cytologic samples; however, amastigotes can be also confirmed by immunohistochemistry [52].

- Serological techniques: such as immunochromatographic tests, enzyme-linked immunosorbent assays (ELISA) and immunofluorescent antibody tests (IFAT) are the most common methods used to detect infected dogs. There are techniques such as Western blotting and the latex agglutination test that are not used in the routine practice. These techniques can detect anti-*Leishmania* antibodies produced by the infected host [52,53,71]. Furthermore, since a vaccine is available in Europe, serological screening is mandatory prior to vaccination in dogs [53]. In addition, annual screening of dogs is frequently performed in endemic areas to diagnose both dogs progressing towards disease and subclinical infections [53].

- Molecular techniques: include conventional polymerase chain reaction (PCR), nested-PCR and quantitative real-time PCR (rt-PCR) [52]. Some other methods such as the loop-mediated isothermal amplification (LAMP) or the use of probes labeled with gold nanoparticles are not used in the routine practice [72,73]. These techniques can detect the presence of the parasite by detection of *Leishmania* DNA [52,71].

These diagnostic techniques must be used understanding the basis of each test and its limitations and making a correct interpretation [28,52,53].

Interestingly, these techniques used for diagnosis of *L. infantum* can be performed using different types of samples such as blood, sera, urine and other infected tissues [74–76]. But the use of alternative samples such as hair [77], saliva [78–82] and conjunctival swabs [78] has also been studied with interesting positive results.

Preventive measures against *L. infantum* infection

The use of preventive measures against *L. infantum* infection has expanded over the last decades [83]. However, there are still two main ways to prevent the infection:

- Measures against the vector: such as physical barriers and insecticides. For example, it is recommended to avoid outdoor activities from dusk till dawn (when the vector is highly present), to use fine mesh nets in windows and to use topical insecticides such as synthetic pyrethroid-based compounds, which have both repellent and anti-feeding effects [5,83,84] (Table 1.3). Topical insecticides are commercially available in different forms such as impregnated collars, spot-on and sprays, each of which have different onset and maximum duration [83,85] (Table 1.3).
- Measures against the parasite: such as vaccines and immunomodulators. Currently, few products are marketed in Europe as immunoprophylaxis against *L. infantum*. The only marketed immunomodulator for the prevention of CanL is Leisguard® which has been marketed since 2012 [86]. On the other hand, the only marketed vaccine against *L. infantum* in Europe is Letifend® which was introduced commercially in 2016 [53,83]. Previously, another vaccine with the name of Canileish® was also available but it is not marketed anymore (withdrawn from the market in 2021) [53,83].

Many studies have investigated the efficacy and safety of the recommended preventive measures [86–88]. For example, the efficacy of topical insecticides can range from 80 to 100% depending on the active ingredients, the formulation and the duration of the product [83,88] (Table 1.4) while the efficacy of the vaccine Letifend® is around 70% [87]. Regarding Leisguard®, an efficacy of 80% has been detected [86].

Table 1.4 Insecticide molecules with efficacy to prevent sand fly bite. Adapted from Miró et al. 2017 [83].

Active ingredient	Formulation	Efficacy (%)	Onset	Duration	Brand name (Company)	References
Permethrin Indoxacarb	Spot-on	84-99	24-48 h	3-4 weeks	Activyl® Plus (MSD)	89
Permethrin Imidacloprid	Spot-on	74-98	24-48 h	3-4 weeks	Advantix® (ELANCO)	90,91
Permethrin	Spray	100	Instant	2-3 days	Duowin® (VIRBAC)	92
Permethrin Fipronil	Spot-on	87-98	24-48 h	4 weeks	Effitix® (VIRBAC)	93
Permethrin	Spot-on	90-100	24-48 h	2 weeks	Ex-spot® (MSD)	94
Permethrin Fipronil	Spot-on	90-99	24-48 h	4 weeks	Frontline Tri-Act® (BOEHRINGER INGELHEIM)	95,96
Deltamethrin	Collar	94-96	7 days	1 year	Scalibor® (MSD)	97–99
Flumethrin Imidacloprid	Collar	91-100	-	8 months	Seresto® (ELANCO)	100
Permethrin Dinotefuran Piriproxyfen	Spot-on	84-100	24-48 h	4 weeks	Vectra 3D® (CEVA)	101,102

Immunotherapy in CanL (adapted from Baxarias et al. 2019 [103])

As previously mentioned, the outcome of infection by *Leishmania* depends largely on the host's immune response [54,66,104,105]. Thereby, treatment that can enhance the host's immune system could provide an alternative direction to combating the infection [106,107].

The use of immunotherapy does not directly attack the pathogen, as other drugs would, but it modulates the host's immune response increasing its protection from the disease [106]. Following this idea, researchers have been looking for different compounds that could improve the immune response against *Leishmania* infections (Fig 1.2).

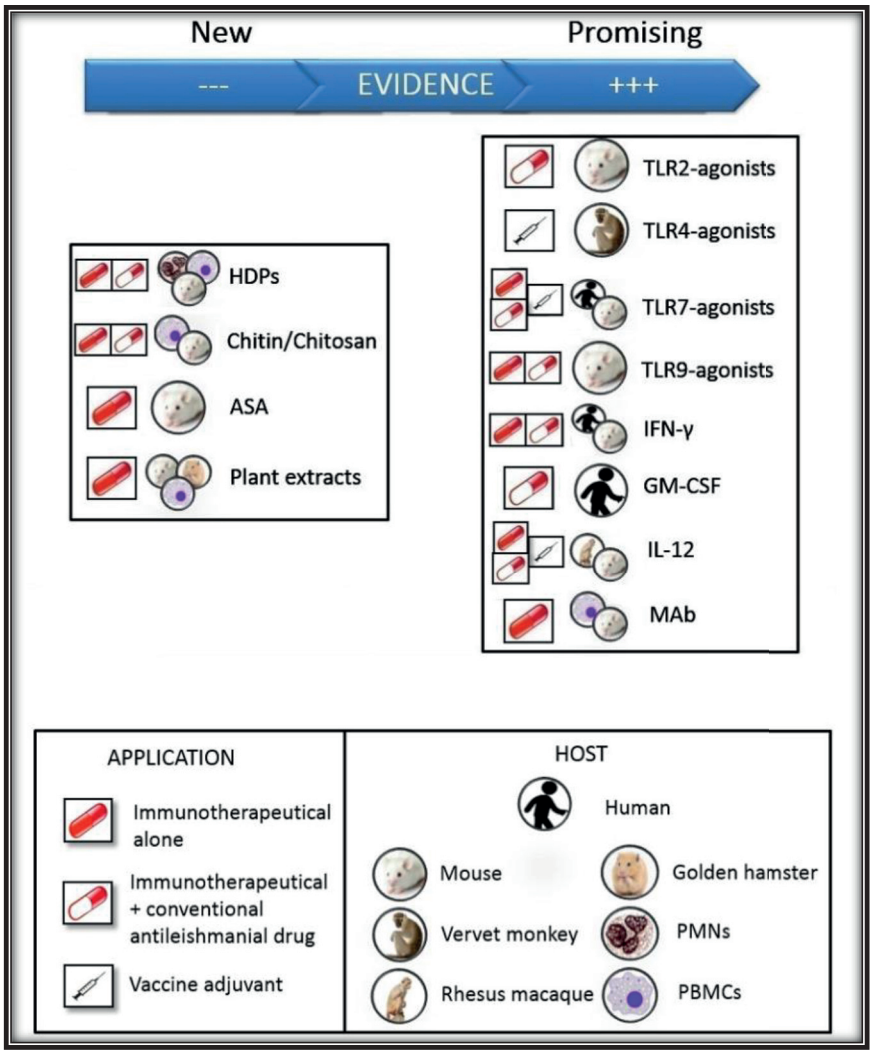

Fig 1.2 Summary of compounds studied for their effect on the immune response against leishmaniosis in mice, primates and humans. Abbreviations: ASA: acetyl salicylic acid; GM-CSF: granulocyte/macrophage colony stimulating factor; HDP: host defense peptide; IFN-γ: interferon gamma; IL: interleukin; MAb: monoclonal antibodies; PBMC: peripheral blood mononuclear cell; PMN: polymorphonuclear cell; TLR: toll like receptor.

The only immunotherapeutic commercialized specifically for usage in CanL is domperidone (Leisguard®). Domperidone is a dopamine D2 receptor antagonist that can potentiate the immune response through modulating the effect of prolactin [86,108–110]. Other compounds such as dietary nucleotides and active hexose dietary compound (AHCC) (Impromune®) are commercialized and have been investigated also as immunotherapeutics for usage in CanL, although they are still commercialized only as food supplements with capacity to strengthen the immune system and to boost the innate and adaptive immune responses. Dietary nucleotides appear to promote the phagocytic activity of macrophages and T lymphocytes in human infants [111,112] and in experimentally infected rodents [113–115]. The active hexose dietary compound has been reported to promote the activity of natural killer (NK) cells, proliferation of macrophages and differentiation of T lymphocytes to the Th1 cell subset in human and rodent peripheral blood mononuclear cells (PBMCs) [116,117]. Furthermore, several

studies have evaluated alternative immunotherapeutical compounds not commercialized yet including several cytokines [118,119], toll like receptor (TLR) agonists [120,121] or chitosan [122], which yielded promising results (Fig 1.3).

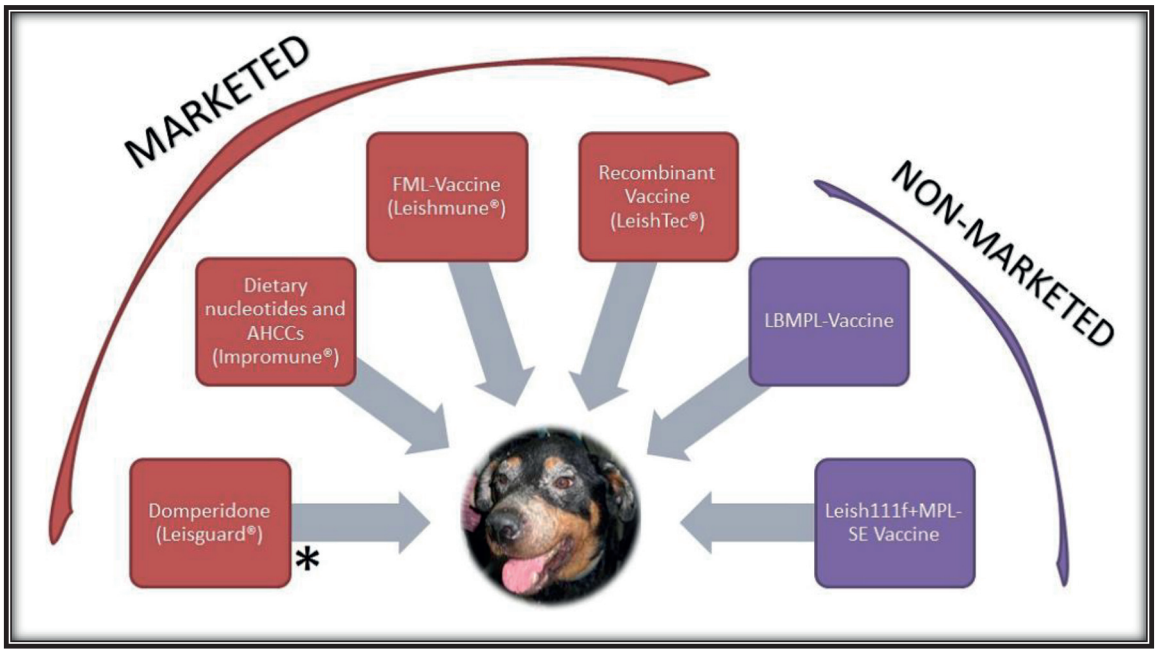

Fig 1.3 Summary of the commercially available and non-available compounds and vaccines studied for the immunotherapy of clinical canine leishmaniosis. * Domperidone is the only compound registered for treatment of sick dogs with mild CanL in some European countries (Spain, Portugal, Italy and Greece). Abbreviations: AHCC: active hexose dietary compound; FML: fucose mannose ligand; LBMPL: *L. braziliensis* promastigote protein+monophosphoryl A; Leish111f+MPL-SE: Trifusion recombinant protein of TSA, LmSTI1 and LeIF+monophosphoryl A stable emulsion.

Commercially-marketed immunotherapy compounds (adapted from Baxarias et al. 2019 [103])

Domperidone (Leisguard®)

Domperidone is a dopamine D_2 receptor antagonist developed and synthesized in 1974 by Janssen Pharmaceutica (Beerse, Belgium) and patented in the USA in 1978.

Domperidone (Leisguard®) was approved for use for both prevention and treatment of CanI due to *L. infantum* by the Heads of Medicine Agencies (HMA) in 2011 [123]. Specifically, it is indicated to reduce the risk of developing an active infection in seronegative healthy dogs as preventive measure and improving mild clinical disease, through the enhancement of the CMI response [123]. This is due its capacity to potentiate the activity of phagocytic cells such as monocytes, macrophages and neutrophils, and potentially contributing to the establishment of a predominantly Th1

immune response [123]. The origin of these effects is related to the release of serotonin in the hypophysis which causes a transitory increase in blood levels of prolactin [108,124]. Prolactin has been classified as a pro-inflammatory lymphocyte-derived cytokine [125]. Hence, increasing the production of prolactin induces a boost of T $CD4^+$ lymphocytes, in addition to the release of cytokines such as IL-2 and IL-12, IFN-γ and TNF-α, producing an activation of NK cells and macrophages, followed by a decrease of $CD4^+$ Th2 cytokines and TNF-β [126–128]. It is accepted that a predominantly Th1 immune response including IL-2, IL-12, IFN-γ and TNF-α is able to control leishmaniosis while susceptibility to the disease has been associated with IL-4 secretion and the Th2-type immune response [54,129,130].

In a study performed by Gómez-Ochoa et al. [131], healthy dogs that received a 30-day course of domperidone treatment showed a rapid increase of the percentage of activated neutrophils when compared with untreated dogs. In a previous study [108], a clinical trial was performed with 98 dogs with mild clinical signs of leishmaniosis. In this study [108], domperidone was suggested as effective in controlling and reducing clinical signs of leishmaniosis in dogs together with a reduction of the anti-leishmanial antibody titer. Furthermore, domperidone has also been reported to improve serum creatinine and reduce anti-*L. infantum* antibody titers, globulins, gamma globulins and C-reactive protein in dogs with leishmaniosis and chronic kidney disease (CKD) [132]. In another study [110], treatment with furazolidone and domperidone was administered to twelve dogs naturally infected with *L. braziliensis* with good results related to the decrease of skin lesions associated with this infection. On the other hand, the control group of this same study [110] was not treated, thus, it was not possible to determine if the results were produced by domperidone, furazolidone or a combination of both.

However, all studies mentioned above present some limitations such as lack of an appropriate control group [108,110,131,132], short follow-up periods [131] and small numbers of dogs studied [110,131,132]. These limitations highlight the need for more complete studies to support the above-mentioned results.

Domperidone is marketed commercially in the clinical practice as treatment for CanL in several European countries with a high frequency of use in Spain, Portugal and Italy [133]. In a study performed in north-eastern Spain [109], 7% of the clinics used domperidone alone or combined with allopurinol as a first-line treatment for CanL while 3.5% employed domperidone alone or combined with allopurinol as second-line treatment. Additionally, domperidone was the third most used compound as a preventive measure against leishmaniosis (50% of the investigated clinics) after topical insecticides (98%) and vaccination (67%) [109]. Importantly, some adverse effects associated with domperidone are sporadically observed in treated dogs and include mammary gland disorders, which disappear after treatment discontinuation, lethargy

and digestive disorders [109,134]. Behavioural disorders have also been documented rarely with domperidone treatment [134].

Domperidone is also used in human medicine as treatment of nausea and vomiting, but a restriction on the use of domperidone-containing medicines was issued in 2014 by the European Medicine Agency (EMA) [135] because of its multiple and dangerous side effects related to cardiopathies in humans. These side effects have not been studied in dogs yet, most likely because the recommended dose of domperidone falls within the safe range, but co-administration of this medication with other drugs with similar side effects or the use of products that could enhance the absorption of domperidone may induce effects similar to those observed in humans [136].

Dietary nucleotides and active hexose correlated compounds (AHCC) (Impromune®)

Dietary nucleotides are low molecular weight intracellular compounds which are naturally present in all types of food of plant and animal origin, although higher levels are found in meat, fresh seafood, seeds and dried legumes [137–140]. Dietary nucleotides have been widely studied and used to increase lipid metabolism, immune responses as well as the development and repair of tissue growth in human infants [111,112] and rodents [113–115]. Active hexose correlated compounds (AHCC) are alpha-glucan-rich dietary supplements extracted from mushrooms that have been reported to have antioxidant activity and induce improvement of the Th1 immune response associated with increment of NK cells, T cells, B cells and cytokines such as IL-12 and TNF-α in human PBMCs and rodents [116,117].

A study performed with BALB/c mice investigated the effect of dietary nucleotides on the immune function [141] demonstrating that dietary nucleotides could enhance the innate and adaptive immune responses of mice through stimulation of Th cells and cytokines [141]. Another study [142] also reported that nucleotides and AHCC could enhance an effective Th1 immune response in *vitro* by increasing TNF-α, IFN-γ, monocyte chemoattractant protein (MCP)-1, regulated upon activation, normal T-cell expressed and secreted (RANTES) and IL-1α levels and reducing IL-6 and IL-9 cytokine levels in *Leishmania*-stimulated murine immune cells.

Several studies have investigated how dietary nucleotides could improve the treatment of CanL [143–145]. Cortese et. al [143] analyzed T cell populations including $CD3^+$ $CD4^+$ $Foxp3^+$ Treg, and $CD3^+$ $CD4^+$ IFN-γ^+ Th1 cells in the blood of dogs after treatment with an immune-modulating diet. A group of dogs treated with a standard anti-leishmanial drug treatment supplemented with an immune-modulating diet and a group of dogs with the same drug treatment but supplemented with a standard diet

were studied [143]. The results showed that dogs which received the immune-modulating diet presented an increase in Treg population and a decrease in Th1 inflammatory response in addition to a mild improvement in the decrease of clinical signs [143]. The effects of dietary nucleotides and AHCC were investigated in another clinical trial in dogs with CanL [145]. A standard treatment of meglumine antimoniate (Glucantime®) and allopurinol was administered to the first group while the second group received a combination of meglumine antimoniate and AHCC and dietary nucleotides [145]. The study results showed that both treatments presented similar efficacy and, therefore, this new treatment modality could be a good alternative for dogs with CanL suffering from adverse effects of allopurinol treatment such as urolithiasis and renal mineralization [61,140,145,146]. Despite these results, there is need for more studies on the treatment of CanL with dietary nucleotide due to the use of different diets used in the same group [143,145], short follow up period [145], lack of a control group [145] in the studies and the use of a non-standardized clinical scoring system [145]. In another study, clinically healthy *Leishmania*-infected dogs, most of which were seropositive, were also treated with dietary nucleotides and AHCC to prove the effect of the diet in delaying the progression of the disease [144]. The outcome of this study showed that the oral administration of dietary nucleotides and AHCC is safe and can reduce the rate of disease progression from a clinically healthy infected status into clinical disease, although it was also stated that additional clinical trials with other drug combinations and larger sample sizes are needed to confirm these observations [144].

Therapeutic vaccines

Four vaccines have been marketed for CanL [53,106]. Two of them have been marketed commercially in Europe: CaniLeish® [147–150] and Letifend® [87,151]; while the other two have been available in Brazil: Leishmune® [152,153] and Leish-Tec® [154,155]. Only the Letifend® vaccine is currently available in Europe while the Leish-Tec is the only vaccine marketed in Brazil. Both CaniLeish® and Leishmune® are not available in the market anymore.

Some of the canine vaccines, and other vaccines which are still in early stages of investigation, have been studied for use as treatment of clinical CanL. The major outcomes were similar in all studies: clinical improvement in treated dogs, which was more relevant in mild or moderate disease than in severe disease, and, in some of them, a reduction of the parasitic load was also observed. These studies are summarized in Table 1.5.

Non-commercially available immunotherapy (adapted from Baxarias et al. 2019 [103])

Toll like receptor (TLR) agonists

Toll like receptors (TLRs) are type I transmembrane proteins which comprise one of the first defense lines against pathogens [156]. There are ten TLRs (TLR1–TLR10) described in dogs [157] as well as in humans, and 12 in mice (TLR1–9, TLR11–13) [158,159]. TLRs are located in the plasma or internal membranes of inflammatory cells including macrophages, dendritic cells (DC), NK cells and lymphocytes (T and B) as well as other types of cells such as keratinocytes.

Their function is to bind conserved molecular structures found in large groups of pathogen-associated molecular patterns (PAMPs) and induce the secretion of inflammatory cytokines such as type-1 interferon, chemokines and co-stimulatory molecules [160]. TLR agonists are natural and synthetic PAMPs [161] that bind to TLRs to activate signaling pathways to manage innate and acquired immune responses [162]. They amplify immune reactions against parasites by stimulating the production of pro-inflammatory cytokines playing an important role in controlling *Leishmania* infection [163]. TLR agonists are promising compounds for prevention and immunotherapy in human leishmaniasis and CanL [164]. However, limited information is available on their potential treatment benefits to both species while the majority of research on this topic has been carried out in rodents or non-human primates.

TLR2 agonists

The immunotherapeutic potential use of the protein aggregate of magnesium–ammoniumphospholinoleate–palmitoleate anhydride (P-MAPA) was evaluated in dogs with leishmaniosis by Santiago et al. [165]. P-MAPA is a compound obtained from the fungus *Aspergillus oryzae* and it has been demonstrated to activate TLR2 in human embryonic kidney cells [166]. The clinical improvements observed in sick dogs with leishmaniosis treated with the immunomodulatory P-MAPA were accompanied with diminution of the skin parasite load, increased levels of IFN-γ and low IL-10 production after P-MAPA treatment [165]. PBMCs and macrophages from *Leishmania* infected dogs were also studied to investigate the immunomodulatory effect of P-MAPA [167]. Macrophages from infected dogs treated with high concentrations of P-MAPA increased TLR2 expression when compared to controls. In addition, the concentration of ROS was increased in PBMCs from infected dogs suggesting the immunomodulator role of P-MAPA associated with restoring the immune balance [167]. The prophylactic action of N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4 hydrochloride (Pam3Cys), a TLR2 agonist, in preventing pathogen infection and reducing their establishment was demonstrated using a murine model of *L. donovani* infection [121]. This study is reviewed in Table 1.6.

Table 1.5 Vaccines with proven immunotherapeutic activity against *L. infantum* in dogs. Adapted from Baxarias et al. 2019 [103].

Type of vaccine (Brand name)	Vaccine composition	Type of study	Number of dogs treated	Parasite species*	Outcome	Reference
FML-vaccine (Leishmune®)	FML + Riedel de Haen saponin	Multi-center, controlled, double-blind and randomized	31	*L. infantum*	Reduction of proportion of symptomatic dogs (from 100% to 38%) and deaths (from 54% to 12%)	168
FML-vaccine (Leishmune®)	FML + Riedel de Haen saponin	Single-center, controlled and randomized	12	*L. infantum* (experimental infection)	Improvement of the clinical profile and reduction of parasite load	169
FML-vaccine (Leishmune®)	FML + QuilA saponin	Single-center and open-label	5	*L. donovani* (experimental infection)	Reduction of clinical signs after complete vaccination (3/5). Died without symptoms (1/5). Died of disease (1/5)	170
FML-vaccine (Leishmune®)	FML + saponin R	Multi-center, controlled and open-label	21	*L. infantum*	Treated remained asymptomatic (19/21) or with mild clinical signs (2/21)	170
LaSap-vaccine	Total antigens of *L. amazonesis* + saponin	Single-center and open-label	8	*L. infantum*	Improvement of the clinical profile and reduction of parasitic load	171
LBMPL-vaccine	*L. braziliensis* promastigote protein+ MPL	Single-center, controlled and open-label	10	*L. infantum*	Normalization in RBC parameters, urea, creatinine, AST, ALP and bilirubin. Reduction of parasitic load and clinical signs (75%)	172
LBMPL-vaccine	*L. braziliensis* promastigote protein+ MPL	Single-center, controlled and open-label	10	*L. infantum*	Lower liver inflammation, parasite load reduction, lower expression of IL-10 and TGF-β1 and higher expression of iNOS mRNA	173
Leish-111f+MPL-SE vaccine	Leish-111f (TSA + LmSTI1 + LeIF) + MPL-SE	Single-center, controlled and open-label	18	*L. infantum*	Improvement of the clinical profile (75% considered cured)	174
Leish-111f+MPL-SE vaccine	Leish-111f (TSA + LmSTI1 + LeIF) + MPL-SE	Single-center, controlled, single-blind and randomized	15	*L. infantum*	Improvement of the clinical profile (better in moderate disease than in severe)	174
(Leish-Tec®)	A2-based recombinant protein + saponin	Multi-center, controlled, double-blind and randomized	250	*L. infantum*	Reduction of rate of deaths without treatment	175
rLdccys1-vaccine	Recombinant cysteine proteinase from *L. infantum* + *Propionibacterium acnes*	Single-center, controlled and open-label	10	*L. infantum*	Control of disease development and reduction of parasitic load	176

*All infections except for those marked were natural infections.

Abbreviations: A2: recombinant *L. infantum* and *L. donovani* amastigote-specific antigen; ALP: alkaline phosphatase; AST: aspartate transaminase; FML: fucose mannose ligand; LeIF: *Leishmania* elongation initiation factor; Leish-111f: trifusion recombinant protein of TSA, LmSTI1 and LeIF; LmSTI1: *L. major* stress-inducible protein 1; MPL: monophosphoryl lipid A; QuilA: saponin adjuvant produced with *Quilaja saponaria*; RBC: red blood cell; rLdccys1: recombinant cysteine proteinase from *L. infantum*; SE: stable emulsion; TSA: thiol-specific antioxidant.

TLR4 agonists

The high potency with which TLR4 activates inflammatory pathways makes it an ideal target for therapeutic intervention and adjuvant development [177]. Several studies have explored the use of a TLR4 agonist as adjuvant in vaccines against leishmaniosis [178–180]. Vaccinated vervet monkeys were challenged with virulent *L. donovani* parasites following intradermal inoculation of *L. donovani* sonicated antigen delivered with either alum, montanide ISA 720 (MISA) or the TLR4 agonist monophosphoryl lipid A (MPL) [120]. MPL failed to induce increased IFN-γ production compared to the other two adjuvants [120]. In a similar study described by Mutiso et al. [120], a group of vervet monkeys treated with MPL and *L. donovani* antigen showed significantly lower skin delayed-type hypersensitivity to the sonicate antigen when compared with treatment group exposed to alum as adjuvant with *L. donovani* antigen [120]. The potential use of TLR4 agonists in combination with leishmanial antigen was evaluated for immunotherapy of sick dogs with leishmaniosis [172]. Those sick dogs treated with both *L. braziliensis* antigen and a TLR4 agonist (MPL) showed clinical improvement. Moreover, a reduction in the transmission of the *Leishmania* to sand flies evaluated by xenodiagnosis was observed. This study is also reviewed in Table 1.5.

TLR7 agonists

Imiquimod (IMQ) is a TLR7 agonist that is currently approved as topical treatment of CL in humans [181] and has also been utilized as a vaccine adjuvant in several studies of *Leishmania* infection [182,183]. A successful case of topical use of imiquimod in CL due to *L. infantum* has been described in a 7 year old child from Balearic Islands [184]. This patient's lesions were not improved by previous treatments received by the patient, including liposomal amphotericin [184]. The prophylactic and therapeutic uses of topical resiquimod, a TLR7/8 agonist, were studied in a *L. infantum* VL murine model. Topical resiquimod was applied in conjunction with subcutaneous or intravenous inoculation of *L. infantum* originally isolated from a patient from north-eastern Brazil to vaccinate and challenge experimental animals. High levels of protection (> 90%) were achieved in vaccinated animals accompanied by resiquimod. Furthermore, BALB/c mice which were treated for 4 weeks with resiquimod after infection with virulent *L. infantum*, had a reduction in liver parasite burdens, demonstrating that resiquimod had beneficial immunomodulatory effects in experimental systemic, organ infecting VL in mice [185]. However, a pilot study using IMQ cream (Aldara®) as the only treatment for stage I-CanL papular dermatitis failed to cure lesions in dogs [186].

Another recent study [187] evaluated the anti-leishmanial activity of imidazoquinoline-based TLR7/8 agonists in *L. amazonensis*-intracellular amastigotes using mouse

peritoneal macrophages and reported that the imidazoquinolines were able to inhibit the growth of *L. amazonensis* intracellular amastigotes and induce macrophages to produce ROS, NO and pro-inflammatory cytokines such as IL-12 and TNF-α [187].

Cytokines

Cytokines have crucial roles in the control of infection as well as in the progress of disease manifestation [188,189]. For this reason, many studies have focused on the use of cytokines as treatment for leishmaniosis. The mechanisms of action of the major cytokines involved in the disease outcome of leishmaniosis are those which influence the balance between the Th1 and Th2 cytokines. Various pro-inflammatory Th1 cytokines such as IL-12, IFN-γ, TNF-α and IL-2 have been identified as associated with the control of the disease [130,188,189]. Contrarily, non-protective Th2 cytokines such as IL-4 and IL-10 have been related to susceptibility to the development of *Leishmania* infections [130,188,189].

The fact that IFN-γ activates macrophages to kill intracellular amastigotes and that it is mainly produced by antigen stimulated T lymphocytes has been repeatedly demonstrated in several animal species including mice [190], dogs [54] and also in humans [191]. However, recombinant human IFN-γ in conjunction with pentavalent antimonial (Glucantime®) therapy has been shown to induce an increase in treatment success and clinical cure; possibly due to the effect of IFN-γ induced macrophage activation [118]. Several studies in human patients from Brazil, Kenya, and India, have demonstrated that the use of IFN-γ therapy in VL accelerates the anti-parasitic effect of pentavalent antimonials compared with use of this cytokine alone [118,192–194].

Chemokine (C-X-C motif) ligand 10 (CXCL10), a chemokine that recruits and activates Th1 cells, NK cells, macrophages, dendritic cells and B lymphocytes, has also been investigated as a potential alternative to treat *Leishmania* infection in mice [195]. Following experimental infection with antimony-resistant isolate of *L. braziliensis*, mice were treated with CXCL10 which controlled lesion progression and parasites burden more efficiently than non-treated mice [195]. Furthermore, mice treated with CXCL10 presented an increased IFN-γ, IL-10, transforming growth factor (TGF)-β and low IL-4 production [195].

Monoclonal antibodies (MAb)

The therapeutic monoclonal antibody (MAb) market has increased exponentially since the first MAb was commercialized in 1986. MAb products are currently approved for

treatment of a large variety of diseases [196]. The advantages of therapeutic MAbs as a treatment usually include low toxicity, high specificity and versatility of activity [197].

IL-10 has been identified in the murine model as a potent suppressor of CMI during *Leishmania* infection (Kane and Mosser, 2001). A study of IL-10 antibody neutralization in cell cultures of splenic aspirates from human VL patients showed a decrease in the number of amastigotes concomitantly with an increased production of IFN-γ and TNF-α [198]. Moreover, anti-IL-10R MAbs used for the reduction of IL-10 levels to treat experimental *Leishmania* infection have been widely studied [198–200]. Those studies performed in murine models experimentally infected with *L. donovani* are summarized in Table 1.6.

Furthermore, a recent study [205] investigated the potential of anti-canine IL-10R-blocking MAb to control and reduce *in vitro* infectivity of *Leishmania* in PBMCs isolated from dogs naturally infected by *L. infantum*. Overall, *Leishmania* showed lower capacity of *in vitro* infectivity in the presence of anti-canine IL-10R-blocking MAb, and an enhancement of T CD4 and CD8 proliferative response with IFN-γ production was also observed [205]. The treatment with anti-canine IL-10R-blocking MAb was further investigated in dogs naturally infected by *L. infantum* in another study [206] and was also compared to chemotherapy using meglumine antimoniate. The study demonstrated that both treatments (anti-canine IL-10R-blocking MAb and meglumine antimoniate) were able to maintain hematological and biochemical parameters and also increase circulating T lymphocytes, IFN-γ production and improve the clinical status [206]. However, these improvements did not remain until the end of the follow-up (180 days) [206].

Other MAbs targeting PD-1 receptor and its ligand (PD-L1) have been also investigated with interesting results [207]. Briefly, the therapeutic potential of anti-PD-1 and anti-PD-L1 MAbs against a non-healing *L. amazonensis* infection in BALB/c mice was tested and the treatments increased significantly IFN-γ producing CD4 and CD8 T cells and decreased parasite load, although treated mice displayed larger lesions than non-treated mice [207]. Interestingly, treatment with anti-PD-1 and anti-PD-L1 did not affect anti-*Leishmania* antibody or IL-10 production [207]. Moreover, mice treated with anti-PD-1 MAb reduced both IL-4 and TGF-β production [207].

However, the use of MAbs is not always beneficial. For example, the treatment of rheumatoid arthritis with MAbs in human clinical practice has been associated with an increased risk for VL [208–210]. This is because the MAbs that were used for rheumatoid arthritis are TNF-α inhibitors and this cytokine is essential for granuloma formation and maintenance, which is an important defense mechanism against intracellular pathogens such as *Leishmania* spp. [208,210].

Table 1.6 Non-commercially available compounds which induce proven immune response activity against *L. donovani*. Adapted from Baxarias et al. 2019 [103].

Compound	Type of study	Experimental model	Drug route of administration	Immune response effect	Outcome	Reference
HDP[1] LL-37, E6, L-1018 and RI-1018	Single-center, controlled, open-label and *in vitro*	THP-1 human macrophages	Culture	Reduction of redox activity	Reduction of amastigote infection	201
Mab Anti-IL-10R	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of nitric oxide synthase activity and production of IL-12 and IFN-γ	Reduction parasite burden	200
Mab Anti-IL-10R and anti-GITR	Single-center, controlled, open-label and *in vivo*	C57BL/6J mice	Intraperitoneal injection	Increase of production of IFN-γ and TNF-α	Reduction parasite burden	199
Plant extract *Grifola frondosa*	Single-center, controlled, open-label and *ex vivo*	BALB/c murine macrophages	Culture	Increase of production of IL-12, IL-1β, TNF-α. Downregulation of IL-10 and TGF-β. Increase of NO production	Inhibition amastigotes replication	202
Plant extract *Sterculia villosa*	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of production of IL-12 and IFN-γ. Downregulation of IL-10 and TGF-β	Reduction parasite burden	203
Plant extract *Withania somnifera*	Single-center, controlled, open-label and *in vivo*	Golden hamsters (*Mesocricetus auratus*)	Oral	Increase of production of IFN-γ and IL-12, inducible NO synthase mRNA transcript and suppressed levels of IL-4, IL-10 and TGF-β	Inhibition parasite multiplication	204
TLR2 agonist[2] Pam3Cys	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of production of IFN-γ.	Reduction infection rate	121

[1]This compound was also studied in *L. major*.

[2]This compound was administered before and after *L. donovani* infection

Abbreviations: anti-GITR: anti-glucocorticoid-induced TNF receptor related protein; HDP: host defense peptide; IL: interleukin; IFN-γ: interferon gamma; MAb: monoclonal antibody; NO: nitric oxide; Pam3Cys: N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Cys-Ser-Lys4 hydrochloride; Redox: reduction-oxidation; TGF: transforming growth factor; TNF-α: tumor necrosis factor alpha.

Host defense peptides (HDPs)

Host defense peptides (HDPs) are short peptides which can vary in length from 12 to 50 amino acids and have been detected in a wide range of animal, plant, fungal and bacterial species. HDPs are induced in response to specific stress situations such as inflammation or infection [211–213]. They also play a crucial role in the innate immunity and have a broad range of different activities that can vary from angiogenesis or cytokine induction to histamine release or chemotactic functions [212,213]. HDPs have been widely studied for their antimicrobial properties, such as topical treatment of wound infections for promoting healing [211–213]. However, the cost of manufacturing HDPs is currently too expensive to be applied in the clinical practice [212].

In relation to *Leishmania* infection, different HDPs appear to have leishmanicidal activities through the activation of the immune defense. A study performed in vitro in promastigote cultures and in a mouse model in vivo [214] investigated the efficacy of two peptides (RP-1 and AA-RP-1) against *Leishmania* infection. Both peptides had a significant antileishmanial effect against three different *Leishmania* species (*L. infantum*, *L. major* and *L. braziliensis*). RP-1 and AA-RP-1 triggered immediate effects on promastigotes while in the experimental infection, BALB/c mice presented a reduction in the *Leishmania* infection rate [214]. In another study performed in Canada [201], four peptides were investigated against *L. major* and *L. donovani*-infected THP-1 human macrophages (Table 1.6).

Plant extracts

Many different plant extracts have been studied for their leishmanicidal activity in promastigote cultures of *L. donovani* [215], *L. infantum* [216], *L. braziliensis* [216], *L. major* [217,218] and *L. amazonensis* [219]. However, studies on antileishmanial plant extracts are still in early stages prior to verification of their efficacy and safety in animals. Three plant extract compounds have shown a potential to be beneficial against murine leishmaniosis due to *L. donovani* and they are summarized in Table 1.6.

Other compounds studied in other *Leishmania* species

Other compounds such as TLR 9 agonists, chitin and chitosan nanoparticles and acetyl salicylic acid (ASA) have been investigated in *L. major* and *L. panamensis* infections mainly in rodent models and showed promising results. However, these compounds have not yet been applied as immunotherapeutical agents in *L. infantum* and *L. donovani* infections.

TLR9 agonists

TLR9 is an intracellular TLR involved in the recognition of un-methylated cytosine triphosphate deoxynucleotide followed by phosphodiester link with guanine triphosphate deoxynucleotide (CpG) oligonucleotides generally of bacterial and viral origin but also of self-DNA in immune-complexes. TLR9 is expressed by B cells, plasmacytoid DCs and also on some activated monocytes [177]. Treatments with CpG alone or in combination with other products in experimental murine *L. major* [220,221] and *L. panamensis* [222] infections have been studied with promising results.

Chitin and chitosan nanoparticles

Chitin, a polymer formed by repeating units of β-(1–4)-poly-N-acetyl-D-glucosamine, is the second most abundant polysaccharide in nature [223]. Its transformation to chitosan is produced through its deacetylation [122]. Thus, the term chitosan is used for chitin with more than a 50% degree of deacetylation [122]. Chitin preparations have powerful effects on immune responses and have been found to be nontoxic, non-allergenic, biodegradable and biocompatible [223,224]. The immunomodulatory effects of chitin and/or chitosan have been investigated in *L. major*-infected murine macrophages [225] and experimental murine *L. major* infection [122,226]. The results of these studies showed that these microparticles induced the production of cytokines such as TNF-α, IFN-γ and IL-10 and reduced the size of skin lesions.

Acetyl salicylic acid (ASA)

ASA is a nonsteroidal anti-inflammatory drug that can inhibit cyclooxygenase-derived prostaglandins, inflammatory reactions and platelet aggregation [227–229]. ASA can induce the production of NO [228]. Nahrevanian and collaborators [229] investigated the oral administration of ASA after lesion appearance in *L. major*-infected BALB/c mice and found a decline in proliferation of amastigotes and reduction of lesion size together with an increase of NO in the blood of treated infected mice [229].

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Chapter 2

Hypotheses and objectives

Hypotheses

Many studies have been performed to determine the reasons of the wide range and variability of the different clinical presentations of CanL [1–5]. Several factors have been linked to the presentation of the disease such as age [6,7], breed [7–9], the host's immune response [10,11] and co-infections with other pathogens [1,12,13]. Thus, with the knowledge that the host's immune response is an important factor for the development of clinical illness [14,15], the most promising prophylactic and therapeutic approach includes the use of immunotherapy to enhance the specific immune response against *L. infantum* infection in dogs. Currently, the only immunotherapeutic compound registered and marketed for prevention and control of clinical progression in mild disease in some countries in Europe for *L. infantu*m infection is domperidone (Leisguard®) [16]. Leisguard® is specifically indicated to reduce the risk of developing an active infection and clinical disease after contact with *L. infantum*, through the enhancement of the cell-mediated immune response, and also for the control of clinical progression of CanL at early stages of the disease (dogs with low to moderate positive antibody levels and mild clinical signs such as peripheral lymphadenomegaly or papular dermatitis) [16]. The active compound of Leisguard® is domperidone which is an immunomodulator that induces a reversible increase of blood prolactin levels that increases CD4+ lymphocyte activity and IL-2, IL-12, IFN-γ and TNF-α production, activating natural killer (NK) and macrophages and a decrease of CD4+ Th2 and TNF-β [17–21]. There are few published studies that have evaluated the use, efficacy and safety of Leisguard® in dogs [22–26]. Sabaté et al. [22] suggested that the implementation of a quarterly repeated 30-day treatment with Leisguard® effectively reduces the risk to develop clinical disease in areas with high prevalence of the disease. Moreover, Gómez-Ochoa et al. [23] described a rapid increase of the percentage of activated neutrophils in healthy dogs that received a 30-day course of Leisguard® when compared with untreated dogs. In another study, Gómez-Ochoa [24] also suggested that Leisguard® was effective in both controlling and reducing the clinical signs of leishmaniosis and reducing the anti-*Leishmania* antibody titers when treating dogs with mild clinical signs of leishmaniosis. Similar results were described in a study performed by Cavalera et al. [25] where dogs with leishmaniosis and CKD were treated with Leisguard®. The study provided preliminary results on the ability of Leisguard® to improve serum creatinine and reduce anti-*Leishmania* antibody titers, globulins, gamma globulins and C-reactive protein [25]. Furthermore, domperidone has also been administered in combination with other products such as furazolidone with good results related to the decrease of skin lesions associated with CanL [26]. However, some of the studies mentioned above present some limitations such as lack of an appropriate control group [23–26], short follow-up periods [23] and small numbers of dogs studied [23,25,26]. Hence, these limitations highlight the need for more complete studies to support the use, efficacy and safety of

Leisguard® as an immunotherapeutic treatment for *Leishmania* infection in dogs and the development of clinical illness.

Thus, the first hypothesis of this doctoral thesis was that the current use of immunotherapy (domperidone, Leisguard®) and other preventive measures to prevent *L. infantum* infection in dogs living in high endemic regions has increased in the last decade. Repellents have always been recommended in the clinical setting and are available in different forms such as collars, spot-on and sprays. Other new products such as domperidone (Leisguard®) and vaccines (CaniLeish®, Letifend®) have been marketed in the last decade and also recommended in combination with repellents [27]. The increase in use of preventive measures improves the control of *L. infantum* infection in endemic regions and assists on decreasing the prevalence of both infection and clinical disease [27].

Additionally, the second hypothesis of this doctoral thesis was that the use of immunotherapy (Leisguard®) alone could also stop or delay the development of clinical illness in *Leishmania*-seropositive healthy dogs. Furthermore, Leisguard® has less severe and shorter-lasting adverse effects than other treatments against CanL such as meglumine antimoniate and allopurinol [28,29]. For these reasons, the use of immunotherapy in *Leishmania*-seropositive healthy dogs might improve the dogs' immune response and, thus, the dogs would present neither clinical signs nor laboratory findings while treated only with Leisguard®. Moreover, *Leishmania*-seropositive dogs treated only with Leisguard® might serorevert faster than non-treated dogs.

Finally, early detection of *L. infantum* infection is highly important to control and prevent the disease in endemic countries [30]. Several diagnostic techniques are available to diagnose *L. infantum* infection such as parasitological diagnosis (that includes the direct observation of the parasite), serological techniques (such as ELISA and IFI) and molecular techniques (such as quantitative PCR) [30–32]. These diagnostic techniques are usually performed with blood, serum, urine and other infected tissues [33–36]. However, the use of alternative samples, such as OT, hair or conjunctival swabs, has also been investigated, with promising results [37–40].

For this reason, the last hypothesis was focused on OT as a promising sample to diagnose *L. infantum* infection. OT is a very interesting type of sample because it has a cheap and non-invasive collection that can be performed by non-trained personnel and could be used to detect anti-*Leishmania* antibodies. Furthermore, OT could be collected faster than serum and a higher number of dogs could be screened by *L. infantum* infection in a shorter period of time. Additionally, OT could also be used as a sample used in follow-up techniques of dogs with CanL and under treatment.

Objectives

The general objectives of this doctoral thesis were:

1. to investigate the past and current use of serological screening tools and preventive measures against *L. infantum* infection in dogs from European *Leishmania*-endemic countries.
2. to investigate and validate new diagnostic techniques for the early detection and follow-up of *L. infantum* infection and immune response in dogs in the clinical setting.
3. to evaluate the efficacy and safety of Leisguard® as an immunotherapeutic for *Leishmania*-seropositive healthy dogs to stop or delay the development of clinical illness.

The specific objectives were:

1. to investigate the most used serological screening tools and preventive measures against *L. infantum* infection in dogs from Europe and how their use changed through the years (chapter 3).
2. to determine the seroprevalence of *L. infantum* infection in apparently healthy dogs in Spain (chapter 4).
3. to investigate and validate new diagnostic methods for the detection of *L. infantum* infection and immune response in dogs (chapters 5 and 6).
4. to investigate the signalment and serological status of *Leishmania*-seropositive healthy dogs (chapter 7).
5. to assess the efficacy and safety of Leisguard® as an immunotherapeutic for *Leishmania*-seropositive healthy dogs (chapter 8).

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Chapter 3

Use of preventive measures and serological screening tools for *Leishmania infantum* infection in dogs from Europe

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Abstract

Background: There are several screening tools for detecting *Leishmania infantum* infection in dogs and various preventive measures to protect against it. Some studies have investigated them, but not many have described their current use. The aim of this study was to investigate which preventive measures and serological screening tools for *L. infantum* infection were employed from 2012 to 2018 in dogs from different endemic European countries.

Methods: A set of electronic datasheets was completed for each dog from several veterinary centres. Classification of preventive measures included: (1) repellents, (2) vaccines and (3) immunomodulators. Classification of serological tests included the: (1) direct agglutination test (DAT), (2) enzyme-linked immunosorbent assay (ELISA), (3) indirect immunofluorescence (IFI), (4) rapid tests and (5) other assays. Dogs were also classified depending on their risk of exposure and living area.

Results: Information from 3762 dogs was gathered. Preventive measures were applied in 91.5% of dogs and the most frequently used were repellents (86.2%) followed by vaccines (39.8%) and Leisguard® (15.3%). The different types of repellents (collar and spot-on) were used similarly. A combination of a vaccine and repellents was preferred in the high-risk group while the low-risk preferred a combination of Leisguard® and a repellent (Chi-square test: X^2=88.41, df=10, p<0.001). Furthermore, all preventive measures were similarly used through the years except for repellents, which were predicted to have a small increase of use each year. Regarding serological screening tools, the most used were rapid and ELISA tests. Rapid tests, ELISA tests and DAT were used similarly through the years, but a significant change was found in the use of IFI and other assays whose use decreased a little each year.

Conclusions: Repellents were the preferred measure, while vaccines and Leisguard® were second-line options. Some dogs were not treated by any measures, which highlights the need for dog owner education. Moreover, there seems to be a preference for rapid tests in the clinical setting to detect specific *L. infantum* antibodies while ELISA or IFI are less often employed. This underlines an increasing problem, as qualitative rapid tests have a variable diagnostic performance limiting the adequate diagnosis of seropositive dogs in endemic areas.

Keywords: canine, Europe, leishmaniosis, prevention, screening diagnostic tools.

Background

Canine leishmaniosis (CanL) caused by the protozoan *Leishmania infantum* is a zoonotic and endemic disease in the Mediterranean basin [1,2]. This protozoan is transmitted by the bite of a female phlebotomine sand fly following a digenetic life cycle which consists of two different phases: (i) a promastigote phase, which is an extracellular and motile form that colonizes the middle gut of the sand fly, and (ii) an amastigote phase, which is an intracellular and non-motile form that colonizes macrophages of infected hosts [3,4]. There are also other potential routes of transmission such as venereal [5,6], transplacental [6,7] and through blood transfusion [8,9], which may play a marginal role compared to the vector transmission [10]. The dog (*Canis lupus familiaris*) is considered the main domestic reservoir for *L. infantum* infection in the Mediterranean basin [2,10], while other mammals such as wild canids [11], rodents [12] and lagomorphs [13] may be able to maintain a wild life cycle.

The use of preventive measures against *L. infantum* infection has expanded over the last decades [14]. However, there are still two main ways to prevent this infection: (i) physical barriers and insecticides against the vector and (ii) immunoprophylaxis. Regarding the vector, it is recommended to avoid outdoor activities during dawn and dusk (when the vector is highly present), to use fine mesh nets in windows and to use topical insecticides such as synthetic pyrethroid-based compounds, which have both repellent and anti-feeding effects [1,14,15]. Topical insecticides are commercially available in different forms: impregnated collars, spot-on and sprays, each of which has different onset and maximum duration [3,14]. Immunoprophylaxis can be divided into vaccines and immunomodulators. Domperidone (Leisguard®) is the only marketed immunomodulator for the prevention of CanL since 2012 [16]. Two commercial vaccines have been available for dogs in Europe: Canileish®, which was first launched in 2011 but is not marketed anymore (withdrawn from the market in 2021), and Letifend®, which was introduced commercially in 2016 and is currently the only available vaccine in Europe [3,14,17].

Moreover, CanL is a complex infection due to its variable clinical manifestations and a wide spectrum of clinical signs and laboratory findings, and several diagnostic techniques are available for its screening and diagnosis [17,18]. Since a vaccine is available in Europe, serological screening is mandatory prior to vaccination of dogs [17]. In addition, annual screening of dogs is frequently performed in endemic areas to diagnose both dogs progressing towards disease and subclinical infections [10,17]. The diagnostic methods used in the clinical setting include parasitological diagnosis (direct observation of the parasite), serological techniques (such as ELISA, IFI and rapid chromatographic immunoassay) and molecular techniques (PCR and quantitative PCR) [1,17,18].

Some studies have investigated the use of preventive measures in *L. infantum* endemic countries, although their focus was the efficacy and safety of those measures [16,19,20] or the veterinary recommendations for their use to dog owners [21–25]. In addition, the development and marketing of new preventive measures such as Letifend® may change the use of the already marketed products. Regarding serological screening tools, several studies have compared their sensitivity and specificity [18,26,27] or the use of different types of samples such as saliva [28]. However, the current use of the different preventive measures and serological screening tools available for *L. infantum* infection is relatively unknown.

For all these reasons, the aim of this study was to investigate the most used serological screening tools and preventive measures against *L. infantum* infection in dogs from 2012 to 2018 and how their use changed through the years.

Methods

Veterinary clinics and cases

Veterinary clinics from Spain (n=84), Portugal (n=3), Italy (n=17) and Cyprus (n=2), which implemented at least two different preventive measures against *L. infantum* in dogs, were selected for a database search of clinical records by the authors from their contacts and client lists and were contacted to participate. Fig 3.1 shows the veterinary clinics that enrolled in the study including 67 from Spain, 3 from Portugal, 10 from Italy and 1 from Cyprus. These veterinary clinics provided information of dogs with the following inclusion criteria: (1) apparently healthy dogs and (2) a previous screening serological test for the detection of antibodies against *L. infantum* antigen before the initial use of the preventive measures.

Study design

Each veterinary clinic received a code to access a website with a set of electronic datasheets that allowed easy data entry. Once the datasheets were completed, their data were automatically uploaded to a common database from which the results were analysed.

The online questionnaire permitted gathering relevant clinical data about dog characteristics (sex, weight, age, breed, risk of exposure and living area) and types of serology tests and preventive measures used. Data of preventive measures were obtained from 2012 to 2017 while data of screening tools were collected from 2012 to 2018.

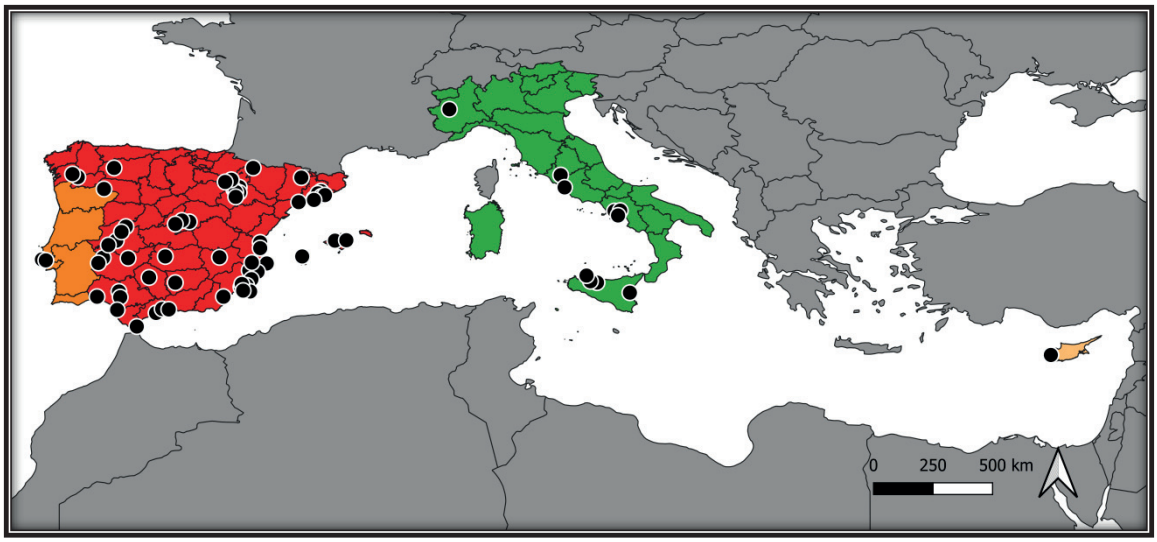

Fig 3.1 Geographical distribution of all participating veterinary clinics from Europe. Spain is marked in red, Portugal in orange, Italy in green and Cyprus in yellow. Black dots represent each enrolled clinic in each country location.

Case removal

After collection of cases, removal of inadequate cases was performed. A case was defined as inadequate when: (i) it did not comply with the previously established inclusion criteria or (ii) a duplicate case detected. When a duplicate case was detected, a thorough search was performed to confirm its duplicity as to not lose any information. Information about the same dog with two different preventive measures and non-overlapping timelines was not defined as a duplicate.

Preventive measures

Dogs were classified considering the combined use of preventive measures. Eleven groups were considered: (i) no preventive measures applied (NON), (ii) only repellents applied (REP), (iii) only Canileish® vaccine (CAN), (iv) only Letifend® vaccine (LET), (v) only Leisguard® (LEI), (vi) Canileish® vaccine + repellent (CAN + REP), (vii) Letifend® vaccine + repellent (LET + REP), (viii) Leisguard® + repellent (LEI + REP), (ix) Canileish® vaccine + Leisguard® (CAN + LEI), (x) Canileish® vaccine + Leisguard® + repellent (CAN + LEI + REP) and (xi) Letifend® vaccine + Leisguard® + repellent (LET + LEI + REP).

Another classification considered the individual use of each product. These four groups were defined as (i) repellent group, which included dogs that used repellent alone or in combination with other products (REP, CAN + REP, LET + REP, LEI + REP, CAN + LEI + REP and LET + LEI + REP), (ii) Canileish®, which included dogs that used Canileish® alone or in combination with other products (CAN, CAN + REP, CAN + LEI and CAN + LEI + REP), (iii) Letifend®, which included dogs that used Letifend® alone or in combination with other products (LET, LET + REP and LET + LEI + REP), and (iv) Leisguard®, which included dogs that used Leisguard® alone or in combination with other products (LEI, LEI + REP, CAN + LEI, CAN + LEI + REP and LET + LEI + REP).

Dogs that used repellent were classified in three different groups based on type of repellent employed: (i) collar, (ii) spot-on and (iii) collar + spot-on.

Classification of exposure risk and living area

Dogs were classified in two different groups depending on their exposure risk to *L. infantum* infection. High risk was considered when dogs lived outdoors or when dogs that despite living indoors went frequently for a walk in plot of land or forest areas at times when the vector was highly present, for example at dawn and dusk. Low risk classification included those dogs which lived indoors and went only for a walk in urban area or just at times when the vector was barely present.

Another classification depending on living area was also performed. Dogs were classified in three groups: urban area (living in cities or big towns with paved streets and small green areas), periurban area (city outskirts or towns surrounded by large green areas) and rural area (small towns or buildings built far away from human settlements like farms, usually agricultural areas and forests).

Screening tools

The screening tools were classified in five groups: (i) direct agglutination test (DAT), (ii) enzyme-linked immunosorbent assay (ELISA), (iii) indirect immunofluorescence (IFI), (iv) rapid tests and (5) other assays.

Additionally, a screening campaign by Ecuphar veterinaria SLU was performed in 2018 using Leiscan® and ELISA in house [29] to increase the number of enrolled dogs; therefore, a bias was to be expected.

Statistical analysis

A descriptive study of all collected data was performed. Quantitative variables (age, weight) were assessed using a non-parametric Mann-Whitney *U* test when two groups were compared (high and low risk) while the Kruskal-Wallis *H* test was used when three groups were compared (living area: urban, periurban or rural). Qualitative variables (sex, breed, preventive measures and serological screening tools) were

assessed using a Chi-square test. A simple linear regression was calculated to predict the proportion of use for each preventative measure or serological test based on time (from 2012 to 2017 or from 2012 to 2018, respectively).

A *p*-value<0.05 was considered statistically significant. The Shapiro-Wilk test was performed to detect normal distribution of quantitative variables. The statistical analysis was performed using the package Stats for the software R i386 3.5.1 for Windows. Maps were created using the Free and Open Source QGIS 3.10.4 for Windows. Graphics were plotted using Graphad Prism version 5.00 for Windows.

Results

Dog characteristics

Dogs from Spain (3603 dogs), Portugal (64 dogs), Italy (69 dogs) and Cyprus (26 dogs) were enrolled in this study with a total of 3762 dogs. Dog characteristics such as sex, age, weight, breed, risk of exposure and living area are displayed in Table 3.1. The most common breeds were Yorkshire terrier (7.1%), Labrador retriever (6.7%), German shepherd (6.2%), Maltese (3.9%), Boxer (3.8%), Golden retriever (3.7%) and French bulldog (3.5%).

No statistically significant differences were found between risk of exposure to the vector (low *vs* high risk of exposure) when sex, age and breed were compared. A significant difference (Mann-Whitney *U* test: U=1,876,996, Z=–13.46, n_1=2613, n_2=1125, p<0.0001) was noted when weight was compared between groups of risk of exposure to the vector. Large size dogs (21.9±13.7 kg) were included in the high-risk group while small size dogs (15.7±12.6 kg) were included in the low-risk group.

Quantitative and qualitative characteristics of dogs depending on their living area are listed in Table 3.2. No differences between groups were found when sex and breed were compared. In the case of age and weight, dogs living in rural areas were younger than dogs living in periurban or urban areas (Kruskal-Wallis *H* test: X^2=10.73, df=2, p=0.005) while dogs living in urban areas were smaller in size than dogs living in rural or periurban areas (Kruskal-Wallis *H* test: X^2=176.06, df=2, p<0.0001) (Table 3.2). Moreover, rural area dogs had a higher risk of exposure to *L. infantum* followed by periurban dogs and finally urban dogs (Chi-square test: X^2=314.67, df=2, p<0.001).

Table 3.1 Qualitative and quantitative clinical characteristics of the dogs.

Qualitative clinical characteristics		n	% (95% CI)		
Sex	Male	2006	53.4 (51.8-55)		
	Female	1753	46.6 (45-48.2)		
	Total	3759			
Breed	Purebred	2711	72.3 (70.9-73.8)		
	Mixed-breed	1037	27.7 (26.2-29.1)		
	Total	3748			
Risk of exposure	High	2620	69.9 (68.4-71.4)		
	Low	1127	30.1 (28.6-31.6)		
	Total	3747			
Living area	Urban area	1585	55.5 (53.6-57.3)		
	Periurban area	818	28.6 (27-30.3)		
	Rural area	455	15.9 (14.6-17.3)		
	Total	2858			
Quantitative clinical characteristics		**n**	**Mean (±SD)**	**Minimum**	**Maximum**
Age (years)		3755	7 (±3.3)	0.5	18.5
Weight (kg)		3753	20 (±13.7)	1.4	110

Abbreviations: CI: confidence intervals; n: number of dogs; SD: standard deviation.

Preventive measures

General results

Preventive measures were applied for 3444 dogs (91.5%) of all the dogs enrolled. Younger dogs (6.9±3.3 years) were more likely to be treated with preventive measures than older dogs (7.7±3.5 years) (Mann-Whitney *U* test: U=614,890.5, Z=–3.79, n_1=317, n_2=3438, p=0.0002).

Table 3.2 Qualitative and quantitative clinical characteristics of the dogs depending on their living area.

Qualitative clinical characteristics		Urban area (n=1585)		Periurban area (n=818)		Rural area (n=455)		*p*-value (Chi-square test)
		n	% (95% CI)	n	% (95% CI)	n	% (95% CI)	
Sex	**Male**	842	53.1 (50.6-55.6)	461	56.4 (52.9-59.8)	241	53 (48.3-57.6)	0.284
	Female	743	46.9 (44.4-49.4)	357	43.6 (40.2-47.1)	214	47 (42.4-51.7)	
Breed	**Purebred**	1174	74.1 (71.8-76-2)	576	70.4 (67.2-73.5)	317	69.7 (65.2-73.9)	0.064
	Mixed-breed	411	25.9 (23.8-28.2)	242	29.6 (26.5-32.8)	138	30.3 (26.1-34.8)	
Risk of exposure	**High**	925	58.4 (55.9-60.8)	676	82.6 (79.9-85.2)	436	95.8 (93.6-97.5)	<0.001[1]*
	Low	660	41.6 (39.2-44-1)	142	17.4 (14.8-20.1)	19	4.2 (2.5-6.4)	
Quantitative clinical characteristics		**n**	**Mean (±SD)**	**n**	**Mean (±SD)**	**n**	**Mean (±SD)**	***p*-value (Kruskal-Wallis *H* test)**
Age (years)		1585	7.2 (±3.3)	817	7.1 (±3.3)	455	6.6 (±3.1)	0.005[2]*
Weight (kg)		1585	17 (±13.1)	818	23 (±13.5)	455	23.9 (±13.8)	<0.0001[3]*

*p-value < 0.05 (statistically significant)

[1]X^2=314.67, df=2, p<0.001, [2]X^2=10.73, df=2, p=0.005, [3]X^2=176.06, df=2, p<0.0001

Abbreviations: CI: confidence intervals; n: number of dogs; SD: standard deviation.

The individual use of each preventive measure in the 3444 dogs is plotted in Fig 3.2. Repellents (alone or in combination with other products) were the most used preventive measure followed by vaccines (Canileish® or Letifend®) and Leisguard® (Fig 3.2a). The different types of repellents (collar, spot-on or a combination of both) were used similarly (Fig 3.2b) while, in the case of vaccines, Canileish® (60.8%) was more frequently used than Letifend® (39.2%) (Fig 3.2c). No statistical differences were observed when the individual use of the different preventive measures depending on sex and breed were compared except for Canileish®, which was more often used in purebred dogs (Chi-square test: X^2=9.26, *df*=1, *p*=0.002) than in mixed-breed dogs.

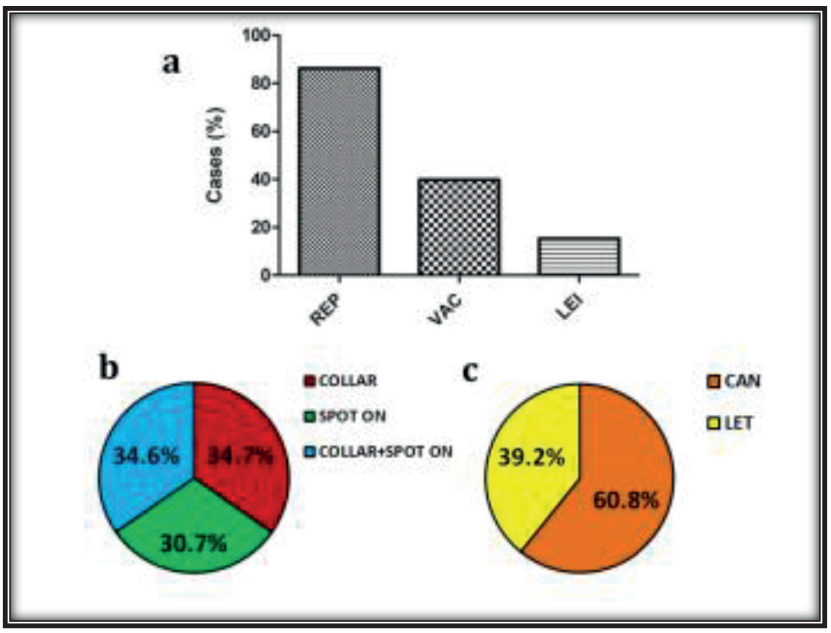

Fig 3.2 Proportions of (a) the individual use of each preventive measure, (b) the type of repellent used and (c) the vaccine used. Preventive measures represented are repellent group (REP), which included dogs that used repellent alone or in combination with other products, vaccine group (VAC), which included dogs that used vaccine alone or in combination with other products, Leisguard® group (LEI), which included dogs that used Leisguard® alone or in combination with other products, Canileish® group (CAN) and Letifend® group (LET).

Regarding age, younger dogs were more likely to use repellent (Mann-Whitney *U* test: *U*=900,141.5, *Z*=– 2.7, n_1=518, n_2=3237, *p*=0.007), Letifend® (Mann-Whitney *U* test: *U*=1,084,731, *Z*=-6.42, n_1=3168, n_2=587, *p*<0.0001) or Leisguard® (Mann-Whitney *U* test: *U*=963,611.5, *Z*=-2.29, n_1=3184, n_2=571, *p*=0.02) than older dogs. As for weight, larger dogs were more likely to use Canileish® (Mann-Whitney *U* test: *U*=1,213,325, *Z*=–2.72, n_1=2846, n_2=907, *p*=0.006) while smaller dogs were more likely to use Leisguard® (Mann-Whitney *U* test: *U*=1,043,852.5, *Z*=-5.56, n_1=3180, n_2=573, *p*<0.0001).

Fig 3.3 shows the combined preventive measures used in all the dogs. The most used preventive measure was repellent alone (Fig 3.3). When comparing the proportions of sex, CAN + LEI and CAN + LEI + REP presented the highest proportion of females (58.6%) while REP presented the highest proportion of males (55.9%) (Chi-square test: X^2=4.78, *df*=1, *p*=0.029), but no other differences were found between the other groups (Table 3.3). Regarding breed, only CAN + REP was found to have a significantly higher proportion of purebred dogs (77%) when compared to the other preventive measures (44.4%) (Chi-square test: X^2=16.53, *df*=6, *p*=0.011) (Table 3.3). When comparing their age, LEI was found to be the oldest group (Table 3.3).

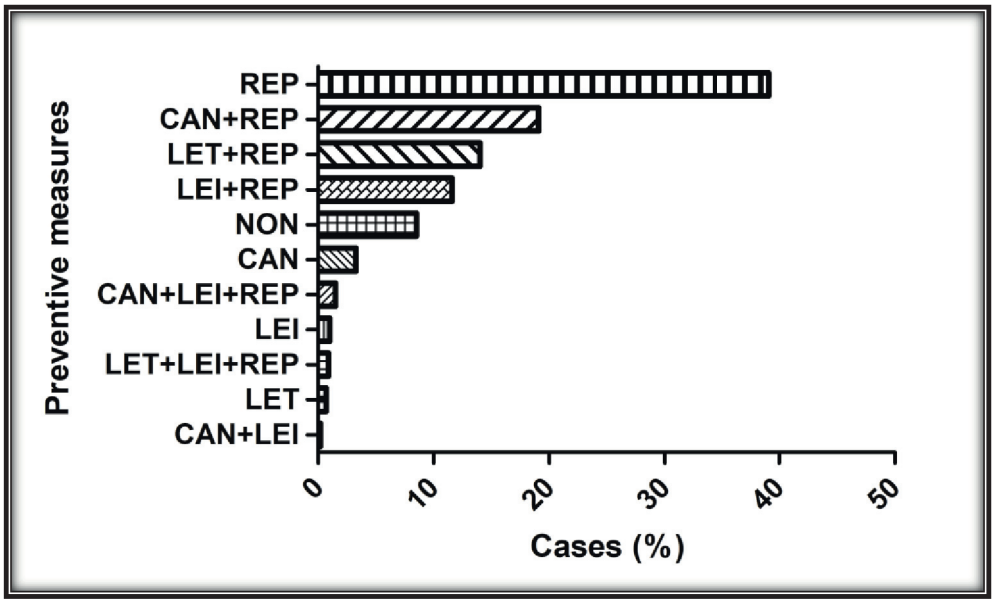

Fig 3.3 Proportions of preventive measures used against *L. infantum* in all dogs studied. Preventive measures represented are only repellents applied (REP), Canileish® vaccine + repellent (CAN + REP), Letifend® vaccine + repellent (LET + REP), Leisguard® + repellent (LEI + REP), no preventive measures applied (NON), only Canileish® vaccine applied (CAN), Canileish® vaccine + Leisguard® + repellent (CAN + LEI + REP), only Leisguard® applied (LEI), Letifend® vaccine + Leisguard® + repellent (LET + LEI + REP), only Letifend® vaccine applied (LET) and Canileish® vaccine + Leisguard® (CAN + LEI).

Regarding weight, LEI + REP and LEI were the groups with smaller dogs and significantly different when compared to the other groups (Kruskal-Wallis *H* test: X^2=45.82, *df*=10, *p*<0.0001) (Table 3.3).

Table 3.3 Qualitative and quantitative clinical characteristics of the dogs depending on the preventive measures used.

Preventive measures	Sex (%, 95% CI)		Breed (%, 95% CI)		Age (years, mean ±SD)	Weight (kg, mean ±SD)	Risk of exposure (%, 95% CI)	
	Male	Female	Purebred	Mixed-breed			High	Low
NON (n=318)	50 (44.4-55.6)	50 (44.4-55.6)	69.1 (63.7-74.1)	30.9 (25.9-36.3)	7.1 (±3.5)	20 (±14.3)	71.9 (66.6-76.8)	28.1 (23.2-33.4)
REP (n=1468)	55.9 (53.3-58.5)	44.1 (41.5-46.7)	71.8 (69.4-74.1)	28.2 (25.9-30.6)	7 (±3.4)	18.8 (±13.2)	66.4 (63.9-68.8)	33.6 (31.2-36.1)
CAN (n=125)	52 (42.9-61)	48 (39-57.1)	75 (66.4-82.3)	25 (17.7-33.6)	6.5 (±2.8)	21.8 (±14.6)	69.4 (60.4-77.3)	30.6 (22.7-39.6)
LET (n=28)	53.6 (33.9-72.5)	46.4 (27.5-66.1)	71.4 (51.3-86.8)	28.6 (13.2-48.7)	4.4 (±3.7)	19 (±10.1)	60.7 (40.6-78.5)	39.3 (21.5-59.4)
LEI (n=39)	41 (25.6-57.9)	59 (42.1-74.4)	61.5 (44.6-76.6)	38.5 (23.4-55.4)	8.8 (±3.3)	11.9 (±14.1)	43.6 (27.8-60.4)	56.4 (39.6-72.2)
CAN+REP (n=719)	53.8 (50.1-57.5)	46.2 (42.5-49.9)	77 (73.7-80)	23 (20-26.3)	6.3 (±3.1)	18 (±14.7)	72.4 (69-75.6)	27.6 (24.4-31)
LET+REP (n=527)	51.4 (47.1-55.8)	48.6 (44.2-52.9)	71.9 (67.9-75.7)	28.1 (24.3-32.1)	5.8 (±3.3)	19 (±13.1)	89.9 (87.1-92.4)	10.1 (7.6-13)
LEI+REP (n=436)	52.5 (47.7-57.3)	47.5 (42.7-52.3)	70 (65.5-74.3)	30 (25.7-34.5)	6.1 (±3.2)	12 (±13.5)	53.8 (49-58.6)	46.2 (41.4-51)
CAN+LEI (n=9)	22.2 (2.8-60)	77.8 (40-97.2)	44.4 (13.7-78.8)	55.6 (21.2-86.3)	4.4 (±3.5)	10 (±13.2)	88.9 (51.8-99.7)	11.1 (0-48.3)
CAN+LEI+REP (n=57)	41.4 (28.6-55.1)	58.6 (44.9-71.4)	75 (61.6-85.6)	25 (14.4-38.4)	6 (±3)	17.8 (±15.7)	71.9 (58.5-83)	28.1 (17-41.5)
LET+LEI+REP (n=32)	53.1 (34.7-70.9)	46.9 (29.1-65.3)	68.8 (50-83.9)	31.2 (16.1-50)	6 (±1.2)	22.5 (±16.8)	78.1 (60-90.7)	21.9 (9.3-40)
***p*-value**	$p<0.0001$*[1]		$p<0.0001$*[2]		$p<0.0001$*[3]	$p<0.0001$*[4]	$p<0.0001$*[5]	

*p-value<0.05 (statistically significant)

[1]Chi-square test: $X^2=39.63$, $df=10$, [2]Chi-square test: $X^2=38.72$, $df=10$, [3]Kruskal-Wallis H-test: $X^2=84.15$, $df=10$, [4]Kruskal-Wallis H-test: $X^2=45.82$, $df=10$, [5]Chi-square test: $X^2=88.41$, $df=10$

Abbreviations: CAN: only Canileish® vaccine; CAN+LEI: Canileish® vaccine + Leisguard®; CAN+LEI+REP: Canileish® vaccine + Leisguard® + repellent; CAN+REP: Canileish® vaccine + repellent; ; CI: confidence intervals; LEI: only Leisguard®; LEI+REP: Leisguard® + repellent; LET: only Letifend® vaccine; LET+LEI+REP: Letifend® vaccine + Leisguard® + repellent; LET+REP: Letifend® vaccine + repellent; n: number of dogs; NON: no preventive measures applied; REP: only repellents applied; SD: standard deviation.

Fig 3.4 shows the use of the different marketed brands of each type of repellent: collar (Fig 3.4a) and spot-on (Fig 3.4b). The most used products were the Scalibor® collar (70%) and the Advantix® spot-on (61%). Significant differences were found regarding breed, age and weight. In detail, purebred dogs used more frequently a combination of both collar and spot-on, while mixed-breed dogs used collars alone more frequently (Chi-square test: X^2=8.03, *df*=2, *p*=0.018). Dogs using collars alone were younger (6.8 years) than dogs using spot-on alone (7.1 years) (Kruskal-Wallis *H* test: X^2=6.27, *df*=2, *p*=0.044) while dogs using spot-on alone were smaller in size (14.5 kg) than dogs using collars alone (22.2 kg) or a combination of collar and spot-on (22.5 kg) (Kruskal-Wallis *H* test: X^2=299.11, *df*=2, *p*<0.0001).

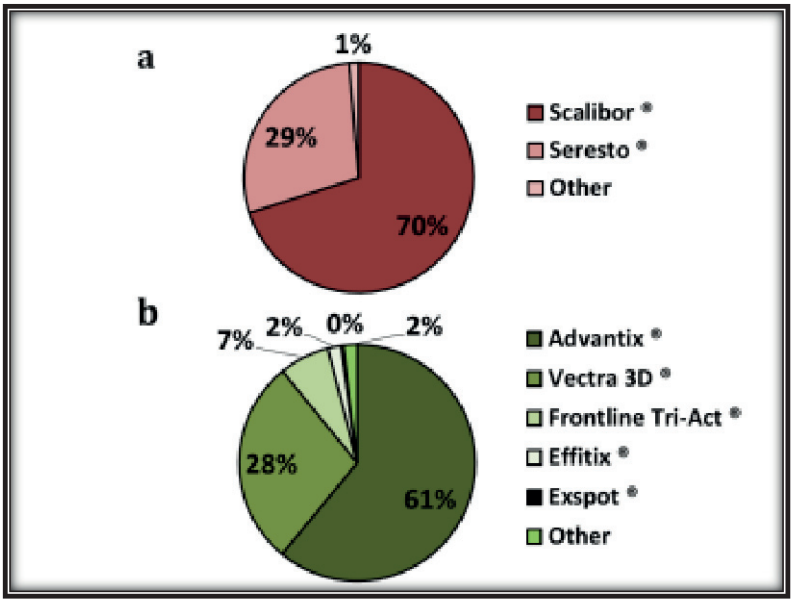

Fig 3.4 Proportions of (a) the use of collar marketed brands and (b) the use of spot-on marketed brands in all dogs studied.

Preventive measures by risk of exposure

The use of preventive measures against *L. infantum* was similar when risk of exposure was compared (91.3% high-risk group and 92.1% low-risk group). Letifend® was used more frequently in the high-risk group (Chi-square test: X^2=107.02, *df*=1, *p*<0.001) while Leisguard® was used more often in the low-risk group (Chi-square test: X^2=54.69, *df*=1, *p*<0.001). Regarding the type of repellents used, the high-risk group had a higher rate of using both types of repellents together (collar and spot-on) while the low-risk group had a higher rate of using collar or spot-on alone (Chi-square test: X^2=92.80, *df*=2, *p*<0.001).

Most of the preventive measures were more frequently used in the high-risk group except for LEI + REP and LEI, which were similarly used in both groups. In fact, LEI + REP and LEI were found to have a significantly higher proportion of use in the low-risk of exposure group than other preventive measures (Chi-square test: X^2=88.41, *df*=10, *p*<0.0001) (Table 3.3). On the other hand, LET + REP was found to have the highest proportion of use in the high-risk group and was significantly different when compared to the other groups (Table 3.3).

Preventive measures by living area

Preventive measures were applied differently depending on the living area showing a higher rate of use in urban area (93.2%) followed by periurban (91.6%) and rural (87.9%) areas (Chi-square test: X^2=13.34, *df*=2, *p*=0.001). The use of collar, spot-on and a combination of both was also compared between urban, periruban and rural areas and significant differences were found (Chi-square test: X^2=194.23, *df*=4, *p*<0.001) with a higher use of collar alone in rural and periruban areas while a combination of both collar and spot-on was preferred in urban areas (Fig 3.5).

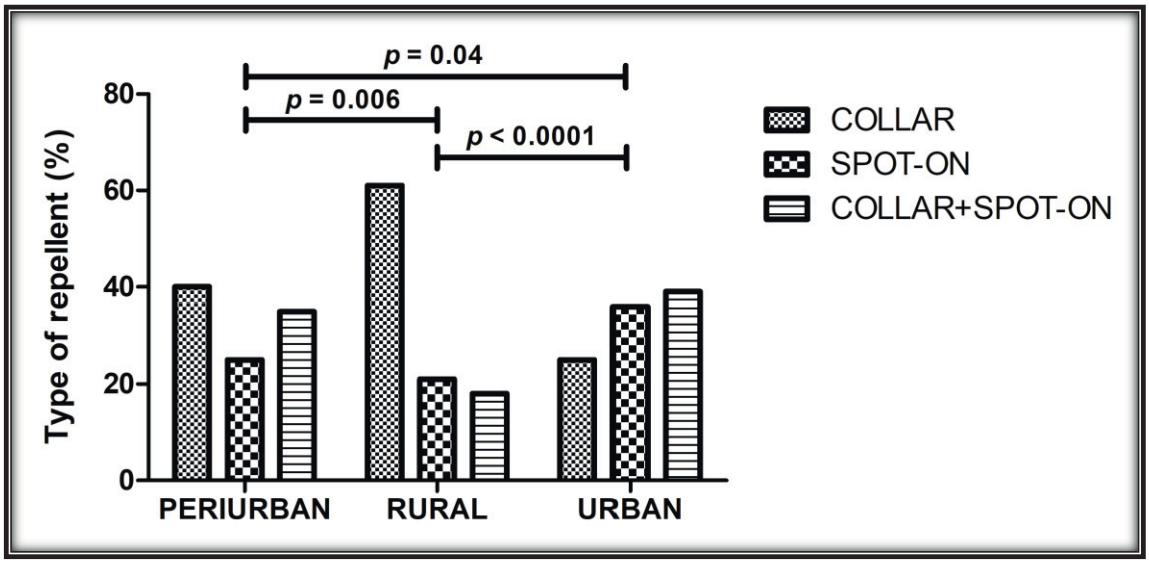

Fig 3.5 Proportions of the type of repellent used depending on the living area. Statistical significance was found in the following comparisons: Periurban vs. rural (Chi-square test: X^2=10.01, *df*=2, *p*=0.006) and urban areas (Chi-square test: X^2=6.07, *df*=2, *p*=0.04) and rural vs. urban areas (Chi-square test: X^2=26.75, *df*=2, *p*<0.0001).

Furthermore, REP was the preventive measure used at the most similar frequency in all areas with 47.5% of use in the urban followed by 30.4% in the periurban and 22.1% in the rural areas. CAN + REP and LET + REP were significantly more used in urban areas with 64% and a 78% frequency, respectively (Chi-square test: X^2=170, *df*=20,

p<0.0001). Moreover, LET + REP was significantly more used in urban areas than CAN + REP (Chi-square test: X^2=30.35, df=2, p<0.001).

Preventive measures trends

The use of the different products from 2012 to 2017 is plotted in Fig 3.6. Repellents were the most used always by > 80% of the dogs studied (Fig 3.6). A significant regression was only found in the use of repellents with an R^2 of 0.75 (Fig 3.6). The predicted use of repellents was equal to -3252.31 + 1.66 of percentage of the use of repellents when time was measured in years, so the percentage of use of repellents increased 1.66% for each year.

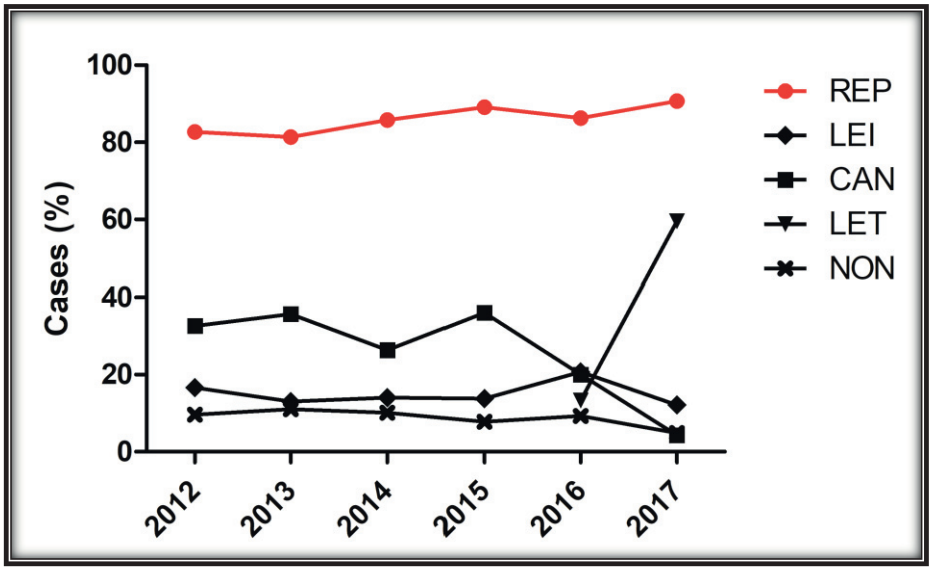

Fig 3.6 Proportions of the use of the different products through the years studied (2012–2017). Preventive measures represented are repellent group (REP), which included dogs that used repellent alone or in combination with other products, Leisguard® group (LEI), which included dogs that used Leisguard® alone or in combination with other products, Canileish® group (CAN), which included dogs that used Canileish® alone or in combination with other products, Letifend® group (LET), which included dogs that used Letifend® alone or in combination with other products, and no preventive measures applied (NON). Data in red present a significant regression: REP (F(1,4)=12.15, p=0.0252).

Serological screening tools

General results

The different types of serological screening tests employed are shown in Fig 3.7 while the different brands of serological screening tests are shown in Fig 3.8. Rapid tests were the most used (SNAP Idexx) followed by ELISA tests (Leiscan®). IFI and DAT were used in < 10% of the cases (Fig 3.7, Fig 3.8).

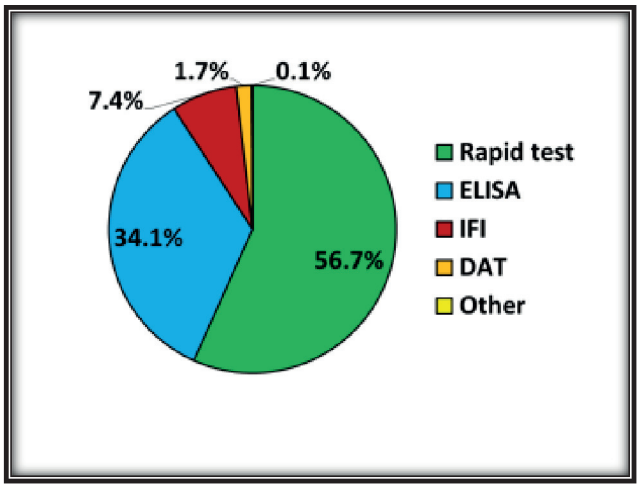

Fig 3.7 Proportions of the different types of serological screening tests. Screening tools represented are the direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IFI), rapid tests and other assays.

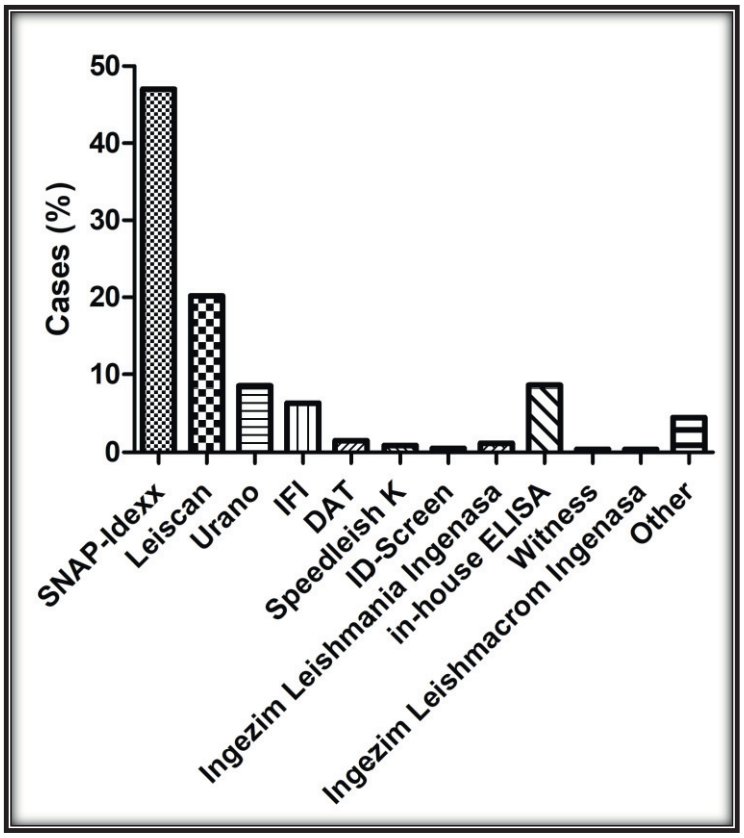

Fig 3.8 Proportions of the different brands of serological screening tests.

Screening tools trends

The use of the different types of serological screening tests from 2012 to 2018 is displayed in Fig 3.9. Rapid tests followed by ELISA were the most frequently used techniques (Fig 3.9). A significant regression was found on the use of IFI tests and other tests with an R^2 of 0.88 and 0.65, respectively. The predicted use of IFI tests was equal to 2066.12—1.02 of percentage of the use of IFI tests when time is measured in years, so the percentage of use of IFI tests decreased 1.02% for each year. The predicted use of other tests was equal to 172.86–0.09 of percentage of the use of other tests when time was measured in years, so the percentage of use of other tests decreased 0.09% for each year.

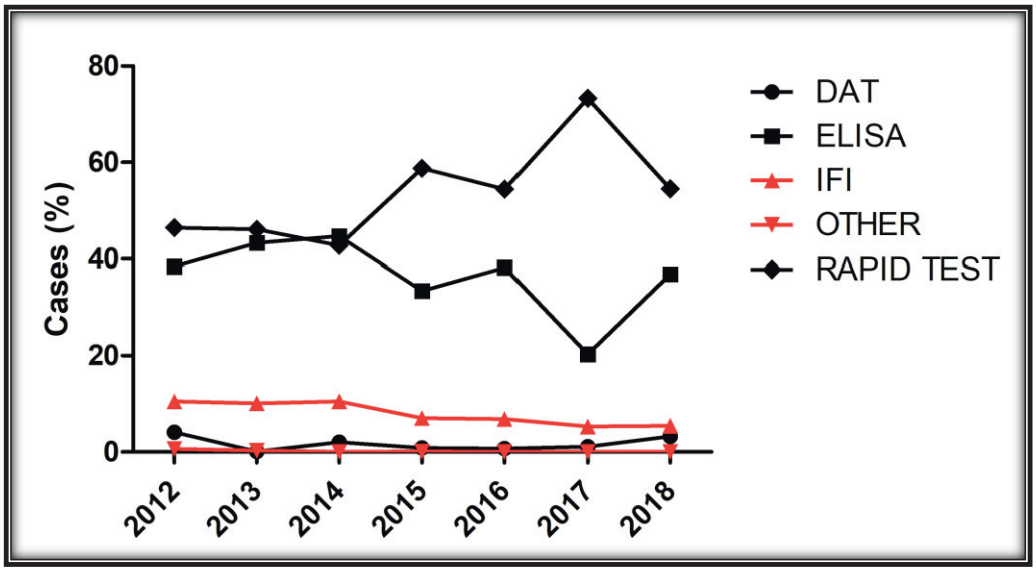

Fig 3.9 Proportions of the use of the different types of serological screening tests through the years studied (2012–2018). Screening tools represented are direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IFI), rapid tests and other assays. Data in red present a significant regression: IFI ($F(1,5)=35.08$, $p=0.002$) and other ($F(1,5)=9.23$, $p=0.0288$).

Discussion

Previous studies have investigated the veterinary recommendations for the use of preventive measures to dog owners in Spain and other European countries and found out that most veterinarians recommend preventive measures against *L. infantum* to their clients [21–25]. These recommendations can be linked directly to the results of the present study as at least one preventive measure was applied in > 90% of the dogs. Furthermore, veterinary recommendations seem to prioritize the use of repellents over vaccines or Leisguard® [22,23], which is also highlighted by the results of the present study where a repellent was used in > 80% of the dogs while vaccines and Leisguard® were used by < 50% throughout all years studied. As expected, these

recommendations are in line with the published guidelines [14], which endorse the use of repellents in both endemic and fringe areas, while vaccines and Leisguard® are described as optional.

Regarding repellent brands, a previous study [23] reported that the most frequently recommended were Seresto®, Advantix® and Scalibor®. Both the present study and an additional study [19] showed similar results with the most used collar being Scalibor® while Advantix® was the most used spot-on. Interestingly, a study performed in north-eastern Spain [22] described a preference for recommending collars (98% of the veterinarians recommended collars to their clients) over spot-on (67% of the veterinarians recommended spot-on), in disagreement with the present results in which there was no difference between the use of collar or spot-on, although the reason for these results could be related to the higher use of collars in periurban and rural areas compared to urban areas found in this study. Regarding vaccines, Montoya et al. [23] reported a higher use of Letifend® than Canileish®. However, the present study differs as a higher use of Canileish® was found when compared with Letifend®. This discrepancy is due to the fact that data on dogs were included from 2012 when Canileish® was still on the market and Letifend® was not marketed yet [3,14,17].

Interestingly, Leisguard® was more frequently administered to smaller dogs [19], as observed in this study. One of the reasons for this result is that the Leisguard® dose administration is linked to body weight so large dogs need a high daily dose and therefore a higher expenditure than when being used for small dogs [16]. Another explanation is the fact that small size dogs are more prone to adverse effects after vaccination [30,31].

An association between socioeconomic status of the dog owner and CanL has been previously documented [32]. Owners with a low income cannot afford some products and that may affect the disease control and even the nutrition and survival of the dog [32]. The presence of a backyard at the residence with a predominance of land and/or vegetation was also associated with CanL [32], which could be a consequence of not only an environmental factor but also of the smaller use of preventive measures in periurban and rural areas as described in the present study, among other factors. Another study from Brazil [33] went further and associated CanL with not just rural areas (small farms) but also the larger size of the dogs (usually used as guard dogs) and lack of owner knowledge about CanL. Coincidentally, in this study, larger dogs were more frequently classified in the high-risk exposure group and living in rural or periurban areas, which could explain its association with CanL.

The use of screening tools was also widespread as stated previously by other studies [19,22–25]. Concerning serological tests, rapid tests and ELISA seem to be preferred by clinicians in the present study as previously reported [19,22–25]. Rapid tests (56.7%) are being used more in the clinical setting probably because of their fast results, low price

and easy performance, while other types of tests such ELISA (34.1%) and IFI (7.4%) are employed less because of increased time of performance and mainly because they need to be conducted in laboratories by trained personnel.

However, ELISA is used more than IFI because IFI's interpretation is subjective and its result depends on the operator's experience and skill to interpret the test while ELISA is interpreted objectively using an ELISA reader to quantify the result [26]. These results highlight an increasing problem in the clinical setting as qualitative rapid tests have a good specificity but are less sensitive than quantitative laboratory tests such as IFI and ELISA and therefore rapid tests can misdiagnose seropositive cases [10,17,18,34]. It is important to remark that rapid tests have a low sensitivity in detecting apparently healthy seropositive dogs [26]. This fact is extremely concerning when testing apparently healthy infected dogs as further investigations will not be performed and therefore infection will not be detected.

The limitations of the study are that, even as the study was expected to collect information from different countries, a limited number of dogs from Portugal, Italy and Cyprus were included, so the information received was mainly from Spain. Furthermore, just a small sample of the vast dog population of Spain (> 7.5 million registered dogs) [35] was included and the use of preventive measures might be overestimated.

Conclusions

In conclusion, dog owners in Spain follow the veterinarian's recommendations for the use of preventive measures against *L. infantum* infection as endorsed by the published guidelines. Repellents were the preferred measure, while vaccines and Leisguard® were second line options. However, there are still dogs that do not use preventive measures in endemic regions. Regarding serological screening tools, there seems to be a preference for the use of rapid tests in the clinical setting to detect specific *L. infantum* antibodies while other types of tests such ELISA and IFI are less often employed.

The results of this study reinforce the need to sensitize owners about the importance of protecting dogs against the parasite and clinicians about the limitations that qualitative serological techniques can present in the diagnosis of seropositive animals in endemic areas.

Abbreviations

CAN: only Canileish® vaccine; CanL: canine leishmaniosis; CAN + LEI: Canileish® vaccine + Leisguard®; CAN + LEI + REP: Canileish® vaccine + Leisguard® + repellent; CAN + REP: Canileish® vaccine + repellent; DAT: direct agglutination test; ELISA: enzyme-linked immunosorbent assay; IFI: indirect immunofluorescence; LEI: only Leisguard®; LEI + REP: Leisguard® + repellent; LET: only Letifend® vaccine; LET + LEI + REP: Letifend® vaccine + Leisguard® + repellent; LET + REP: Letifend® vaccine + repellent; NON: no preventive measures applied; PCR: polymerase chain reaction; REP: only repellents applied.

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Authors' contributions

JH, LSG and CM prepared study protocols and datasheets. MB, JH, CM, CA and LSG contacted veterinary clinics to participate in the study. CA also collected cases for the study. MB performed case removal, statistical analysis and prepared tables and figures. MB and LSG contributed with data analysis and interpretation. MB wrote the manuscript. LSG revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

No ethics approval was needed for this study as only retrospective information was collected. Signed informed consent about information used was obtained from veterinarians.

Competing interests

JH and CM have financial competing interests as they receive a salary from Ecuphar veterinaria SLU which markets both Leisguard® and Leiscan®.

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Chapter 4

Serological survey of *Leishmania infantum* in apparently healthy dogs in different areas of Spain

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Abstract

Background: Canine leishmaniosis caused by *Leishmania infantum* is an endemic disease in Spain. The dog is considered the main reservoir and the detection of specific serum antibodies against *L. infantum* antigens has been the most used technique for diagnosing this infection. The LEISCAN® LEISHMANIA ELISA test is a commercialized enzyme-linked immunosorbent assay (ELISA) for the detection and measurement of canine anti-*Leishmania* serum antibodies. The aim of this study was to assess seroprevalence results of apparently healthy dogs in different areas of Spain using LEISCAN®.

Methods: Collection of sera from 5451 apparently healthy dogs was performed between 2020 and 2021 in different areas of Spain. Dogs were of adult age (≥12 months), were not previously diagnosed with clinical leishmaniosis or vaccinated against *Leishmania*, and did not present clinical signs. LEISCAN® was performed following the manufacturer´s protocol.

Results: The overall seroprevalence was 5.5%. The highest seroprevalences were found in the Southeast of Spain: Comunidad Valenciana (14%) and Región de Murcia (14%); while the lowest seroprevalences were found in Northern Spain: Galicia (1%), Navarra (2%) and Castilla y León (2%) (*p*-value<0.001).

Conclusions: The seroprevalence for *L. infantum* in apparently healthy dogs in Spain varied from almost no infection to being over 10%.

Keywords: canine, diagnosis, ELISA, leishmaniosis, seroprevalence.

Background

Canine leishmaniosis (CanL) caused by the protozoan *Leishmania infantum* is a zoonotic and endemic disease in Spain [1–3]. *L. infantum* is usually transmitted by the bite of a female phlebotomine sand fly following a digenetic cycle that alternates between two differentiated phases: (a) an extracellular and motile promastigote that colonizes the digestive tract of the vector sand fly, and (b) an intracellular and non-motile amastigote that colonizes the monocyte-macrophage system of the vertebrate host [4]. The dog is considered the main domestic and peridomestic reservoir for *L. infantum* infection in Spain [5,6], while other mammals such as wild canids [7], rodents [8] and lagomorphs [9] may be able to maintain a wild cycle.

Detection of serum specific antibodies against *Leishmania* has been the most frequently used technique for detecting infected dogs [10–12]. Furthermore, since a vaccine is available in Europe, serological screening is mandatory prior to vaccination

in dogs [13]. Several commercial serological techniques are available such as immunochromatographic tests, enzyme-linked immunosorbent assays (ELISA) and immunofluorescent antibody tests (IFAT) [10–12,14,15].

The LEISCAN® LEISHMANIA ELISA test is an enzyme immunoassay for the detection and measurement of canine serum anti-*Leishmania* antibodies [15,16]. Previous studies have evaluated LEISCAN® and obtained good diagnostic sensitivity and specificity [15,16].

The aim of this study was to assess seroprevalence results of apparently healthy dogs in different areas of Spain using LEISCAN®.

Methods

Dogs

Collection of sera from 5451 apparently healthy dogs was performed between June of 2020 and June of 2021 by veterinarians from 80 veterinary practices in different areas of Spain. The inclusion criteria of dogs enrolled were adult age (≥12 months), not have been previously diagnosed with clinical leishmaniosis nor vaccinated against *Leishmania*, and absence of clinical signs based on clinical history and a full clinical examination.

Detection of anti-*Leishmania* antibodies using LEISCAN®

LEISCAN® (Ecuphar veterinaria SLU, Spain) was performed to detect anti-*L. infantum* antibodies in serum following the manufacturer´s protocol. Briefly, samples were diluted using the dilution solution included in the kit and incubated for 10 min at room temperature in 96-well plates. Then, washes were performed five times with the diluted washing solution and, afterwards, 100 µL of conjugate were added in each well. After incubating the plate for another 5 min at room temperature, washes were repeated and 100 µL of substrate were added to each well. Finally, after an incubation of 10 min at room temperature in the dark, stop reaction solution was added to the plate and the results were read at 450 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China).

LEISCAN® results were calculated using the following formula: ratio sample = optical density (OD) sample/OD low control positive. Samples were classified following the protocol as positive (when the ratio sample was ≥1.1), dubious (when the ratio sample was ≥0.9 and <1.1) and negative (when the ratio sample was <0.9).

Statistical analysis

The statistical analysis was performed using the package Stats for the software R i386 3.6.1 for Windows, using t-test to compare the altitudes of the centres between the LEISCAN® results (positive or negative) and using Chi-square tests to compare seroprevalence between autonomous communities, the different areas of Spain (North, South, East and West) and the type of centre that collected the samples (veterinary practice or dog shelter). A *p*-value of <0.05 was considered statistically significant. Maps were created using the Free and Open Source QGIS 3.10.4 for Windows. Information about altitudes of the centres were collected from Google Earth Web (https://earth.google.com/web/).

Results

The overall seroprevalence and dubious results of the 5451 dogs and their geographical distribution are shown in Table 4.1. The highest seroprevalences were found in the Southeast of Spain: Comunidad Valenciana and Región de Murcia; while the lowest seroprevalences were found in Northern Spain: Galicia, Navarra and Castilla-León (Table 4.1) (Fig 4.1) (Chi-square: X^2=88.96, *df*=1, *p*<0.001).

The majority of centres that collected samples were veterinary practices (68/80, 85%), while only a few were dog shelters (12/80; 15%). These practitioners collected samples from 4733 apparently healthy dogs (86.8% of the total); while dog shelters collected 718 samples (13.2%). No differences in seroprevalence were detected between dogs from veterinary practices and dogs from shelters (*p*>0.05).

The mean altitude depending on the geographical distribution of the veterinary practices is also shown in Table 4.1. The highest altitudes were found in Castilla-León (over 800 m) and Aragón (over 600 m), while the lowest altitudes were found in Galicia (under 40 m), Islas Baleares (under 70 m) and Comunidad Valenciana (also under 70 m) (Table 4.1). No differences in altitude were detected when comparing between seropositive and seronegative dogs (*p*>0.05).

Table 4.1 Seroprevalence and dubious results of *L. infantum* infection classified by Spanish autonomous community.

Autonomous community (number of dogs)	Number of veterinary practices	Mean of the metres of altitude (±SD)	Percentage of seroprevalence (95%CI)	Percentage of dubious results (95%CI)
Andalucía (1234)*	13	468.8 (±287.4)	4.5 (3.4-5.9)	2 (1.3-2.9)
Aragón (516)	8	625.3 (±356)	6.7 (4.7-9.2)	1.6 (0.7-3)
Islas Baleares (189)	3	64.9 (±40.6)	7.4 (4.1-12.1)	3.7 (1.5-7.5)
Castilla-La Mancha (18)**	2	552.2 (±10)	41.2 (18.4-67.1)	5.6 (0.1-27.3)
Castilla y León (216)	3	873.2 (±259.4)	1.9 (0.5-4.8)	2.3 (0.8-5.3)
Cataluña (851)*	13	240.2 (±221.7)	3 (1.9-4.3)	0.8 (0.3-1.7)
Comunidad deMadrid (358)	7	599.4 (±45.9)	5.7 (3.5-8.6)	1.1 (0.3-2.8)
Comunidad Valenciana (325)	6	65.6 (163.9)	13.9 (10.3-18.2)	2.5 (1.1-4.8)
Extremadura (472)	4	298 (±89.8)	3.8 (2.3-6)	0.8 (0.2-2.2)
Galicia (235)	3	38.8 (±16.3)	0.9 (0.1-3.1)	0.4 (0-2.4)
Navarra (414)	6	362 (± 67.9)	2.4 (1.2-4.4)	0.2 (0-1.3)
País Vasco (35)**	1	500 (±0)	5.9 (0.7-19.7)	2.9 (0.1-14.9)
Región de Murcia (468)	8	254.7 (±176.9)	13.7 (10.7-17.3)	3.6 (2.1-5.8)
La Rioja (120)	3	383.3 (±66.7)	2.5 (0.5-7.1)	0 (0-3)
Total (5451)	80	373.3 (±294)	5.5 (4.9-6.1)	1.6 (1.3-2)

*The Spanish autonomous communities from which high numbers of dogs were included were Andalucía and Cataluña.

**The significance of results obtained in País Vasco and Castilla-La Mancha remains to be further studied due to the limited number of dogs collected in these regions.

Abbreviations: CI: confidence interval, SD: standard deviation.

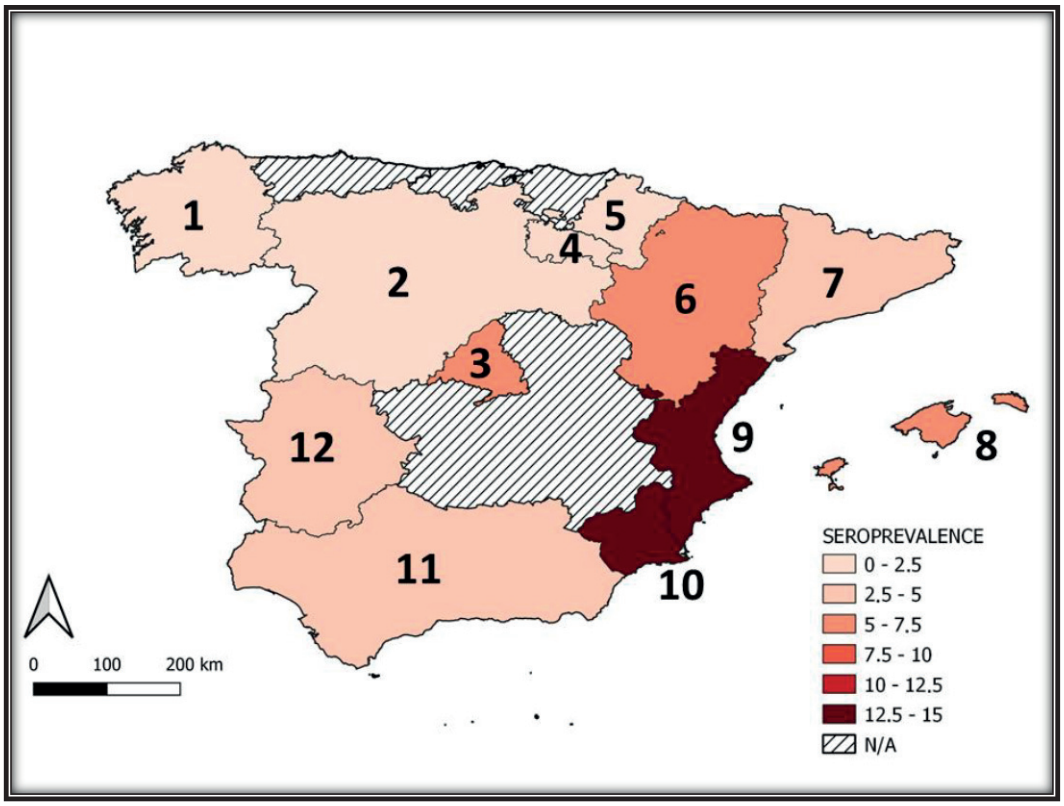

Fig 4.1 Geographical distribution of *L. infantum* seroprevalence in Spain: *1* Galicia, *2* Castilla y León, *3* Comunidad de Madrid, *4* La Rioja, *5* Navarra, *6* Aragón, *7* Cataluña, *8* Islas Baleares, *9* Comunidad Valenciana, *10* Región de Murcia, *11* Andalucía, *12* Extremadura.
Abbreviations: N/A: not applicable.

Discussion

The seroprevalence of *L. infantum* infection in dogs in Spain has been previously investigated and seroprevalences of around 10% between 2011 and 2020 have been described [1–3]. The seroprevalence in the present study (5.5%) is lower than expected; however, the reason could be explained by the inclusion criteria as only apparently healthy dogs with no clinical signs were included in the study. Furthermore, dubious results were not considered as positive results and, therefore, the seroprevalence was lower in all Spanish regions. In addition, it is likely that the differences observed on seroprevalences might also be due to variable diagnostic performance of previous studies performed [11,15,16].

In terms of specific investigated regions, previous serological surveys in Spain have documented similar results, detecting lower seroprevalences in the north of Spain and higher in the Southeast [1–3] which is also the nearest region to the Mediterranean. However, the results found in Islas Baleares are lower than expected (7.4%) when compared to previous studies that found seroprevalences of around 20% [2,3,17,18]. This could be explained with the same reasons as the lower overall seroprevalence: only

sampling apparently healthy dogs, not considering dubious results as positive and the test performed. Furthermore, the samples were collected all year around in the present study while in previous studies all the samples were collected in a specific time of the year [17,18] or, conversely, were collected for various years [2,3]. These results highlight the need to use preventive measures against *L. infantum* in any region of Spain [10,14] as well as to perform an annual health check-up and serology for the detection of anti-*Leishmania* antibodies in dogs living in *L. infantum*-endemic countries [13,14].

Interestingly, it has been described that owned dogs usually have a lower risk of infection than dogs living in dog shelters or kennels [19], which could be associated to environmental factors such as living outdoors, although it has also been described the opposite, being dogs living in kennels less likely to present *L. infantum* infection [20]. In the present study, no differences in seroprevalence were detected between owned dogs from veterinary practices and dogs from shelters. Another important factor could be that owned dogs are more frequently tested in the clinical setting (for clinical suspicion or annual health check-up) than dogs living in dog shelters (usually only sampled at kennel admittance) [19]. In endemic areas, it is appropriate to screen dogs for *L. infantum* antibodies at least every 6-12 months [13,21].

In recent studies, phlebotomine sand flies have been described to be able to maintain *L. infantum* infection in regions above 1,300 m [22], which are higher altitudes than previously reported [23,24]. In the present study, all sampled areas were under 1000 m of altitude and no differences were detected between different altitudes.

Conclusions

The seroprevalence for *L. infantum* as detected by the ELISA technique used in apparently healthy dogs in Spain varied from almost no infection in the Northern areas of Spain to being over 10% in the Southeast close to the Mediterranean basin. These results highlight the need to use preventive measures against *L. infantum* in any region of Spain.

Abbreviations

CanL: canine leishmaniosis; CI: confidence interval; ELISA: enzyme-linked immunosorbent assay; IFAT: immunofluorescent antibody test; OD: optical density.

Consent to participate

Study authorization was obtained from the Spanish authority, Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) with the authorization number 008/EPA-2383ESP.

Consent was obtained from the owner or the tutor of the dog(s) to collect the sample and perform Leiscan®.

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Chapter 5

Detection of specific antibodies against *Leishmania infantum* in canine serum and oral transudate using an in-house ELISA

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Abstract

Background: Canine leishmaniosis caused by the protozoan *Leishmania infantum* is a complex infection due to its variable clinical signs and laboratory findings. Therefore, a broad range of techniques is available for diagnosis. Testing for specific antibodies in serum is the most commonly used technique, although the testing of other body fluids, such as oral transudate (OT), can be an alternative as its collection is non-invasive and testing can be performed by untrained personnel. The aim of this study was to assess and compare the detection of *L. infantum*-specific antibodies in paired samples of serum and OT collected from apparently healthy dogs and dogs with clinical leishmaniosis using an in-house enzyme-linked immunosorbent assay (ELISA).

Methods: Serum and OT were collected from 407 dogs, which varied in breed, sex, age, lifestyle and clinical status, by many practicing veterinarians in Spain. The main geographical areas of sampling included Barcelona (n=110), Mallorca (n=94), Cadiz (n=54) and Asturias (n=47). The majority of infected dogs were apparently healthy (89.9%) while 41 presented clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection and subsequently diagnosed with leishmaniosis (10.1%). An in-house ELISA was performed to quantify the anti-*Leishmania* antibodies in serum and OT.

Results: The *L. infantum* infection rate determined by the in-house ELISA was 37.1% in serum samples and 32.7% in OT samples. Serum and OT ELISA results showed a positive correlation (Spearman's correlation coefficient r_s=0.6687, p<0.0001). The percent agreement between the serum and OT ELISA results was 84%, while agreement according to Cohen's kappa statistic (κ) was substantial (0.66) when all samples were analyzed. The highest percent agreement (92.1%) between both tests was found in dogs from low endemicity regions and from sick dogs, with both groups presenting almost perfect agreement according to Cohen's κ agreement test (0.84). Few seronegative dogs (n=23) tested positive by the OT ELISA. The agreement between serum and OT went from almost perfect to moderate when the geographical distribution and clinical status were analyzed.

Conclusions: The results of this study demonstrated an almost perfect to moderate agreement between OT and serum samples tested using the in-house ELISA. These results are particularly promising in sick dogs with high antibody levels while the results seem less optimal in apparently healthy dogs with low antibody levels.

Keywords: diagnosis, dog, leishmaniosis, oral transudate, serology, Spain.

Background

Canine leishmaniosis (CanL), a zoonotic and endemic protozoan disease caused by *Leishmania infantum*, is endemic in the Mediterranean basin [1,2]. Transmission is mostly through the bite of a female phlebotomine sand fly following a digenetic life-cycle which consists of two different phases: an extracellular and mobile promastigote in the sand fly, and an intracellular and non-motile amastigote in the mammalian host [3]. Other confirmed transmission routes, such as venereal [4,5] and transplacental [5,6] transmission and through blood transfusion, also occur [7,8]. The dog is considered to be the main domestic reservoir for *L. infantum* infection in the Mediterranean basin [2,9], while other mammals may be able to maintain a wild-life cycle [10–12].

The seroprevalence of *L. infantum*-infected healthy dogs in western Europe was 23% between 1971 and 2006 [13]. In Spain, the seroprevalence has been reported to be around 10%, although it can vary from 0 to 57% depending on the region [14]. Moreover, the prevalence of dogs that develop the clinical disease is usually lower than 10% [15,16]. CanL is a complex infection due to its variable clinical manifestations and wide spectrum of clinical signs and laboratory findings [9,17,18]. One factor underlying this variability is the dog's immune response, which requires a balance between inflammatory and regulatory responses to control *L. infantum* infection [19]. For example, neutrophils and macrophages play distinctive roles in the dog's initial immune ability to control the infection or to allow progression towards disease. Both neutrophils and macrophages phagocytize the parasite which can lead either to the elimination of the parasite through the production of reactive oxygen species (ROS), or to the survival of parasites within macrophages, leading to parasite persistence and dissemination [19]. T lymphocytes also play an integral role in preventing parasite growth and disease development as these T cells produce interferon gamma (IFN-γ) among other cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-2 or chemokines, which results in the differentiation, recruitment and activation of macrophages. However, as the infection progresses towards disease, there is a decrease in T cell proliferation and IFN-γ production and a lack of macrophage activation, resulting in a reduction of parasite elimination [19]. Many other factors can also affect the development of the disease, such as age, sex, and host genetics, among others. To date, however, the mechanisms responsible for the dog's resistance or susceptibility are still unknown [15,17].

Due to this complexity, CanL diagnosis often requires an integrated approach, including a clinicopathological examination and specific laboratory tests [9,15,18]. A full clinical history, thorough physical examination and several routine diagnostic tests, such as a complete blood count (CBC), biochemical profile, urinalysis and serum electrophoresis, are necessary when there is a suspicion of CanL [15,18]. In addition, several diagnostic techniques are available that enable a definitive diagnosis of *L.*

infantum infection, such as parasitological diagnosis (direct observation of the parasite), serological techniques (such as the enzyme-linked immunosorbent assay [ELISA] and indirect fluorescent antibody test [IFAT]) and molecular studies (such as quantitative PCR) [1,17,18,20]. Parasitological methods and molecular studies can detect the presence of the parasite, by direct observation or detection of DNA, respectively, while serological techniques detect serum anti-*Leishmania* antibodies. The diagnostic techniques must be used with full knowledge of the basis of each test and its limitations, as well as how to correctly interpret the results [15,17,18].

Interestingly, these diagnostic techniques can be performed using different types of samples, such as blood, serum, urine and other infected tissues [15,21–23]. The use of alternative samples, such as oral transudate (OT), hair or conjunctival swabs, has also been studied, with interesting results [24–27]. Immunoglobin A (IgA) can be found in OT as it is secreted in the salivary glands by plasma cells, along with immunoglobin G (IgG) and immunoglobin M (IgM), both of which are derived from plasma [28]. Specific antibodies against *L. infantum* have been previously detected in saliva samples of infected sick dogs only by means of a time-resolved immunofluorometric assay (TR-IFMA) [24,29–31]. However, to the authors' best knowledge, the detection of antibodies against *L. infantum* by ELISA in OT from apparently healthy dogs has not been previously documented. The advantages of using OT instead of serum include a non-invasive, cheap and painless collection of the sample, which can also be performed by untrained personnel.

The aim of this study was to assess and compare the detection of *L. infantum*-specific antibodies in paired samples of serum and OT from apparently healthy dogs and from dogs with clinical leishmaniosis, using an in-house ELISA.

Methods

Dogs

A minimum sample size of 310 dogs was calculated [32] using an expected seroprevalence of *L. infantum* infection of 10% [14] and a power of 80%. Both serum and OT samples from 407 dogs varying in breed, sex, age, lifestyle and clinical status were collected between January of 2018 and June of 2021 by several veterinarians practicing in different areas of Spain (Fig 5.1), a country endemic for CanL [14]. Dogs were chosen randomly from veterinary clinics, dog shelters and groups of hunting dogs. The clinical data recorded included the signalment and clinical status of all dogs (Table 5.1). None of the dogs were vaccinated against CanL. Dogs were considered young if they were aged ≤ 1.5 years, while dogs aged > 1.5 years were considered to be

adult. Dog characteristics, such as sex, age, breed and clinical status, and the significant differences between dogs are shown in Table 5.1.

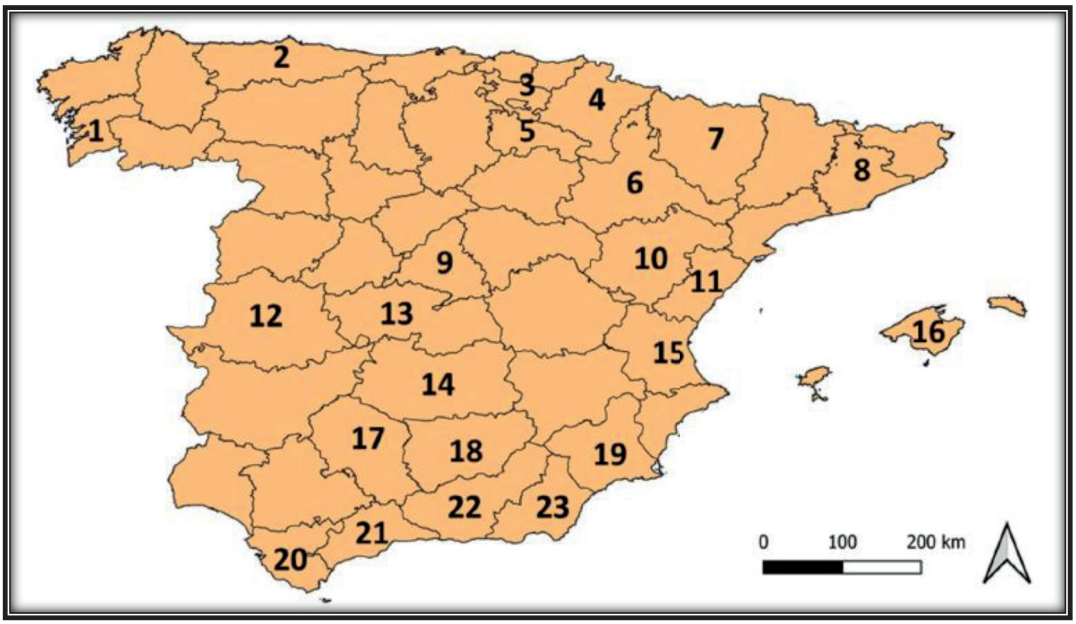

Fig 5.1 Geographical distribution of dogs sampled in Spain: 1 Pontevedra (n=5), 2 Asturias (n=47), 3 Álava (n=3), 4 Navarra (n=3), 5 La Rioja (n=1), 6 Zaragoza (n=10), 7 Huesca (n=1), 8 Barcelona (n=110), 9 Madrid (n=8), 10 Teruel (n=3), 11 Castellón (n=19), 12 Cáceres (n=3), 13 Toledo (n=1), 14 Ciudad Real (n=6), 15 Valencia (n=15), 16 Mallorca (n=94), 17 Córdoba (n=6), 18 Jaén (n=2), 19 Murcia (n=10), 20 Cádiz (n=54), 21 Málaga (n=4), 22 Granada (n=1), 22 Almería (n=1).

The main sampling areas included Barcelona (n=110 dogs), Mallorca (n=94), Cádiz (n=54) and Asturias (n=47) (Table 5.1). In the additional sampling areas, fewer than 20 dogs were sampled per area, with a total of 102 dogs (Fig 5.1). Dogs were also classified according to their clinical status. The majority of dogs were apparently healthy (89.9%) while 41 presented clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection and were diagnosed with leishmaniosis (10.1%) [9] (Table 5.1). Most dogs were sampled at the time of diagnosis and had not previously been treated with anti-*Leishmania* drugs, with the exception of three dogs that had been recently treated with allopurinol. Dogs from Asturias, an area with very low endemicity [14,33], were classified as negative controls, while samples from sick dogs that were diagnosed with leishmaniosis were classified as positive controls.

Table 5.1 Signalment and geographical distribution of dogs enrolled in the study.

Geographical distribution (number of dogs)	Sex (%, number of dogs)		Breed (%, number of dogs)		Most common breeds (%, number of dogs)	Age (%, number of dogs)*		Age median (years, min-max)[d]*	Clinical status (%, number of dogs)	
	Female[a]	Male[a]	Purebred[b]	Crossbreed[b]		Young[c]	Adult[c]		Healthy[e]	Sick[e]
Asturias (47)	51.1, 24	48.9, 23	89.4, 42	10.6, 5	English setter (17, 8) and Mastiff (10.6, 5)	8.9, 4	91.1, 41	5.5, 0.5-12	100, 47	0, 0
Barcelona (110)	46.4, 51	53.6, 59	20.9, 23	79.1, 87	German shepherd (4.5, 5) and Labrador retriever (3.6, 4)	25.5, 24	74.5, 70	4, 0.3-12	87.3, 96	12.7, 14
Cádiz (54)	44.4, 24	55.6, 30	29.6, 16	70.4, 38	Spanish sighthound (11.1, 6)	17.5, 7	82.5, 33	3.5, 0.5-16	100, 54	0, 0
Mallorca (94)	68.1, 64	31.9, 30	80.9, 18	19.1, 76	Ibizan hound (54.3, 51), Mallorca shepherd dog (5.3, 5) and Andalusian wine-cellar rat-hunting dog (5.3, 5)	38, 35	62, 57	3, 0.5-14	92.6, 87	7.4, 7
Total of provinces of origin (407)	51.4, 209	48.6, 198	46.7, 190	53.3, 217	Ibizan hound (12.8, 52), German shepherd (3.9, 16) and Mastiff (3.4, 14)	22.8, 79	77.2, 267	4, 0.3-16	89.9, 366	10.1, 41

[a]Mallorca had a higher rate of female dogs (Chi-square test: X^2=11.7, *df*=3, *p*=0.008), [b]Asturias and Mallorca had a higher rate of purebred dogs (Chi-square test: X^2=110.9, *df*=3, *p*<0.001), [c]Mallorca had significantly more young dogs than Asturias (Fisher's Exact test: *p*<0.0001) and Cádiz (Fisher's Exact test: *p*=0.025), while Asturias had significantly more adult dogs than Barcelona (Fisher's Exact test: *p*=0.024), [d]Mallorca dogs were significantly younger than Asturias (Mann-Whitney *U* test: *U*=2740, n_1=45, n_2=92, *p*=0.002) and Barcelona (Mann-Whitney *U* test: *U*=5106, n_1=94, n_2=92, *p*=0.032) dogs, [e]Barcelona and Mallorca had some sick dogs (Chi-square test: X^2=13.4, *df*=3, *p*=0.004) while all dogs in Asturias and Cádiz were apparently healthy.

*Age was not recorded in 2 dogs from Asturias, 16 dogs from Barcelona, 14 dogs from Cádiz, 2 dogs from Mallorca and 27 dogs from other Spanish regions.

Abbreviations: max: maximum; min: minimum.

Sampling

Blood samples were obtained by jugular or cephalic venepuncture and later centrifuged (Heraeus Labofuge 400R Centrifuge; Thermo Fisher Scientific, Waltham, MA, USA) at 789 g for 10 min to obtain serum.

OTs were collected by foam swabs (Ecouvillon PP; Dominique Dutscher, Bernolsheim, France) impregnated with hypertonic saline (NaCl 7.5%; B. Braun Melsungen AG, Melsungen, Germany) mainly as described previously [34] but with some modifications. The swabs were kept in the dog's mouth between the gum and the inner mucosa of the upper or lower lip for around 2 min and later centrifuged (Eppendorf Centrifuge 5418; Merck KGaA, Darmstadt, Germany) at 16,000 g for 1 min. After that, OTs were collected.

All samples, including both serum samples and OTs, were identified and stored at – 80 °C until further use.

Quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies

Serum ELISA

The in-house ELISA was performed on serum samples of all dogs studied as previously described [21]. Briefly, samples were diluted to 1:800 in phosphate buffered saline (PBS)-Tween with 1% dry milk and incubated at 37 °C for 1 h, following which they were washed three times (3 min each wash) with PBS-Tween and once (1 min) with PBS. The samples were then incubated for 1 h at 37 °C with peroxidase-conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.16 ng/µl. After incubation, the plates were washed three times with PBS-Tween followed by an additional wash with PBS. Then, *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) were added to the plates and the reaction was finally stopped with 5 M H2SO4. The results were read at 492 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China) and were defined as ELISA units (EU) in relation with a positive canine serum sample used as a calibrator set at 100 EU. The cut-off of the serum in-house ELISA was already determined to be 35 EU using the ELISA results of 80 dogs from a non-endemic area, as previously described [35]. Cut-off was established by the standard deviation (SD) method, consisting of multiplying the SD of the results by four and adding up the mean of the results obtained by the ELISA (mean + 4 SD). Serum was classified as high positive when the result was ≥ 300 EU, medium positive when the result was ≥ 150 EU and < 300 EU, low positive when the result was ≥ 35 EU and < 150 EU and negative when the result was < 35 EU [35].

Oral transudate ELISA

The in-house ELISA was performed on OTs of all dogs studied as previously described [21] with some modifications. OT samples were diluted to 1:5 in PBS-Tween with 1% dry milk and incubated at 37 °C for 1 h. Washes were performed as described for the serum samples, and peroxidase conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.5 ng/µl was added and then incubated at 37 °C for 1 h. Washes were repeated and *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) were added to the samples. The reaction was stopped with 5 M H_2SO_4. As described for the serum samples, the results were read in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd.) at 492 nm and were quantified as EU relative to a positive canine OT sample used as a calibrator set at 100 EU. The cut-off of the OT in-house ELISA was established using the ELISA results of 30 non-infected healthy Beagles. With the values of these 30 dogs, the SD was calculated and multiplied by 4, and then added up to the mean of all the results (mean + 4 SD), resulting in a cut-off value of 28 EU. The OTs were then classified as positive when the result was ≥ than 28 EU and negative when it was < 28 EU.

Statistical analysis

A descriptive analysis of all collected data was performed. Qualitative variables (sex [female/male], breed [purebred/mixed breed], age [young/adult] and ELISA results [positive/negative]) were assessed with a Fisher's exact test when only two groups were compared and with a Chi-square test when there were more than two groups. Quantitative variables (age, EU) were assessed using a non-parametric Mann–Whitney *U* test when two groups were compared (clinical status: apparently healthy/sick), and the Kruskal-Wallis *H* test was used when more than two groups were compared (geographical distribution). Spearman's correlation test was carried out to detect a relationship between ELISA quantitative results of the serum and OT.

The agreement between the interpretation of the results of serum and OT ELISAs was calculated by percent agreement and by Cohen's kappa statistic (κ) for agreement (kappa agreement test). When evaluating kappa agreement, the agreement was considered to be slight when it ranged from 0.00 to 0.20, fair when at range 0.21-0.40, moderate at range 0.41-0.60, substantial at range 0.61-0.80 and almost perfect at range 0.81-1.00 [36].

A *p*-value of < 0.05 was considered to be statistically significant. The Shapiro–Wilk test was performed to detect normal distribution of quantitative variables. Areas where < 20 dogs were sampled were excluded from the geographical distribution analysis. The statistical analysis was performed using the package Stats for R software version i386 3.6.1 for Windows. Cohen's κ statistic for agreement was calculated using free on-line GraphPad software (https:// www. graph pad. com/ quickcalcs/ kappa1/). Graphs were plotted using Graphad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Serum ELISA results

The rate of *L. infantum* infection determined by serum ELISA and the serological status of dogs (negative, low positive, medium positive or high positive) are shown in Table 5.2. The infection rate was significantly higher in adult dogs than in young dogs (42.7 *vs* 21.5%; Fisher's exact test: p=0.001), and lower in apparently healthy dogs than in sick dogs (29.5 *vs* 100%; Fisher's exact test: p<0.0001) (Table 5.2). No significant differences were observed between dogs of different sex and breed (Table 5.2). When dogs from different geographical locations were compared, a significantly lower rate of infection was found in Asturias when compared to the other locations (Chi-square test: χ^2=23.7, df=3, p<0.001) (Table 5.2).

Regarding the quantitative ELISA results shown in Table 5.3, adult and sick dogs presented significantly higher median EU values than young and apparently healthy dogs, respectively (Fig 5.2; Mann–Whitney *U* test: U=12,389, n_1=267, n_2=79, p=0.018; Mann-Whitney *U* test: U=829, n_1=366, n_2=41, p<0.0001). No significant differences were observed when different sexes and breeds were compared (Table 5.3). When groups from different geographical locations were compared (Table 5.3; Fig 5.3a), Asturias (3.7 EU) presented a significantly lower median value than Barcelona (11.4 EU), Cádiz (6.3 EU) and Mallorca (25.3 EU) (Kruskal-Wallis *H* test: χ^2=99.2, df=3, p<0.0001) while Barcelona and Mallorca had significantly higher median values than Cádiz.

Table 5.2 Rate of *L. infantum* infection, percent agreement and Cohen's Kappa agreement test between serum and OT ELISA results.

Classification (number of dogs)		Number of positive dogs (%)		Agreement (%)	Cohen's Kappa agreement number (interpretation)	95% CI of Cohen's Kappa
		Serum ELISA	OT ELISA			
Total of dogs (407)		149 (36.6)	133 (32.7)	345 (84.8)	0.66 (substantial agreement)	0.59-0.74
Sex	**Female (209)**	78 (37.3)	71 (34)	174 (83.3)	0.64 (substantial agreement)	0.53-0.75
	Male (198)	71 (35.9)	62 (31.3)	171 (86.4)	0.7 (substantial agreement)	0.59-0.8
Age*	**Young (79)**	17 (21.5)[a]	15 (19)[b]	69 (87.3)	0.61 (substantial agreement)	0.39-0.83
	Adult (267)	114 (42.7)[a]	103 (38.6)[b]	224 (83.9)	0.67 (substantial agreement)	0.58-0.76
Breed	**Purebred (190)**	63 (33.2)	63 (33.2)	158 (83.2)	0.62 (substantial agreement)	0.5-0.74
	Mixed breed (217)	86 (39.6)	70 (32.3)	187 (86.2)	0.7 (substantial agreement)	0.6-0.8
Geographical distribution	**Asturias (47)**	0 (0)[c]	3 (6.4)[d]	44 (93.6)	**	**
	Barcelona (110)	30 (27.3)[c]	23 (20.9)[d]	99 (90)	0.73 (substantial agreement)	0.58-0.88
	Cádiz (54)	9 (16.7)[c]	7 (13)[d]	48 (88.9)	0.56 (moderate agreement)	0.25-0.87
	Mallorca (94)	33 (35.1)[c]	28 (29.8)[d]	74 (79.6)	0.54 (moderate agreement)	0.36-0.72
Clinical status	**Sick (41)**	41 (100)[e]	37 (90.2)[f]	37 (90.2)	**	**
	Apparently healthy (366)	108 (29.5)[e]	96 (26.2)[f]	308 (84.2)	0.61 (substantial agreement)	0.52-0.7
	Negative control (Asturias) and positive control dogs (Sick) (88)	41 (46.6)	40 (45.5)	81 (92.1)	0.84 (almost perfect agreement)	0.73-0.95
Serological status	**High positive (26)**	26 (100)	26 (100)	26 (100)	**	**
	Medium positive (40)	40 (100)	34 (85)	34 (85)	**	**
	Low positive (83)	83 (100)	50 (60.2)	50 (60.2)	**	**
	Negative (258)	0 (0)	23 (8.9)	235 (91.1)	**	**
	Negative control (Asturias) and high and medium positive dogs (113)	66 (58.4)	63 (55.8)	104 (92)	0.84 (almost perfect agreement)	0.74-0.94

[a]Fisher's Exact test: p=0.001, [b]Fisher's Exact test: p=0.001, [c]Chi-square test: X^2=23.7, df=3, p<0.001, [d]Chi-square test: X^2=12.8, df=3, p=0.004, [e]Fisher's Exact test: p<0.0001, [f]Fisher's Exact test: p<0.0001.

*Age was not recorded in 61 dogs.

**The Cohen's Kappa agreement could not be calculated in the Asturias, the seropositive sick and the serological status groups because of the lack of positive to both tests or the lack of negative to both tests.

Abbreviations: CI: confidence interval; OT: oral transudate.

Table 5.3 Median of serum and OT EU according to the degree of reactivity to sera ELISA.

Classification of dogs (number of dogs)		Median of serum EU (min-max)**	Median of OT EU (min-max)**
Total of dogs (407)		17.7 (0-300)	14.9 (0-300)
Sex	**Female (209)**	22.3 (0-300)	13.8 (0-300)
	Male (198)	15.9 (0-300)	15.8 (0-300)
Age*	**Young (79)**	11.0 (1.8-300)[a]	9.9 (0-250.5)[b]
	Adult (267)	22.3 (0-300)[a]	18.1 (0-300)[b]
Breed	**Purebred (190)**	16.9 (0-300)	16.0 (0-300)
	Mixed breed (217)	18.2 (0-300)	13.6 (0-300)
Geographical location	**Asturias (47)**	3.7 (0-7.4)[c]	8.6 (0.2-39.9)[d]
	Barcelona (110)	11.4 (2.7-300)[c]	12.0 (0.2-300)[d]
	Cádiz (54)	6.3 (0-300)[c]	4.1 (0-300)[d]
	Mallorca (94)	25.3 (3.2-300)[c]	14.7 (2.2-166.5)[d]
Clinical status	**Sick (41)**	300.0 (39.3-300)[e]	111.7 (11.6-300)[f]
	Apparently healthy (366)	12.8 (0-300)[e]	12.9 (0-300)[f]
Serological status	**Negative (258)**	7.0 (0–34.7)	9.7 (0–76.4)
	Low positive (83)	59.2 (35–142.9)	38.1 (0–166.5)
	Medium positive (40)	210.4 (150.4–291.8)	80.4 (0–300)
	High positive (26)	300.0 (300)	160.9 (28.5–300)
	Total positives (149)	132.8 (35–300)	59.2 (0–300)

[a]Mann-Whitney *U* test: U=12389, n_1=267, n_2=79, p=0.018, [b]Mann-Whitney *U* test: U=12863, n_1=267, n_2=79, p=0.003, [c]Kruskal-Wallis *H* test: χ^2=99.2, df=3, p<0.0001, [d]Kruskal-Wallis *H* test: χ^2=38.7, df=3, p<0.0001, [e]Mann-Whitney *U* test: U=829, n_1=366, n_2=41, p<0.0001 and [f]Mann-Whitney *U* test: U=1461, n_1=366, n_2=41, p<0.0001.

*Age was not recorded in 61 dogs.

**Samples with a value of 300 EU may be higher as the spectrophotometer is only able to read up to 3 of optical density.

Abbreviations: EU: ELISA units; OT: oral transudate; max: maximum; min: minimum.

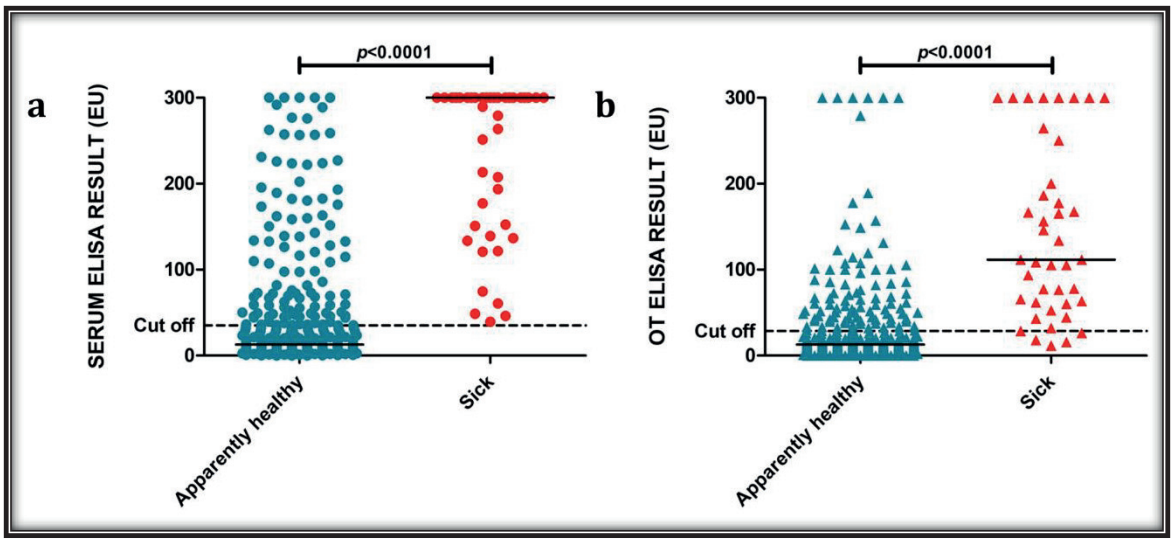

Fig 5.2 Antibody levels against *L. infantum* (EU) as determined by the in-house ELISA performed on serum (a) and OT (b) samples collected from dogs classified according to clinical status (apparently healthy vs sick). Horizontal solid black lines indicate the median. Horizontal black dashed lines indicate the cut-off: 35 EU in serum ELISA and 28 EU in OT ELISA. Abbreviations: ELISA: enzyme-linked immunosorbent assay; EU: ELISA units; OT: oral transudate.

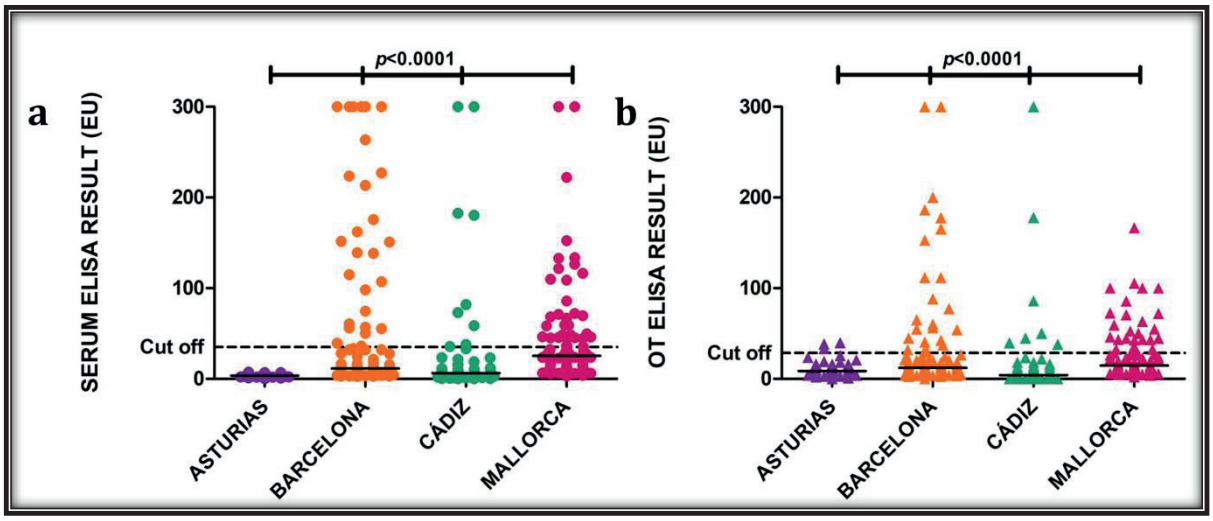

Fig 5.3 Antibody levels against *L. infantum* (EU) by the in-house ELISA performed on serum (a) and OT (b) samples collected from dogs classified according to geographical distribution. Horizontal solid black lines indicate the median. Horizontal black dashed lines indicate the cut-off: 35 EU in serum ELISA and 28 EU in OT ELISA.
Abbreviations: ELISA: enzyme-linked immunosorbent assay; EU: ELISA units; OT: oral transudate.

Oral transudate ELISA results

The rate of *L. infantum* infection determined on OT ELISA is shown in Table 5.2. Similar to the results for the serum samples, the rate of OT sample positivity was also significantly higher in adult (Fisher's exact test: p=0.001) and sick dogs (Fisher's exact test: p<0.0001) (38.6%) when compared to young dogs (19%) while it was lower in apparently healthy dogs than in sick dogs (26.2% *vs* 90.2%) (Table 5.2). No significant differences were observed in terms of sex and breed (Table 5.2). When comparisons were made between groups of dogs from different geographic locations, a significantly lower rate of infection was still found for dogs from Asturias compared to those from other locations (Chi-square test: χ^2=12.8, df=3, p=0.004) (Table 5.2).

Regarding the quantitative ELISA results shown in Table 5.3, as found in the serum results, adult and sick dogs presented a significantly higher mean EU value than young and apparently healthy dogs, respectively (Mann–Whitney U test: U=12,863, n_1=267, n_2=79, p=0.003; Mann–Whitney U test: U=1461, n_1=366, n_2=41, p<0.0001) (Fig 5.2b). No significant differences were observed between different sex and breed (Table 5.3). When groups of dogs from different geographical location were compared (Table 5.3; Fig 5.3b), Asturias (8.6 EU) and Cádiz (4.1 EU) presented a significantly lower mean EU value than Barcelona (12 EU) and Mallorca (14.7 EU) (Kruskal-Wallis H test: χ^2=38.7, df=3, p<0.0001).

Correlation and comparison between ELISA results for serum and OT samples

A positive correlation was established between the results of the in-house ELISA for the serum and OT samples (Spearman's correlation coefficient r_s=0.6687, p<0.0001) when all samples were studied (Fig 5.4). The positive correlation improved when only Asturias dogs (negative control) and sick dogs (positive control) were investigated (Spearman's correlation coefficient r_s=0.7479, p<0.0001) and also when only Asturias seronegative dogs and high and medium seropositive dogs were studied (Spearman's correlation coefficient r_s=0.7585, p<0.0001). On the other hand, when only low seropositive dogs were investigated, the positive correlation was lower (Spearman's correlation coefficient r_s=0.3079, p=0.005).

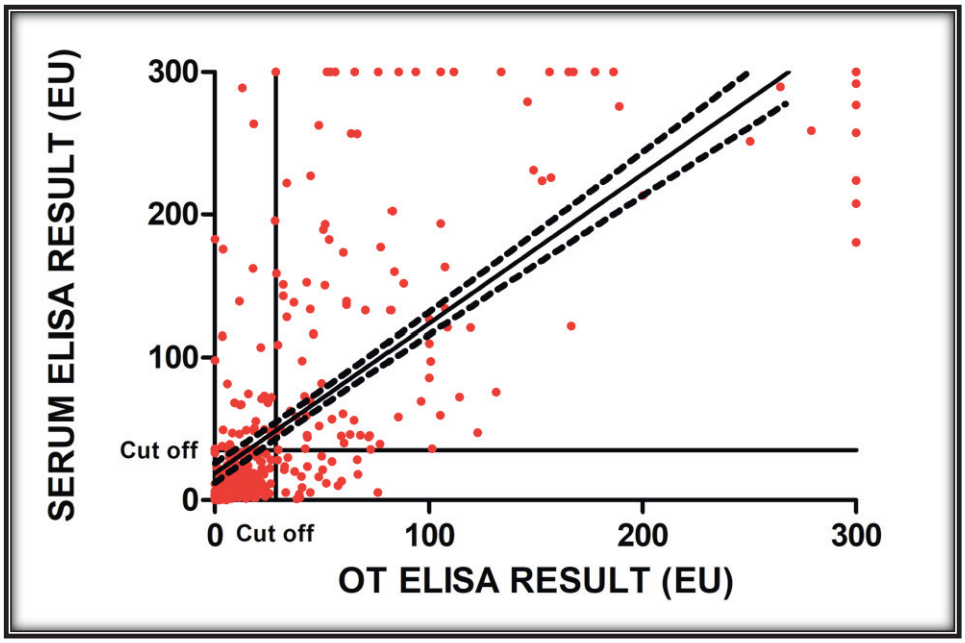

Fig 5.4 Spearman's correlation coefficient (r_s) for the serum and OT ELISA results (r_s[407]=0.6687, p<0.0001). Red filled circles indicate the individual results for each sampled dog. The horizontal solid black line indicates the cut-off: 35 EU in serum ELISA (Y-axis) and 28 EU in OT ELISA (X-axis). Abbreviations: ELISA: enzyme-linked immunosorbent assay; EU: ELISA units; OT: oral transudate.

Of the total of 407 dogs, 235 (57.7%) were negative by both serum and OT ELISA while 110 (27%) were positive to both tests. In contrast, there was disagreement regarding the remaining 62 dogs (15.3%). Six medium seropositive and 33 low seropositive dogs (9.6%) with a median of 55.3 EU (ranging from 35 to 288.9 EU) were negative by OT ELISA with a median of 12.4 EU (ranging from 0 to 27.2 EU) while 23 seronegative dogs (5.7%) with a median of 16.7 EU (ranging from 0.9 to 30.8 EU) were positive by OT ELISA with a median of 43.4 EU (ranging from 29.4 to 76.4 EU) (Fig 5.5). The percentage agreement and Cohen's kappa agreement between serum and OT ELISA results was substantial (0.66) when studying the whole group while it went from almost perfect to moderate depending on the classification studied (Table 5.2).

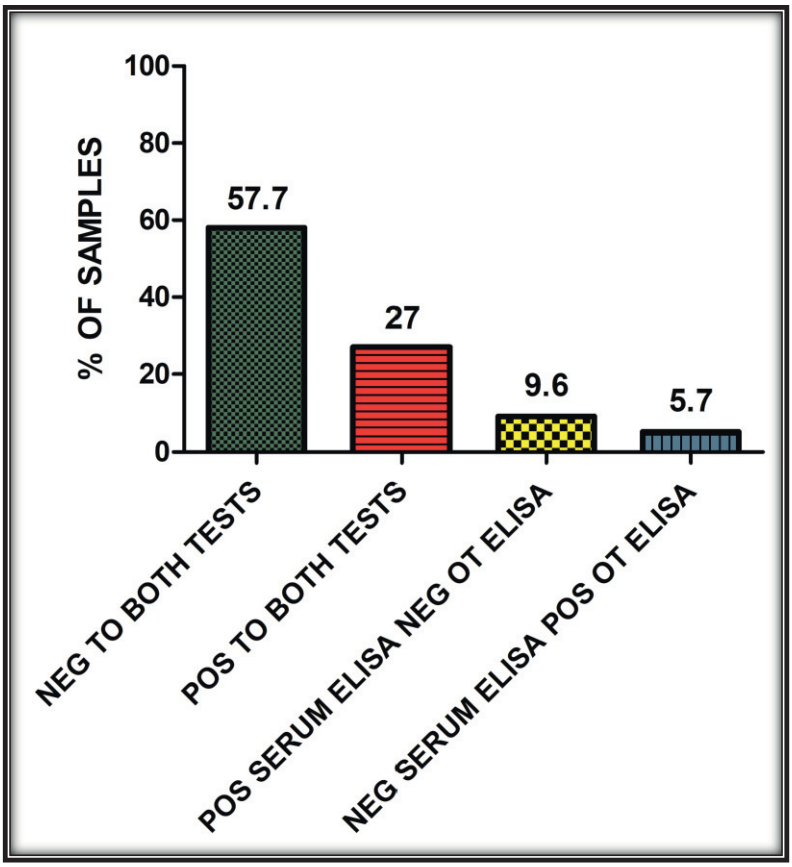

Fig 5.5 Proportion of positive and negative samples based on the results of both the serum and OT ELISAs.
Abbreviations: Neg: Negative; Pos: positive

Comparison of the EU values for the serum and OT samples according to degrees of reactivity is shown in Table 5.3. When comparing the OT EU, antibody levels were found to be significantly higher in OT samples with a high or medium positive EU value for the serum ELISA than in those with a low positive serum ELISA (Kruskal–Wallis *H* test: χ^2=43.2, *df*=2, *p*<0.0001).

Discussion

A quantitative in-house ELISA technique [21] was adapted in the present study to detect specific anti-*Leishmania* antibodies in OT canine samples and to assess the diagnostic performance of this ELISA. This ELISA is currently performed on serum samples to detect specific immunoglobulins as it has been proven that most dogs infected with an active disease show high levels of different isotypes of antibodies [9,18,37]. The presence of several types of immunoglobulins has also been studied in saliva [28]. IgA has been proven to be present in saliva as it is secreted in the salivary glands by plasma cells, and plasma-derived antibodies have been found, such as IgG and IgM [28]. Specific canine anti-*Leishmania* antibodies have also been documented in oral fluid samples by

using a TR-IFMA [24,29–31], which is a technique that has shown a broader range of detection of antibodies in serum than ELISA. These studies showed great success at discriminating between seropositive and seronegative dogs with no overlapping in terms of evaluating IgG2 [24,29–31]. However, the authors of these studies were not successful at correctly differentiating seropositive dogs from seronegative based on IgA evaluation [24,29–31]. These studies provided the first evidence of the potential of oral fluid for the quantification of anti-*Leishmania* IgG2 to diagnose CanL [24,29–31]. Nonetheless, no studies have evaluated the ability to detect anti-*Leishmania* antibodies by using a quantitative in-house ELISA technique in OT samples until now. Additionally, the first study performed on oral fluid samples for the diagnosis of CanL was carried out on a very homogeneous group of dogs, using dogs with advanced clinical leishmaniosis and high antibody levels [24], while in the present study, dogs with subclinical infection and low antibody titers were also included.

In the present study, the agreement between the qualitative interpretation of serum and OT ELISA results was evaluated using two methods: (1) percent agreement and (2) agreement according to the kappa agreement statistic. The percent agreement is easy to calculate and can be interpreted directly, but it does not take into account the agreements made by chance [38]. On the other hand, Cohen's kappa agreement statistic is a statistical value useful for assessing inter-rater or intra-rater reliability and takes into consideration the possibility of chance [38]. A Cohen's kappa agreement of > 0.80 is needed to be able to validate a new test [38]. When Cohen's kappa agreement was interpreted for the 407 dogs, a substantial agreement of 0.66 was found. As stated earlier in this text, this agreement is not sufficient to affirm that OT can be used to correctly differentiate between seropositive and seronegative dogs by means of an in-house ELISA. However, a high number of dogs in this study presented subclinical infection and low seropositive antibody levels, which is a likely explanation of why the agreement was lower than found in previous studies where the dog populations studied were mostly sick dogs with advanced clinical leishmaniosis [24,31]. When Cohen's kappa agreement was obtained only for seronegative dogs from Asturias (a low endemicity area) and for sick dogs with clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection, an almost perfect agreement of 0.84 was obtained. The same result (0.84) was found when Cohen's kappa agreement was obtained for seronegative dogs from Asturias and seropositive dogs with high or medium levels of antibody levels. These findings agree with those reported in previous studies [24,31] and highlight the usefulness of detecting antibodies against *L. infantum* in OT in dogs with clinical leishmaniosis or progressing towards disease.

When the percent agreement was evaluated, an agreement of 84.8% was found. The remaining samples from 15.2% (62) dogs showed disagreements between the serum and OT ELISA. Included in these samples that disagreed, 39 were from seropositive dogs (39/62 dogs; 62.9%) that were negative by the OT ELISA. There are several

reasons that could explain this disagreement in results from the OT and serum ELISA. First, there may be a lesser ability to detect seropositive dogs with a low serum antibody, as detected when comparing the Cohen's κ agreement statistic described above. This seems to be the most plausible reason as when only seronegative dogs from Asturias and sick dogs with clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection were studied, the percent agreement increased to 92.1%. A similar result, i.e. 92%, was obtained when the results from only seronegative dogs from Asturias and seropositive dogs with high or medium levels of antibody levels were considered. This result was to be expected as the sick group presented a higher proportion of high serum antibody levels compared to the apparently healthy group which had a higher proportion of low antibody levels. Another explanation could be a lack of homogenous OT sample collection, as even if untrained personnel can perform this procedure, it is difficult to perform correctly if the standardized protocol is not followed as described [39]. For example, if the impregnated swabs were not kept in the mouth of the dog for at least 2 min, insufficient OT could have been absorbed. As the samples in this study were collected by several veterinarians, even though a standardized protocol was recommended and agreed to, we could not confirm that all samples were always collected in a similar manner. On the other hand, of these 62 disagreements, 23 seronegative dogs (23/62 dogs; 37.1%) turned out to be positive in the OT ELISA. These results were unexpected. One possible explanation is that sand flies mainly feed on skin areas with very little hair, such as the face [15], which could lead to a local expression of parasite-specific immunoglobulins before the parasite disseminates systemically. A second possibility is that there may be an as-yet unknown cross-reactivity with another pathogen, such as oral bacteria, in some dogs with poor dental hygiene and dental disease, such as gingivitis, stomatitis and periodontal disease. Further studies on the diagnostic performance of the OT ELISA are needed to evaluate this hypothesis.

When taking locations of origin into consideration, the percent agreement was higher in Asturias (93.6%), followed by Barcelona (90%), Cádiz (88.9%) and Mallorca (79.6%). In comparison, Cohen's kappa agreement was substantial in Barcelona (0.73), followed by Cádiz with a moderate agreement (0.56) and Mallorca, also with a moderate agreement (0.54).

Despite the OT showing a lower diagnostic value than serum according to the quantitative in-house ELISA used in this study, a good percentage of success was obtained for the OT samples. In addition, OT sample collection is easy, cheap, non-invasive and painless; consequently, OT could be of use in specific cases, such as dogs that do not have easy access to veterinary clinics, dogs that need continued follow-up or aggressive dogs that can only be touched by its owner.

Further studies are needed to increase the reliability of the results of the present study. First, an investigation of the OT quality must be performed to confirm the correct collection of the samples before performing OT ELISA. In addition, a group of dogs with poor dental hygiene and presenting dental diseases could be added to the study population to assess the possibility of poor dental health being a factor of false positivity by OT ELISA. Also, it would be also of interest to perform a longitudinal study of those dogs that were seronegative yet tested positive by OT ELISA, as well as those dogs that tested negative for the OT ELISA yet tested positive by the serum ELISA, to describe antibody kinetics. Finally, other techniques using OT could also be developed and improved. Even ELISA as a serological test has some limitations in terms of the detection of infection as it can detect antibodies elicited by *Leishmania* vaccines in dogs [17].

The seroprevalence of canine *L. infantum* infection was around 10% [14,33,40] between 2011 and 2020 in Spain, which is lower than the seroprevalence detected in the present study (36.6%). In terms of specific Spanish areas, Asturias has always presented one of the lowest seroprevalence rates [14,33,40], usually around 1%, while the rates from Cádiz and Mallorca are usually higher than 15% [14]. These results resemble those found in the present study, with low rates in Asturias (0%) and high rates in Cádiz (16.7%) and Mallorca (35.1%). Regarding the results found in Barcelona (27.3%), a previous study performed in 27 sick and 20 clinically healthy dogs in 2006 [41] documented a 65% seroprevalence of *L. infantum* in Barcelona, but no other studies in this area have been carried out in the last decade. However, seroprevalence rates of around 13% were detected in other areas of Catalonia [33,40]. Interestingly, the seroprevalence rates detected in this study seem to be slightly higher than those described in previous studies [14,33,40,41]. This could be related to the number of sick dogs included in the Barcelona (12.7%) and Mallorca (7.4%) groups. The incidence rate of human leishmaniosis in Spain was 0.62 cases per 100,000 inhabitants between 2005 and 2017, with cases mainly distributed throughout the Mediterranean region [42]. However, asymptomatic infections are also common in humans in Spain and Mediterranean basin countries as recently reviewed elsewhere [43].

We also detected higher serological rates of *L. infantum* infection in both adult and sick dogs. A high rate should be expected in sick dogs that have been already diagnosed with leishmaniosis and still present clinical signs and/or clinicopathological abnormalities [9,15]. In terms of age of dogs, previous studies have found that puppies (< 1 year old) have a lower rate of *L. infantum* infection than dogs aged > 1 year old [33,40] and that the risk of *Leishmania* infection increases with increasing age [40].

Conclusions

In conclusion, the present study demonstrates an almost perfect to moderate agreement between OT and serum samples using a quantitative in-house ELISA for *Leishmania* antibodies. These results are promising for the detection of infection in sick dogs with high antibody levels while they seem to be less optimal in apparently healthy dogs with low antibody levels. Further studies could improve OT serology and its reliability and value as a future diagnostic technique for *L. infantum* infection when compared with other diagnostic methods for CanL.

Abbreviations

CanL: canine leishmaniosis; ELISA: enzyme-linked immunosorbent assay; EU: ELISA units; IFN-γ: interferon gamma; IgA: immunoglobin A; IgG: immunoglobin G; IgM: immunoglobin M; OT: oral transudate; PBS: phosphate buffered saline; ROS: reactive oxygen species; SD: standard deviation; TR-IFMA: time-resolved immunofluorometric assay.

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Authors' contributions

MB, AAF, MMA and LSG collected serum and OT samples. MB and JV performed laboratory work (ELISAs). MB performed statistical analysis and prepared the figures. MB and LSG contributed with data analysis and interpretation. JV wrote the first draft of the manuscript. MB and LSG wrote the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical review and approval were waived for the study, due to non-invasive procedures (OT sampling) and use of residual samples (serum). A signed consent form was obtained from the owner or the person in charge of the dog(s).

Competing interests

The authors declare that they have no competing interests.

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Chapter 6

Effect of storage on nitro blue tetrazolium reduction test in dog blood samples

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Abstract

Background: The nitro blue tetrazolium (NBT) reduction test has been used for measuring metabolic activity of phagocytes of mammals. Activated neutrophils transform NBT into formazan in the cytoplasm. The NBT reduction test can detect activation of neutrophils in peripheral blood and is used to assess neutrophil function in dogs. However, the NBT reduction test is not used frequently in the clinical setting as samples should be processed after blood collection.

Objectives: The aim of this study was to evaluate the effect of storage on NBT reduction test in dog blood samples.

Methods: Residual blood samples of twenty-two dogs were included of different ages, breeds and sex. The buffy coat layer was separated from the blood and incubated with 0.1% NBT. The NBT reduction test was performed at 0, 24, 48 and 72 h after collection of blood. Samples were stored at 4ºC until the tests were performed. Blood smears were evaluated by ordinary light microscopy and NBT reduction rate was reported which represents the percentage of activated neutrophils. The NBT reduction rate was calculated after counting 300 neutrophils in each slide.

Results: The means of NBT reduction rate of neutrophils at 0, 24, 48 and 72 h were 8.3%, 8.5%, 8.7% and 7.8%, respectively. No significant differences were observed between time points.

Conclusions: This study showed that the NBT reduction test can be performed up to 72 h after collection of blood if refrigerated at 4ºC. This finding facilitates the performance of the NBT reduction test in the clinical setting.

Keywords: activated neutrophils, canine, formazan, metabolic measurement, NBT.

Background

The nitro blue tetrazolium (NBT) reduction test has been widely used for measuring metabolic activity of mammals and microbial cells [1–3]. The NBT is a soluble and colourless ditetrazolium salt that can be transformed into insoluble and blue-coloured formazan when reduced [1]. Consequently, the reduction of NBT has been used to measure reactive oxygen species (ROS) production in phagocytes [1].

For example, activated neutrophils and monocytes can reduce NBT into formazan inside the phagocytic vacuole by the NADPH-oxidase enzyme [1–3] and the amount of reduced NBT is directly proportional to the amount of ROS produced in the oxidative burst [4]. Then, the NBT reduction rate can be obtained by calculating the percentage of neutrophils and monocytes containing formazan in their cytoplasm by ordinary light

microscopy [2]. Thus, NBT reduction test can detect activation of neutrophils and monocytes in peripheral blood.

Furthermore, this test has been previously used to assess canine neutrophil function in leishmaniosis [3], monocytic ehrlichiosis [5], diabetes mellitus [6] and transitory immunosuppression following immunization with polyvalent vaccines [7]. However, the NBT reduction test is not used frequently in veterinary clinical setting probably due to the assay's protocol limitations which dictates that the samples should be processed within 2 to 6 h after collection of the sample [8].

Hence, the aim of this study was to evaluate the effect of storage at 4ºC of the samples up to 72 h on the results of NBT reduction test in dog blood samples.

Methods

Dogs

Residual EDTA blood samples of 22 dogs were included in this prospective study. Between one to six millilitres of blood were collected from the dogs by jugular or metatarsal venepuncture for routine laboratory tests. The dogs were from Catalonia (Spain) and were sampled in 2019 for an annual health check-up. Dogs belonged to private owners and written informed consent was obtained from the respective owners (including staff and student volunteers). This includes consent for use of any remaining materials from those initially obtained for diagnostic purpose. Therefore, ethical study approval was not needed due to the use of residual blood samples. A physical examination was performed for all dogs included in the study. Four dogs were considered sick due to dermatological clinical signs, compatible with clinical leishmaniosis [9] and they were also seropositive in a quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies [10]. The other 18 dogs were classified as apparently healthy.

Residual EDTA blood storage

After blood collection, residual EDTA blood samples were placed into separate Eppendorf tubes. One of the tubes was kept at room temperature and immediately used for NBT reduction test while the other three were stored at 4ºC until further use at 24, 48 and 72 h, respectively.

Nitro blue tetrazolium reduction test

The NBT reduction test was performed as described elsewhere [3] with some modifications. Blood was left at room temperature (between 20ºC to 25ºC) for 15 min before NBT reduction test was performed. Afterwards, blood was mildly agitated and

three 40 mm/20 µL hematocrit capillary microtubes (Servopax, Wiesel, Germany) were filled. Then, microtubes were centrifuged at 2910xg for 5 min (Fugevet+ GDC005, Nahita International LTD, London, UK) to obtain the buffy coat layer. After centrifugation, the buffy coat layer from the three microtubes was placed in an Eppendorf tube with an equal volume of 0.1% NBT solution (proportion 1:1) (N6876, Sigma-Aldrich Co., St. Louis, USA). The Eppendorf was mildly agitated and incubated for 15 min in a heater at 37ºC, and another 15 min at room temperature (between 20ºC to 25ºC). After incubation, three blood smears were obtained by placing 3 µL of NBT-stained blood on each slide. Each slide was subsequently stained with Diff-Quick (Sigma-Aldrich Co., ST. Louis, USA) and evaluated by ordinary light microscopy, first at 10 and 20x magnifications to scanner the slide and at 40x (Fig 6.1, Fig 6.2), 60x (Fig 6.3) and 100x (Fig 6.4) magnifications for cell counting. The results of the test were reported as NBT reduction rate which represents the percentage of activated neutrophils/monocytes. The NBT reduction rate was calculated after counting 300 neutrophils/monocytes in each slide, rejecting those aggregated or broken. Thus, the percentage was the number of activated neutrophils/monocytes, as defined by those containing dark blue formazan deposits, divided by the total number of neutrophils/monocytes and multiplied by 100. It is important to highlight that it is difficult to differentiate neutrophils from monocytes when there are blue formazan deposits.

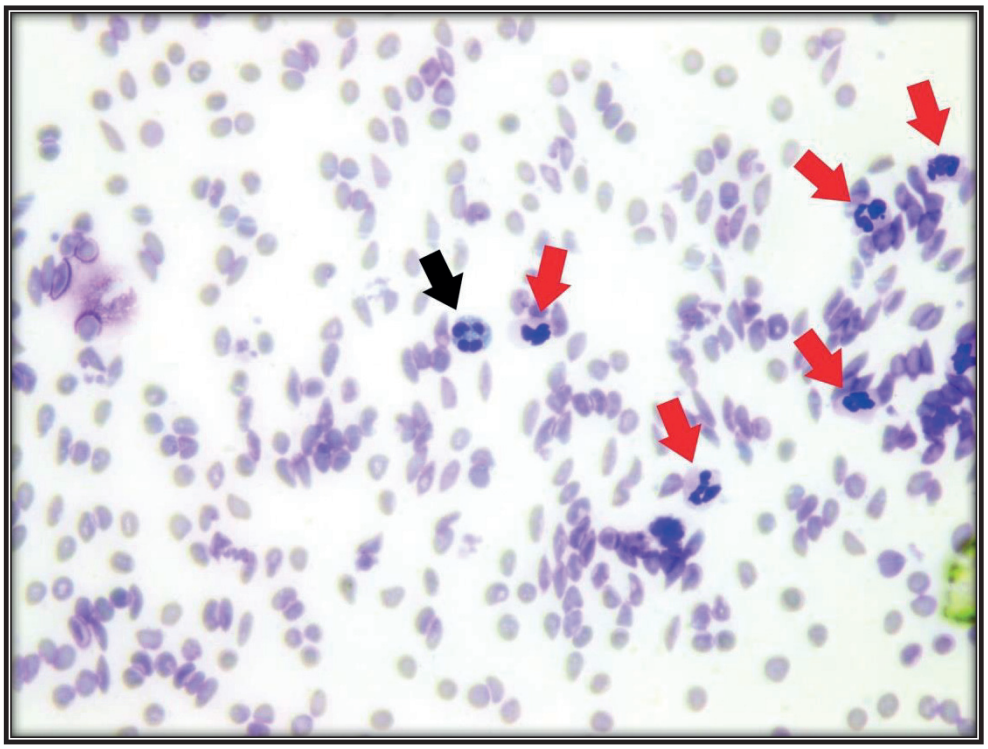

Fig 6.1 Several neutrophils without reduced formazan in the cytoplasm (red arrows) and another neutrophil/monocyte showing reduced formazan in the cytoplasm (black arrow) surrounded by red blood cells (x40 objective) stained with Diff-Quick. Formazan sometimes hinders differentiation between neutrophils and monocytes.

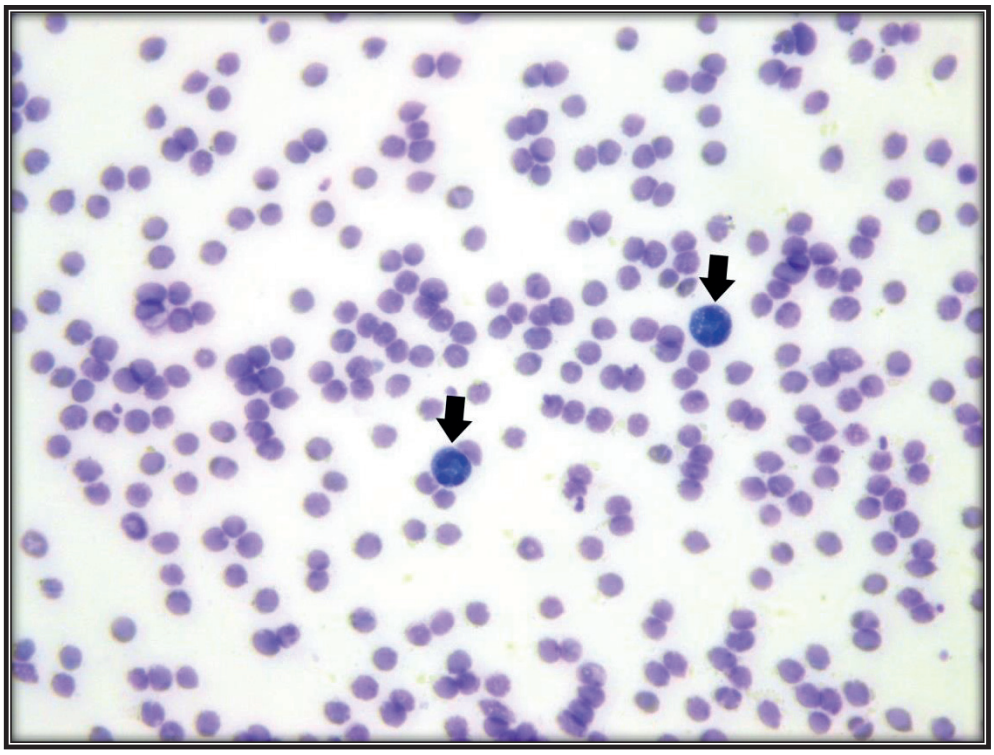

Fig 6.2 Two neutrophils/monocytes showing reduced formazan in the cytoplasm (black arrows) surrounded by red blood cells (x40 objective) stained with Diff-Quick. Formazan sometimes hinders differentiation between neutrophils and monocytes.

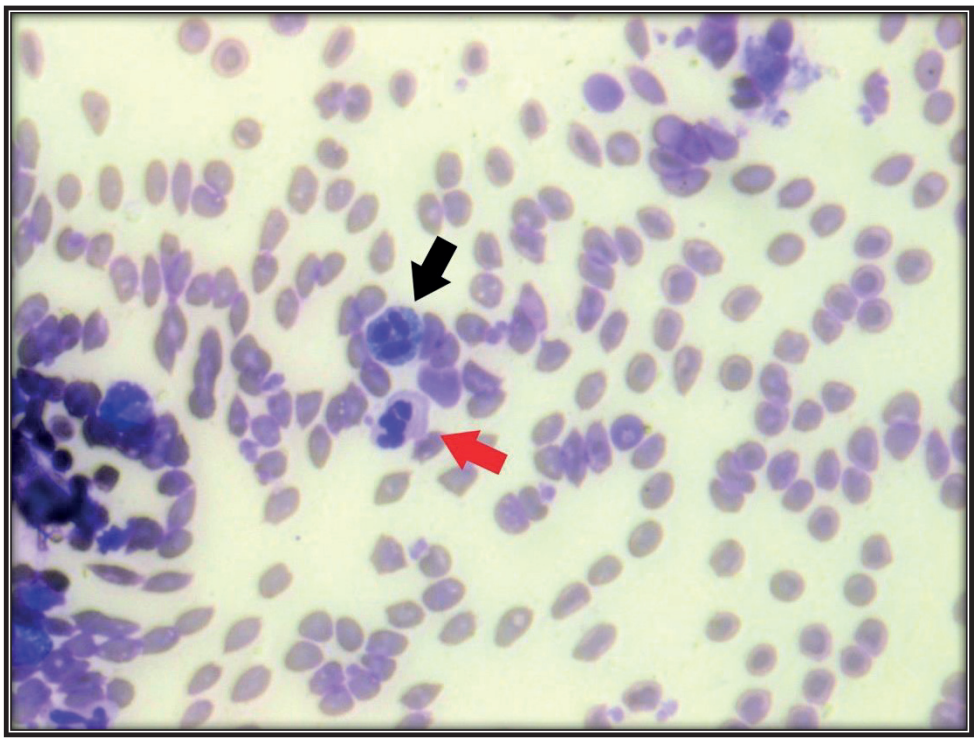

Fig 6.3 One neutrophil without reduced formazan in the cytoplasm (red arrow) and another neutrophil/monocyte showing reduced formazan in the cytoplasm (black arrow) surrounded by red blood cells (x60 objective) stained with Diff-Quick. Formazan sometimes hinders differentiation between neutrophils and monocytes.

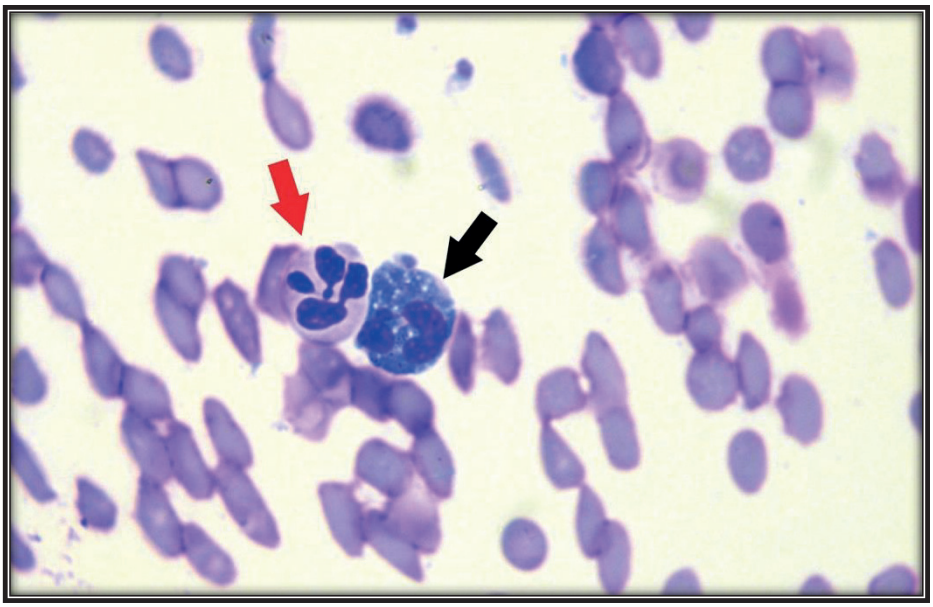

Fig 6.4 One neutrophil without reduced formazan in the cytoplasm (red arrow) and another neutrophil/monocyte showing reduced formazan in the cytoplasm (black arrow) surrounded by red blood cells (x100 objective) stained with Diff-Quick. Formazan sometimes hinders differentiation between neutrophils and monocytes.

The procedure described above was carried out at 0 h (fresh blood), and at 24, 48 and 72 h (blood stored at 4ºC) in 17 blood samples. In the other 5 cases, the procedure was not performed at 72 h due to lack of sample.

Statistical analysis

The statistical analysis was performed using the package Stats for the software R i386 3.6.1 for Windows, using an ANOVA for repeated measures to detect differences between time points (0 h, 24 h, 48 h and 72 h) and t-test was used to compare between groups (crossbreed or purebred, female or male, and healthy or sick). The Shapiro–Wilk test was performed to detect normal distribution of quantitative variables. A *p*-value of < 0.05 was considered statistically significant. Graphs were plotted using Graphad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Most of the dogs were classified as crossbreed (72.7%, 16/22) while the purebred dogs included two Labrador retrievers, two Golden retrievers, one Spanish greyhound and one Ibizan hound. The mean of age was 6 years and ranged from 1 to 13 years. Both sexes were included with 12 females (63.2%) and 7 males (36.8%). Information on age and sex was missing for 3 dogs.

The mean NBT reduction rate at 0, 24, 48 and 72 hours was 8.3%, 8.5%, 8.7% and 7.8%, respectively (Fig 6.5). No significant differences were observed between time points (Repeated measures ANOVA: F=1.58, *p*=0.2055).

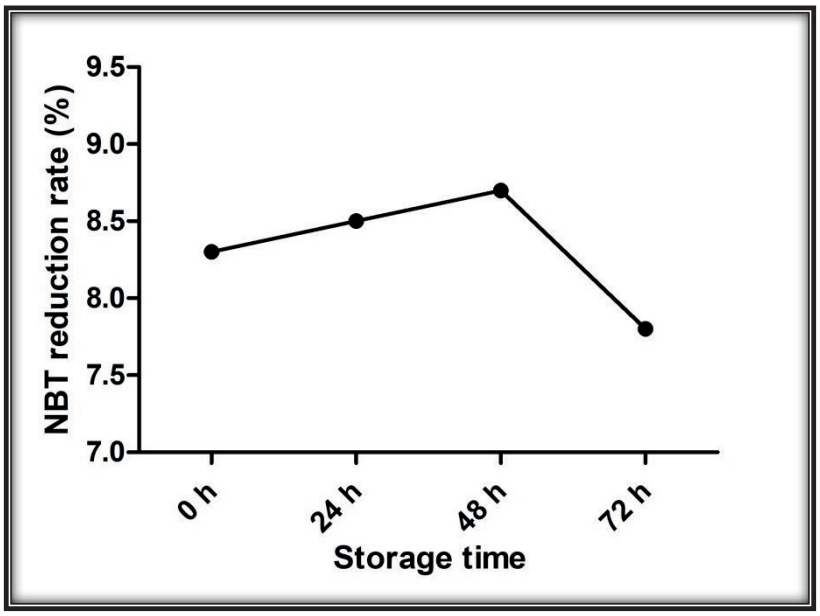

Fig 6.5 Change of NBT reduction rate of neutrophils of peripheral blood in each studied time point. Abbreviations: NBT: nitro blue tetrazolium.

The mean NBT reduction rate depending on breed, sex and clinical status at 0, 24, 48 and 72 hours is shown in Table 6.1. No differences in NBT reduction rates were found when comparing between crossbreed or purebred, female or male, and healthy or sick dogs ($p>0.05$).

Discussion

A previous study performed on human samples [11] described that storing blood samples for up to 8 h at 4ºC or for up to 4 h at 23ºC did not influence the test results. Additionally, when blood samples were stored at 23ºC for 8 h or more, the results of the test increased, but the reasons were not further investigated [11]. In the present study, the storing of the dog blood samples at 4ºC was longer, varying from 24 to 72 h, and the results were similarly not influenced by the time lapse. We believe that this is the first time that such result has been reported, as the NBT reduction test protocol demands sample processing within 2 to 6 h after blood collection [8]. This finding might enable the NBT reduction test to win a place among the standard laboratory tests available to the clinician to assess canine neutrophil and monocyte function in sick dogs [3,5–7]. For example, dogs with canine leishmaniosis presenting a mild disease have a higher NBT reduction rate than healthy dogs [3] and, thus, the NBT reduction test

could be used to detect improvement in these cases. In fact, any abnormalities or diseases that affect phagocytic activity in both inflammatory and bacterial processes could be studied using the NBT reduction rate. In human medicine, the NBT reduction rate had been used in numerous cases such as chronic granulomatous diseases [12,13], tuberculosis [14], viral meningoencephalitis [15] and liver abscess caused by amebiasis [16]. Recently, the NBT has also been used in several other cases such as to investigate the immune profile of human patients with fungus infection [17], gingival fibromatosis [18] and chronic obstructive pulmonary disease [19], the ROS production during the cell differentiation of HL-60 cells [20], and also the ROS production of biofilms [21] and of heat stressed whole blood cultures [22].

Interestingly, other factors such as the choice of anticoagulant and the type of blood sample (capillary or venous) have been previously investigated when performing the NBT reduction test and have been proven to affect the results [11,23–25]. For example, the use of heparin as anticoagulant could produce stimulation of oxidative metabolism and, thus, increase the percentage of activated neutrophils and monocytes in the sample [11,23]. Regarding the type of blood sample when comparing between capillary and venous blood, Randall et al. [24] reported no differences in NBT reduction rate between capillary and venous blood in healthy patients. However, in another study [25], the proportion of activated neutrophils and monocytes was significantly lower in capillary blood than in venous blood in sick patients with increased proportion of NBT rate. Unfortunately, in the present study, only peripheral blood was collected from dogs and investigated with NBT reduction test, thus, it was not possible to investigate the differences between peripheral and capillary blood in canine samples.

Furthermore, dog characteristics such as breed, sex and clinical status do not seem to affect the NBT reduction rate during storage, although more information should be included to confirm these results.

Although the present study reports interesting results regarding the NBT reduction test in dogs, some limitations must be considered. Further studies should include a higher number of dog samples, specifically samples from dogs with diseases that could affect the number of activated neutrophils and monocytes such as dogs with canine leishmaniosis [3], monocytic ehrlichiosis [5] or diabetes mellitus [6]. Moreover, further studies should evaluate if the NBT reduction test can be performed in blood samples stored longer than 72h. In addition, the use of imaging software could be helpful assessing the degree of color change of activated neutrophils and monocytes in canine blood samples as it has been used previously to assess the intensity of ROS production in mouse spermatozoa by NBT reduction test [26].

Table 6.1 NBT reduction test results.

Number of dogs		Mean NBT reduction rate (±SD)			
		0 h	24 h	48 h	72 h*
Total (22)		8.3 (±5.3)	8.5 (±5.4)	8.7 (±4.8)	7.8 (±4)
Breed	Crossbreed (16)	7.7 (±4.7)	7.7 (±4.9)	8.2 (±4.4)	7.1 (±3.7)
	Purebred (6)	10 (±6.7)	10.6 (±6.6)	10.1 (±6.1)	10.1 (±4.7)
Sex**	Female (12)	8.2 (±5.6)	8.6 (±6.3)	8.8 (±5.5)	7.6 (±4.5)
	Male (7)	8.6 (±4.2)	9.5 (±4)	9.6 (±3.6)	8.2 (±3.4)
Clinical status	Apparently healthy (18)	8.5 (±5.6)	8.7 (±5.9)	8.9 (±5.2)	7.9 (±4.4)
	Sick*** (4)	7.3 (±3.3)	7.4 (±2.7)	7.6 (±2)	7.4 (±2.6)

*the procedure was not performed at 72 h due to lack of sample in five dogs.
**information of sex was missing in three dogs.
***four dogs were considered sick due to dermatological clinical signs, compatible with clinical leishmaniosis and they were also seropositive in a quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies.
Abbreviations: NBT: nitro blue tetrazolium, SD: standard deviation.

Conclusions

This study showed that the NBT reduction test can be performed up to 72 h after collection of canine blood if correctly refrigerated at 4ºC. This finding supports the performance of NBT reduction test in the clinical setting.

Abbreviations

NBT: nitro blue tetrazolium; ROS: reactive oxygen species; SD: standard deviation.

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Chapter 7

Signalment, serological status and clinicopathological findings of *Leishmania*-seropositive apparently healthy dogs

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Abstract

Background: Canine leishmaniosis (CanL) is a disease caused by *Leishmania infantum* and is endemic in the Mediterranean basin. This infection is complex and can range from a total absence of clinical signs and clinicopathological abnormalities to a severe fatal clinical illness. The most frequently used technique for screening and diagnosing CanL is the detection of serum specific antibodies against *L. infantum* antigen. Numerous epidemiological serosurveys in apparently healthy dogs have been carried out in Europe. However, most of these studies lack assessment of clinical health status based on a thorough physical examination and routine laboratory tests. Therefore, the aim of this study was to evaluate signalment, serological status, and clinicopathological findings of *L. infantum*-seropositive apparently healthy dogs living in endemic areas.

Methods: Collection of blood from 212 apparently healthy dogs based on full physical examination was carried out by several veterinarians practicing in Spain (179 dogs) and Italy (33 dogs). Complete blood count (CBC), biochemistry profile, serum electrophoresis, urinalysis and endpoint in-house ELISA to quantify the anti-*Leishmania* antibodies were performed. All dogs enrolled were *L. infantum*-seropositive and were classified as healthy (n=105) or sick (n=107) depending on the results in routine laboratory tests.

Results: Most common clinicopathological findings in apparently healthy *L. infantum*-seropositive sick dogs were serum protein alterations (82%) including polyclonal hypergammaglobulinemia (74.5%), hyperproteinemia (71.7%), and decreased A/G ratio (64.1%), followed by renal alterations such as inadequate urinary specific gravity (USG) (46%) and proteinuria (35.6%) and lymphopenia (21.7%). Moreover, most of the sick dogs were classified in LeishVet stage IIa (55.1%) while stage IV had the lowest proportion of dogs (0.9%). Lower levels of red blood cells (RBC), hematocrit, hemoglobin, reticulocytes, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), lymphocytes, eosinophils, albumin and A/G ratio and also higher levels of total proteins, globulins, alkaline phosphatase (ALP), alpha2 globulins, beta globulins, and gamma globulin were associated with increased antibodies levels. Regarding age, older dogs tended to have higher urinary protein/creatinine ratio (UPC) and lower creatinine values. Furthermore, the healthy group had a higher proportion of low seropositive dogs (79%) than the sick group (30%) (p<0.0001).

Conclusions: This report describes the signalment and clinical data of apparently healthy *L. infantum*-seropositive dogs. Serum protein alterations were the most consistent finding followed by proteinuria and lymphopenia, and dogs with higher antibody levels showed a tendency for a higher degree of laboratory alterations.

Keywords: antibody level, canine leishmaniosis, Italy, Spain.

Background

Canine leishmaniosis (CanL) caused by the protozoan *Leishmania infantum*, is a vector-borne and zoonotic disease, which is endemic in the Mediterranean basin [1,2]. The dog is considered the main reservoir of *L. infantum* infection [3]. *Leishmania* completes its life cycle within two hosts, a phlebotomine sand fly vector, which transmits the promastigote form, and a mammal, where the amastigote form develops [3]. This canine disease is widely variable and generally non-specific. The most common clinical signs are skin lesions and lymphadenomegaly [3–7]. Other common clinical signs are reviewed elsewhere [8]. Diagnosis is achieved through an integrated approach considering signalment, history, clinical findings, and results of laboratory tests [3,8]. Laboratory diagnostic methods used to diagnose CanL can be divided into (1) basic diagnostic tests including a complete blood count (CBC), biochemistry profile, urinalysis, and serum protein electrophoresis (SPE), and (2) specific laboratory tests that will aid in the direct (cytology/histology, PCR, and parasite culture) or indirect identification of the parasite (anti-*Leishmania* antibody) [9,10]. Although non-specific, most common laboratory abnormalities in dogs with leishmaniosis are mild to moderate non-regenerative anemia, serum proteins imbalances, and proteinuria. In contrast, prevalence of renal azotemia is low despite the high percentage of dogs with proteinuria, indicating renal involvement [11–13].

Development of clinical disease depends on the immune response of the individual host, and two major opposite patterns have documented: (1) T cell-mediated protective immune response, where dogs remain infected but they do not progress to the development of clinical illness, and (2) marked humoral non-protective immune response with a reduced or absent T cell mediated immunity, where dogs develop overt clinical disease [10,14,15]. Therefore, a wide spectrum of clinical manifestations has been described in dogs with leishmaniosis, ranging from a mild papular dermatitis due to a specific cellular immunity and low humoral responses to a more severe clinical presentations characterized by renal damage due to immune complex deposition associated to a massive humoral response and high parasite burden [10].

Some factors such as age, sex, breed, nutrition, host genetics, coinfections and/or concomitant diseases, immunosuppressive conditions, cytokine environment, parasitic burden, virulence of strain, previous infections and method of transmission have demonstrated to affect the presentation of the clinical picture [16–18]. However, the mechanisms for resistance or susceptibility to CanL are not completely understood [10]. Age seems to be an important factor. While some researchers have documented a highest prevalence of leishmaniosis in dogs younger than 3 years and older than 8 years [19], others have documented more advanced stages of the disease in older dogs [4,20]. There is no agreement about the effect of sex to develop clinical leishmaniosis, although some authors have found an increased prevalence of infections in males [21].

Breed influence has also been documented; crossbreeds, Ibizan hound [22], Maremma sheepdog [21], Poodle and Yorkshire [23] seem to be less affected by clinical leishmaniosis while Boxer, German shepherd, Rottweiler and Cocker spaniel [5,24] have more susceptibility to be affected by the disease. Moreover, small breeds and long-coated breeds are at lower risk of developing clinical disease [21].

In endemic regions, where the density of the vector due to optimal climatological conditions, and hosts is high and there is lack of use of preventive measures [1], high prevalence of *L. infantum* infection in apparently healthy dogs exists [25]. Furthermore, apparently healthy dogs can be divided into two groups: 1) seropositive dogs but with no evidence of clinical signs and 2) seronegative but PCR positive dogs [1]. However, the prevalence of clinical illness is frequently lower than 10% [10]. According to a longitudinal study, apparently healthy PCR and antibody positive dogs living in endemic areas will develop clinical and clinicopathological signs over time [26].

Numerous epidemiological serosurveys in seropositive apparently healthy dogs have been carried out in Europe [6]. However, most of these studies lack assessment of clinical health status based on a thorough physical examination and routine laboratory tests. Therefore, the aim of this study was to evaluate signalment, serological status, and clinicopathological findings of seropositive apparently healthy dogs living in endemic areas of *L. infantum* infection.

Methods

Dogs

Collection of blood from 212 apparently healthy dogs based on a full physical examination was performed between September 2020 and June 2021 by several veterinarians practicing in different areas of Spain (n=179 dogs) and Italy (n=33 dogs). Blood samples were collected by jugular or metatarsian venipuncture and transferred immediately into different tubes: ethylenediaminetetraacetic acid (EDTA) tubes for CBC (Spain: XN1000, Sysmex, Italy: Siemens Advia 2120) and plain serum tubes for serum electrophoresis (Spain and Italy: Capillarys 3, Sebia), biochemistry profile (Spain: Vitros 5600, Ortho, Italy: Beckman Coulter AU 5800), which included urea, creatinine, total proteins, albumin, total globulins, albumin/globulin (A/G) ratio, alanine aminotransferase (ALT) and alkaline phosphatase (ALP), and the detection of anti-*Leishmania* antibodies. Urine samples were collected by “free flow” or cystocentesis for urinalysis which included the study of urinary specific gravity (USG), sediment analysis, urinary protein/creatinine ratio (UPC) (Spain: Vitros 5600, Ortho, Italy: Roche Cobas U601), and urine strip test (Beckman Coulter) detecting pH, proteins, blood, acetone, glucose, nitrites, urobilinogen, urobilin, and leukocytes. Hematological and

biochemical parameters were considered altered when they were outside the reference intervals. Serum protein electrophoretic patterns were defined following previously published guidelines [27]. Chronic antigenic stimulation was considered when normal to increased total proteins, normal to mild hypoalbuminemia, normal to mild hyperglobulinemia and polyclonal beta and/or gammaglobulinemia was present. When increased alpha1 or alpha2 globulins were also detected, an acute phase response with chronic antigenic stimulation pattern was considered.

All dogs enrolled were seropositive to *L. infantum* and classified into two different groups depending on the results of the routine laboratory tests: 1) seropositive healthy dogs (with absence of clinicopathological abnormalities) (n=105), and 2) seropositive sick dogs (with clinicopathological abnormalities) (n=107). Seropositive sick dogs were also classified by the LeishVet clinical staging [28].

Quantitative ELISA for the detection of *L. infantum*-specific antibodies

An in-house ELISA was performed on sera of all dogs studied as previously described [29]. Briefly, samples were diluted to 1:800 in phosphate buffered saline (PBS)-Tween containing 1% dry milk and incubated in *L. infantum* antigen-coated plates (20 µg/ml) for 1 h at 37ºC. Then, the plates were washed three times with PBS-Tween and once with PBS alone and incubated with Protein A conjugated to horseradish peroxidase (Peroxidase Conjugate Protein A; Merck KgaA, concentration 0.16 ng/µL) for 1 h at 37ºC. After that, the plates were washed again as described above. The plates were developed by adding the substrate solution *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KgaA). The reaction was stopped with 50 µl of 2.5M H_2SO_4. Absorbance values were read at 492 nm by an automatic reader (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China). All plates included the serum from a sick dog with confirmed infection as positive control and serum from a healthy dog as a negative control and all samples were analysed in duplicate. The result was quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and arbitrarily set at 100 EU. Sera were classified as: high positive when having a positivity percentage equal or higher than 300 EU; medium positive when having a positive percentage equal or higher than 150 EU and less than 300 EU; and low positive when having a positivity percentage lower than 150 EU and equal or higher than 35 EU [29].

All samples classified as medium or high positive were further studied using a two-fold serial dilution ELISA. Sera two-fold dilutions were started at 1:800 and continued for 7 to 11 further dilutions. The result was quantified as EU related to a calibrator arbitrary set at 100 EU, with an optical density (OD) value of one at 1:800 dilution. The mean values of the dilutions at which the OD was close to one were chosen for the calculation of the EU using the following formula: (Sample OD/Calibrator OD) x 100 x dilution factor [29].

Statistical analysis

A descriptive study of signalment and clinical data of the dogs was performed. Quantitative variables (age, weight, endpoint ELISA, numerical clinical data) were assessed using a t-test (in normal distributed data) or a Mann-Whitney *U* test (in non-normal distributed data) when two groups were compared (healthy or sick, crossbreed or purebred, male or female, young or adult,) while an ANOVA (in normal distributed data) or a Kruskal-Wallis *H* test (in non-normal distributed data) was used when more than two groups were compared (clinical staging). Qualitative variables (sex, breed, ELISA interpretation, categorical clinical data) were assessed using a Fisher's exact test (when there were two nominal variables, and the sample size was small) or a Chi-square test (when there were more than two nominal variables, and the sample size was big). A Spearman's correlation was also performed to investigate the relation between quantitative variables (age, weight, endpoint ELISA, numerical clinical data).

A p-value < 0.05 was considered statistically significant. The Shapiro-Wilk test was performed to detect normal distribution of quantitative variables. The statistical analysis was performed using the package Stats for the software R i386 3.5.1 for Windows.

Results

Signalment

Quantitative and qualitative characteristics of the dogs are displayed in Table 7.1. The most common breeds were Warren hound (6.6%), American Staffordshire terrier (3.3%), German shepherd (3.3%) and Labrador retriever (3.3%). No differences were found between seropositive healthy dogs and seropositive sick dogs between breed, sex, age and weight (Table 7.1).

In-house ELISA results and interpretation are also displayed in Table 7.1. There were significantly a higher percentage of low seropositive dogs included in the seropositive healthy group (79%) when compared to the seropositive sick group (30%) (Fisher's exact test: OR=10, $p<0.0001$) (Table 7.1). Furthermore, the median of EU in endpoint ELISA was significantly lower in seropositive healthy dogs when compared to seropositive sick dogs (Mann-Whitney *U* test: W=9269, $p<0.0001$) (Table 7.1).

Table 7.1 Qualitative and quantitative characteristics of the dogs.

Qualitative characteristics		Total (n=212) % (95% CI)	Seropositive healthy (n=105) % (95% CI)	Seropositive sick (n=107) % (95% CI)	*p*-value (Fisher's exact test)
Breed	**Crossbreed**	43.9 (37.1-50.8)	46.7 (36.9-56.7)	41.1 (31.7-51)	0.49
	Purebred	56.1 (49.2-62.9)	53.3 (43.3-63.1)	58.9 (48.9-68.3)	
Sex	**Female**	43.4 (36.6-50.4)	41 (31.5-51)	45.8 (36.1-55.7)	0.49
	Male	56.6 (49.6-63.4)	59 (49-68.5)	54.2 (44.3-63.9))	
ELISA interpretation at diagnosis	**High or medium positive**	44.8 (38-51.8)	18.7 (11.8-27.4)	70.1 (60.5-78.6)	<0.0001
	Low positive	55.2 (48.2-62)	79.4 (70.5-86.6)	29.9 (21.4-39.5)	
Stage LeishVet*	**II****	-	-	19.6 (12.6-28.4)	-
	IIa	-	-	55.1 (45.2-64.8)	
	IIb	-	-	9.3 (4.6-16.5)	-
	III	-	-	15 (8.8-23.1)	-
	IV	-	-	0.9 (0-5.1)	-
Quantitative characteristics		**Median (min-max)**	**Median (min-max)**	**Median (min-max)**	***p*-value (Mann-whitney *U* test)**
Age (years)		5 (0.5-14)	4 (1-14)	5 (0.5-12)	0.09
Weight (kg)		22 (3-62)	23 (6-62)	20 (3-58)	0.45
Endpoint ELISA (EU)		247 (51-61286)	137 (51-1181)	789 (75-61286)	<0.0001

*Only sick dogs can be classified in LeishVet staging.

**Some dogs (n=21) could not be classified as stage IIa or stage IIb due to lack of urinalysis.

Abbreviations: CI: confidence interval, EU: ELISA units; max: maximum; min: minimum; n: number of dogs.

Clinical data

The CBC findings in the different groups are displayed in Table 7.2. Seropositive sick dogs presented significantly lower red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and lymphocytes concentration compared to seropositive healthy dogs (Table 7.2).

The biochemistry panel, serum electrophoresis and urinalysis findings in the different groups are displayed in Table 7.3. Seropositive sick dogs presented significantly lower albumin, A/G ratio, creatinine in serum and sero-albumin when compared to seropositive healthy dogs (Table 7.3). On the other hand, seropositive sick dogs presented significantly higher total protein, globulin, ALP, alpha1 globulin, alpha2 globulin, beta globulin, gamma globulin and UPC values when compared to seropositive healthy dogs (Table 7.3).

Clinicopathological findings in seropositive sick dogs

The distribution of hematologic and biochemical clinicopathological findings in apparently healthy seropositive sick dogs is represented in Table 7.4. Based on clinicopathological alterations, 107 out of 212 dogs were classified as apparently healthy seropositive sick dogs. A total of 106 complete blood counts and biochemistry panels, respectively, and 88 urinalyses were reviewed.

Forty-two (39.6%) dogs presented at least one hematologic alteration. Most common hematologic clinicopathological findings were lymphopenia (21.7%) followed by leukopenia (7.6%) and anemia (6.6%) (Table 7.4). Half of the leukopenic dogs (3.8%) had lymphopenic leukopenia and of the remaining four dogs, three had concurrent neutropenic and lymphopenic leukopenia and one had neutropenic leukopenia (2.8% and 0.9%, respectively) (Table 7.4). Anemia was mild in all cases, except one that was classified as moderate, and was classified as normocytic and normochromic in all dogs. None of the anemic dogs showed an appropriate regenerative response (reference intervals for reticulocytes <150.1 x 10^9/L) [30]. Other less frequent hematologic findings (frequency less than 5%) were mature neutrophilia (4.6%), eosinophilia (3.7%), and altered platelet concentration (2.8% with thrombocytopenia and 1.9% with thrombocytosis) (Table 7.4).

Almost all dogs (n=105; 98.1%) presented biochemical abnormalities. Protein alterations were the most common clinicopathological findings in the seropositive sick dogs. Of the SPE available (n=106), almost all dogs (82.1%) had serum protein abnormalities. The most common serum protein electrophoretic changes observed were polyclonal hypergammaglobulinemia (74.5%) followed by hyperproteinemia

(71.7%), decreased albumin to globulin ratio (64.1%), and hyperglobulinemia (45.3%) (Table 7.4). Polyclonal hyperbetaglobulinemia and polyclonal hypergammaglobulinemia and hyperbetaglobulinemia alone were seen in 24.5% and 17.9% of the dogs, respectively. Hypoalbuminemia was only detected in 12 dogs (11.3%). All hypoalbuminemic dogs had increased alpha2 globulins and six of them had concurrent proteinuria. The most frequent serum protein electrophoretic pattern seen was consistent with chronic antigenic stimulation alone (65.1%) followed by 18 dogs (17%) with concurrent chronic antigenic stimulation and acute phase response.

Proteinuria was the second most common clinicopathological finding (Table 7.4). Proteinuria was present in 35.6% of the seropositive sick dogs in which UPC was available (n=87). However, renal azotemia was only present in 1.9% of the dogs. Other biochemical alterations observed were increased hepatic enzymes (13.2%), and decreased urea (12.3%) and creatinine (4.7%) concentrations.

Relationship between clinicopathological findings, signalment, antibody levels and clinical staging

Regarding numerical hematology parameters, the eosinophil concentration was the only parameter to show significant differences when compared with the sex of the dogs. Males showed significantly higher eosinophil concentrations than females (Mann-Whitney *U* test: W=4417, p=0.02). Regarding age, young dogs tended to have higher total leukocyte, band neutrophil and monocyte concentrations than adult dogs (Mann-Whitney *U* test: W=2549, p=0.03; W=3270, p=0.04; W=2625, p=0.02, respectively). Concerning breed, crossbreed dogs had a higher RBC (t-test: t=2.14, df=194.43, p=0.03), mean corpuscular volume (MCV) (t-test: t=-2.73, df=199.28 p=0.007) and MCHC values than purebred dogs (Mann-Whitney *U* test: W=6433, p=0.03). No other differences in hematological parameters were found regarding age, sex, breed or clinical staging. When Spearman's correlation between hematological numerical data and signalment and clinical staging was studied, lower levels of RBC, hematocrit, hemoglobin, reticulocytes, MCV, MCH, lymphocytes, and eosinophils were detected with increased antibodies levels, and lower leukocyte and band neutrophil numbers were found with increasing age (Table 7.5).

When hematologic categorical data was studied, the only hematological parameters significantly different were platelet numbers regarding clinical staging where thrombocytosis was found with more frequency in IIa LeishVet clinical stage (Chi-square: X^2=37.76, df=8, p<0.001). No other categorical hematological differences were found regarding signalment or clinical staging.

Table 7.2 Complete blood count (CBC) parameters of the dogs.

CBC parameters	Reference intervals* [30,31]	Total (n=212) Median (min-max)	Seropositive healthy (n=105) Median (min-max)	Seropositive sick (n=107) Median (min-max)	*p*-value
RBC (10^6/µL)	5.1-7.6	6.6 (4-9.1)	6.8 (5-9.1)	6.1 (4-8.3)	<0.0001[a]
Hemoglobin (g/dL)	12.4-19.2	16.2 (10.4-22.5)	17.1 (13.4-22.5)	15.2 (10.4-19.8)	<0.0001[b]
Hematocrit (%)	35-52	47 (29-62)	49 (36-62)	44 (29-59)	<0.0001[c]
MCV (fL)	60-77	71 (59-81)	71 (59-80)	71 (61-81)	0.27
MCH (pg)	21.9-26.3	24.6 (20.2-33.3)	24.9 (20.2-33.3)	24.2 (21.5-28.6)	0.02[d]
MCHC (g/dL)	34.4-38.1	34.4 (29.5-45.8)	35.2 (29.9-45.8)	34 (29.5-39.7)	<0.0001[e]
WBC (10^9/L)	5.6-20.4	9.5 (3.4-23.5)	9.4 (4.8-23.5)	9.6 (3.4-22.7)	0.91
Neutrophils conc (10^9/L)	2.9-13.6	6.1 (2.4-20)	6 (2.7-17.2)	6.2 (2.4-20)	0.52
Lymphocytes conc (10^9/L)	1.1-5.3	1.9 (0.2-4.7)	2.1 (0.4-4.5)	1.7 (0.2-4.7)	<0.0001[f]
Monocytes conc (10^9/L)	0.4-1.6	0.4 (0-2)	0.4 (0.1-1.6)	0.4 (0-2)	0.49
Eosinophils conc (10^9/L)	0.1-3.1	0.4 (0-4)	0.5 (0-4)	0.4 (0-3.3)	0.47
Platelet conc (10^3/µL)**	200-500	Adequate	Adequate	Adequate	-

[a]t-test: t=-6.1; [b]t-test: t=-6.8; [c]t-test: t=-5.9; [d]Mann-Whitney *U* test: W=4566; [e]Mann-Whitney *U* test: W=4062; [f]Mann-Whitney *U* test: W=4066.

*Reticulocytes (reference interval <150.1 x 10^9/L) and basophils conc (reference interval 0-200/µL) are not included in the table due to low numbers and non-significance.

**The majority of platelet con results were done qualitatively due to platelet aggregation.

Abbreviations: CBC: complete blood count; conc: concentration; max: maximum; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; min: minimum, n: number of dogs; RBC: red blood cells concentration; WBC: leukocytes concentration.

Table 7.3 Biochemistry profile, serum electrophoresis and urinalysis parameters of the dogs.

Parameters (units)	Reference intervals [32,33]	Total (n=212) Median (min-max)	Seropositive healthy (n=105) Median (min-max)	Seropositive sick (n=107) Median (min-max)	*p*-value
Total protein (g/L)	54-71	71 (54-117)	67 (54-83)	75 (59-117)	<0.0001[a]
Albumin (g/L)	26-33	33 (24-56)	34 (26-52)	33 (24-56)	0.002[b]
Globulin (g/L)	27-44	37 (26-81)	32 (26-41)	42 (29-81)	<0.0001[c]
A/G ratio	0.86-1.93	0.9 (0.4-1.7)	1.1 (0.7-1.7)	0.8 (0.4-1.5)	<0.0001[d]
ALT (U/L)	21-102	43 (12-1037)	44 (18-132)	42 (12-1037)	0.19
ALP (U/L)	20-156	47 (14-1271)	44 (14-208)	55 (20-1271)	0.001[e]
Creatinine (mg/dL)	0.5-1.5	0.9 (0.4-3.9)	0.9 (0.5-1.4)	0.8 (0.4-3.9)	0.01[f]
Urea (mg/dL)	21.4-59.9	35 (14-155)	35 (14-77)	34 (14-155)	0.81
Serum electrophoresis (g/L)					
- **Sero-albumin**	- 24.4-49.6	- 34.5 (19.1-49.1)	- 36.7 (25.5-45.4)	- 31.5 (19.1-49.1)	- <0.0001[g]
- **Alpha1 globulin**	- 1.7-4.5	- 3.5 (1.7-8.4)	- 3.4 (1.7-4.8)	- 3.7 (2-8.4)	- 0.008[h]
- **Alpha2 globulin**	- 3.8-10.2	- 7.3 (2.9-18.2)	- 6.5 (2.9-12.7)	- 8.1 (4.3-18.2)	- <0.0001[i]
- **Beta globulin**	- 8-18	- 13.2 (7.5-37.9)	- 12.3 (7.5-17.9)	- 14.7 (9.2-37.9)	- <0.0001[j]
- **Gamma globulin**	- 2.6-11.7	- 10.1 (4.4-60.6)	- 7.9 (4.4-11.9)	- 15.1 (5.3-60.6)	- <0.0001[k]
UPC	<0.5	0.1 (0-101.8)	0.1 (0.02 – 0.44)	0.2 (0-101.8)	0.007[l]
USG (g/L)	>1030	1034 (1007-1058)	1036 (1016-1058)	1031 (1007-1056)	0.06

[a]Mann-Whitney *U* test: W=9144; [b]Mann-Whitney *U* test: W=4252; [c]Mann-Whitney *U* test: W=10214; [d]t-test: t=-14.2; [e]Mann-Whitney *U* test: W=4656; [f]Mann-Whitney *U* test: W=4466; [g]t-test: t=-7.7; [h]Mann-Whitney *U* test: W=6673; [i]Mann-Whitney *U* test: W=8060; [j]Mann-Whitney *U* test: W=8188; [k]Mann-Whitney *U* test: W=10126; [l]Mann-Whitney *U* test: W=4913.

Abbreviations: A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine transaminase; max: maximum; min: minimum; n: number of dogs; UPC: urinary protein creatinine ratio, USG: urinary specific gravity.

Table 7.4 Distribution of the most common clinicopathological findings in seropositive sick dogs.

Clinicopathological findings	Number of dogs (%;95% CI)	Clinicopathological findings	Number of dogs (%;95% CI)
Hematological alterations (n=106)	42 (39.6; 30.3–49.6)	**Renal alterations**	40 (46; 35.2–57)
Anemia	7 (6.6; 2.7–13.3)	Proteinuria (n=87)	31 (35.6; 25.7–46.6)[a]
Lymphopenia	23 (21.7; 14.3–30.8)	Inadequate USG	40 (46; 35.2–57)
Leukopenia	8 (7.6; 3.3–14.3)	Isosthenuria (n=87)	10 (11.5; 5.7–20.1)[a]
Lymphopenic leukopenia	4 (3.8; 1–9.4)	Renal azotemia (n=106)	2 (1.9; 0.2–6.7)
Neutropenic leukopenia	1 (0.9; 0.02–5.1)	**Increased hepatic enzymes (n=106)**	14 (13.2; 7.4–21.2)
Neutropenic and lymphopenic leukopenia	3 (2.8; 0.6–8.1)	**Others (n=106)**	23 (21.7; 14.3-30.8)
Neutrophilic leukocytosis	1 (0.9; 0.02–5.1)	Low urea	13 (12.3; 6.7–20.1)
Neutrophilia	3 (2.8; 0.6–8.1)	High urea	6 (5.7; 2.1–11.9)[a]
Neutrophilia and lymphopenia	1 (0.9; 0.02–5.1)	Low creatinine	5 (4.7; 1.6–10.7)
Eosinophilia	3 (2.8; 0.6–8.1)	High creatinine	3 (2.8; 0.6–8.1)[a]
Eosinophilia and monocytosis	1 (0.9; 0.02–5.1)		
Thrombocytopenia	3 (2.8; 0.6–8.1)		
Thrombocytosis	2 (1.9; 0.2–6.7)		
Biochemical alterations (n=107)	105 (98.1; 93.4-99.8)		
Serum protein alterations (n=106)	87 (82.1; 73.4–88.9)		
Hyperproteinemia	76 (71.7; 62.1–80)		
Hypoalbuminemia	12 (11.3; 6-18.9)		
Hyperglobulinemia	48 (45.3; 35.6–55.3)		
Decreased A:G ratio	68 (64.1; 54.3–73.2)		
Polyclonal hypergammaglobulinemia	79 (74.5; 65.1–82.5)		
Polyclonal hyperbetaglobulinemia	26 (24.5; 16.7–33.8)		
Polyclonal hypergammaglobulinemia and hyperbetaglobulinemia	18 (17.9; 11.1–26.6)		
Protein electrophoretic patterns (n=106)	87 (82.1; 73.4–88.9)		
Chronic antigenic stimulation	69 (65.1; 55.2–74.1)		
Chronic antigenic stimulation and acute phase response	18 (17; 10.4–25.5)		
Normal	19 (17.9; 11.2–26.6)		

[a] Two of these dogs were interpreted as having renal azotemia.

Abbreviation: CI, confidence interval; USG: urinary specific gravity.

Table 7.5 Spearman's correlation between hematological parameters and age and ELISA units.

CBC parameters	Age		ELISA Units	
	r_s	*p-value*	r_s	*p-value*
RBC	0.02	0.78	-0.34	<0.0001
Hemoglobin	0.08	0.23	-0.38	<0.0001
Hematocrit	0.05	0.43	-0.43	<0.0001
Reticulocytes	-0.03	0.83	-0.57	<0.0001
MCV	0.05	0.5	-0.17	0.02
MCH	0.09	0.2	-0.21	0.002
MCHC	0.06	0.4	-0.08	0.26
WBC	-0.14	0.04	-0.08	0.22
Neutrophils conc	-0.1	0.17	-0.06	0.43
Band Neutrophils conc	-0.15	0.03	0.09	0.22
Lymphocytes conc	-0.11	0.12	-0.16	0.02
Monocytes conc	-0.1	0.13	0.12	0.08
Eosinophils conc	-0.13	0.07	-0.15	0.03
Basophils conc	-0.07	0.3	-0.22	0.001
Platelets conc*	-	-	-	-

*Most platelet results were done qualitatively due to platelet aggregation.

Abbreviations: CBC: complete blood count; conc: concentration; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; RBC: red blood cells concentration; WBC: leukocytes concentration.

Regarding numerical biochemistry parameters, the only differences found were ALT and UPC regarding sex and alpha2 globulin concentration regarding age. Females showed higher ALT levels (Mann-Whitney *U* test: W=4274, *p*=0.04) while males had higher UPC values (Mann-Whitney *U* test: W=3031.5, *p*=0.01). Adult dogs had higher values of alpha2 globulins (Mann-Whitney *U* test: W=747.5, *p*=0.05). No statistical differences were noted regarding breed. Regarding clinical staging, LeishVet stage II showed higher concentrations of total proteins and lower ALT levels, stage IIb had lower levels of total proteins, albumin, globulins, gamma globulins, A/G ratio and USG, and stage III showed higher levels of globulins, gamma globulins, UPC, ALT, and urea while had lower levels of albumin, A/G ratio and USG (Table 7.6). When Spearman's correlation between biochemical numerical data and signalment and clinical staging was studied, a positive correlation with total proteins, globulins, ALP, alpha2 globulins, beta globulins, gamma globulins and UPC, and negative with albumin and A/G ratio was found regarding EU. Regarding age, a positive correlation with UPC and ALT, and negative with creatinine concentration were found (Table 7.7).

When biochemical categorical alterations were studied, males showed a higher frequency of increased alpha2 globulins (Chi-square: X^2=3.99; *df*=1, *p*=0.05) and decreased A/G ratio (Chi-square: X^2=4.87; *df*=1, *p*=0.03). Regarding the clinical staging, serum protein alterations were found in all stages, however, a higher degree of hyperglobulinemia and hypergammaglobulinemia, decreased A/G ratio, increased alpha2 globulins, and renal alterations (isosthenuria, proteinuria, and renal azotemia) were observed with higher clinical stages (IIb and III) (Table 7.8). In addition, thrombocytosis and SPE pattern corresponding to chronic antigenic stimulation was more frequently observed in clinical stage IIa (Table 7.8).

Discussion

In endemic regions such as Spain and Italy, a high prevalence of *L. infantum* infection in apparently healthy dogs exists [25] while clinical CanL is usually developed by a limited proportion of the infected dogs [10,34]. According to a longitudinal study, apparently healthy but *L. infantum*-seropositive dogs will develop clinical and clinicopathological signs over time [26]. However, these apparently healthy *L. infantum*-seropositive dogs are fairly unknown, usually neglected in the clinical setting and few recommendations have been published regarding their monitoring or treatment [28,35]. In recent years and in endemic areas, due to an increased awareness of the disease and the use of advanced laboratory diagnostic tests that allow earlier diagnosis in the clinical setting, apparently healthy *L. infantum*-seropositive sick dogs without overt clinical signs but with the presence of laboratory abnormalities usually associated with leishmaniosis are a frequent finding. Therefore, this study describes the signalment and clinical data

of apparently healthy *L. infantum*-seropositive dogs and the most common clinicopathological findings in the population of apparently healthy *L. infantum*-seropositive sick dogs without evident clinical signs.

Mild to moderate non-regenerative anemia is a common laboratory finding in dogs with leishmaniosis [5,7,11,13,20]. Although multifactorial, decreased erythropoiesis due to chronic inflammation is thought to be an important pathogenic mechanism leading to anemia of chronic disease [12,36]. Other described factors involved in the pathogenesis of anemia in CanL are renal disease, chronic bleeding (epistaxis, skin lesion and gastrointestinal ulceration), myelodysplastic syndrome, decreased lipid fluidity of the erythrocyte membrane and less likely the production of anti-erythrocyte antibodies immune-mediated mechanism [37–40]. In the present study, the frequency of anemic patients was low and, anemia was classified as mild to moderate normocytic/normochromic non-regenerative in all anemic dogs. The prevalence of anemia in the seropositive sick dogs group is low (6.6%) in contrast with previous studies where anemia ranged between 40% to 70% [5,11,20,41]. However, our study is in concordance with others where anemia was a less frequent finding in those subclinical *L. infantum*-seropositive sick dogs than in dogs showing overt clinical signs [4,13,36,42]. We also found a negative correlation between EU and erythrogram-related parameters including RBC, hematocrit, hemoglobin, reticulocytes and MCV. These findings suggest that apparently healthy *L. infantum*-seropositive sick dogs present more severe clinicopathological findings with increased antibodies levels as previously reported [29].

Leukogram changes are considered infrequent and have shown a great variability between previously published studies [4,7,11,41]. Our results agree with other studies where a normal leukogram pattern is the most common observation [4,11,13,41]. Lymphopenia alone or with concurrent mild leukopenia or neutrophilia was the second most common leukogram change and the third more common clinicopathological alteration. These leukogram changes suggest a stress response due to increased endogenous glucocorticoids usually present in sick animals [7,11,43]. Since other less frequent leukogram changes were also detected, indicating a multifactorial origin of these alterations (enhanced recruitment in several organs, decreased production due to high bone marrow parasitism and inflammation); an individual evaluation of the leukogram changes in dogs with leishmaniosis is recommended to determine the principal ongoing pathogenic mechanism. In addition, we found a negative correlation between lymphocyte, monocyte, and eosinophil concentrations and EU. As published before, these results could be related to a bone marrow dysfunction associated with higher parasitism [12,44]. However, the lack of concurrent cytopenias at the same time and clinical significance of eosinopenia and monocytopenia, decreased lymphocyte numbers with increased antibody levels, could be related to a stress response or enhancement migration of lymphocytes to targeted organs due to *L. infantum* infection in dogs with more severe clinicopathological alterations.

Table 7.6 Relationship between numerical biochemistry parameters and clinical staging of the seropositive sick dogs.

Parameters (units)	Stage II (n=21) Median (min–max)	Stage IIa (n=59) Median (min–max)	Stage IIb (n=10) Median (min–max)	Stage III (n=16) Median (min–max)	*p-value* (Kruskal-Wallis *H* test)
Total protein (g/L)	81.5 (66–117)	74 (59-103)	68 (60–89)	75.5 (71–110)	0.02[a]
Albumin (g/L)	33 (24–47)	34 (26–56)	31 (26–35)	30 (25–39)	0.03[b]
Globulin (g/L)	45.5 (37–81)	40 (29–71)	36 (30–63)	46 (37–75)	0.001[c]
A/G ratio	0.68 (0.3–0.9)	0.82 (0.26–1.3)	0.86 (0.4–1.3)	0.52 (0.3–1.5)	0.04[d]
ALT (U/L)	31.5 (12–278)	44 (17–1037)	38 (18–88)	52 (21–228)	0.02[e]
ALP (U/L)	63.5 (20–107)	55 (20–1271)	48.5 (20–105)	51.5 (20–861)	0.71
Creatinine (mg/dL)	0.94 (0.6–1.2)	0.8 (0.43–1.39)	0.77 (0.6–1.2)	0.81 (0.47–1.9)	0.42
Urea (mg/dL)	35 (14–54)	32 (14–62)	37.5 (24–80)	46.5 (19–150)	0.05[f]
Serum electrophoresis (g/L)					
- **Sero-albumin**	- 31.1 (21.5–38.2)	- 33.3 (21.2–49.1)	- 31.6 (23–36.6)	- 27.2 (19.1–45.2)	- 0.02[g]
- **Globulins**	- 47.6 (37.8–86)	- 40.1 (28–80.8)	- 36.2 (27.4–66)	- 51.1 (29.8–81.5)	- 0.0006[h]
- **Alpha1 globulin**	- 3.9 (2–8.4)	- 3.6 (2–5.9)	- 3.7 (2.5–4.6)	- 3.5 (2.7–5.5)	- 0.93
- **Alpha2 globulin**	- 7.95 (5.2–18.2)	- 8 (4.3–18.1)	- 8.9 (5.4–10.2)	- 8.3 (6.5–13.6)	- 0.69
- **Beta globulin**	- 15.8 (12.2–37.5)	- 13.7 (9.2–37.9)	- 15.5 (9.4–18.3)	- 16 (10.3–24.8)	- 0.32
- **Gamma globulin**	- 19.1 (10.1–60.6)	- 14 (5.4–50.8)	- 10.2 (5.3–34.6)	- 21.9 (8.6–56)	- 0.004[i]
UPC	1.1 (0.2–1.9)	0.11 (0–0.6)	0.96 (0.6–1.8)	1.93 (0.1–101.8)	<0.0001[j]
USG (g/L)	1033.5 (1026–1041)	1034.5 (1007–1056)	1020.5 (1008–1042)	1024 (1008–1046)	0.02[k]

[a] X^2=10.2; [b] X^2=8.85; [c] X^2=16.19; [e] X^2=9.45; [f] X^2=7.99; [h] X^2=17.32; [i] X^2=1.41; [j] X^2=53.9; [K] X^2=11.73

[d,g] One-way ANOVA analysis: [d] F=3.82; [g] F=5.5

Stage IV is not included due to a small number of dogs (n=1)

Abbreviations: A/G: albumin/globulin ratio; ALP: alkaline phosphatase; ALT: alanine transaminase; max: maximum; min: minimum; UPC: urinary protein creatinine ratio, USG: urinary specific gravity.

Table 7.7 Spearman's correlation between biochemical parameters and age and ELISA units.

Parameters (units)	Age		ELISA Units	
	r_s	*p-value*	r_s	*p-value*
Total protein (g/L)	-0.07	0.31	0.4	<0.0001
Albumin (g/L)	-0.06	0.39	-0.22	0.001
Globulin (g/L)	-0.01	0.91	0.51	<0.0001
A/G ratio	-0.01	0.13	-0.55	<0.0001
ALT (U/L)	0.24	0.0004	-0.1	0.18
ALP (U/L)	0.13	0.07	0.14	0.04
Creatinine (mg/dL)	-0.18	0.007	-0.08	0.26
Urea (mg/dL)	-0.09	0.19	-0.06	0.4
Serum electrophoresis (g/L)				
- **Sero-albumin**	- -0.13	- 0.06	- -0.46	- <0.0001
- **Globulins**	- 0.03	- 0.64	- 0.53	- <0.0001
- **Alpha1 globulin**	- -0.03	- 0.69	- 0.11	- 0.12
- **Alpha2 globulin**	- 0.13	- 0.07	- 0.31	- <0.0001
- **Beta globulin**	- 0	- 0.96	- 0.16	- 0.02
- **Gamma globulin**	- 0.05	- 0.49	- 0.6	- <0.0001
UPC	0.15	0.04	0.2	0.007
USG (g/L)	-0.08	0.32	-0.05	0.48

Abbreviations: A/G ratio: albumin/globulin ratio; ALP: alkaline phosphatase; ALT: alanine transaminase; UPC: urinary protein creatinine ratio, USG: urinary specific gravity.

Table 7.8 Significant differences between categorical clinicopathological alterations and clinical staging.

Categorical alterations (number of dogs)	Stage II (n=21) Number of dogs (%)	Stage IIa (n=59) Number of dogs (%)	Stage IIb (n=10) Number of dogs (%)	Stage III (n=16) Number of dogs (%)	Stage IV (n=1) Number of dogs (%)	*p-value* (Chi-Square; *df*)
Thrombocytosis (n=2)	0 (0)	2 (3.4)	0 (0)	0 (0)	0 (0)	<0.001 (37.8; 8)
SPE pattern: Chronic antigenic stimulation (n=69)	15 (75)	40 (67.8)	4 (40)	10 (62.5)	0 (0)	0.004 (22.9; 8)
Hyperglobulinemia (n=48)	12 (60)	21 (35.6)	3 (30)	12 (75)	0 (0)	0.02 (11.5; 4)
Increased alpha2 globulins (n=14)	3 (15)	5 (8.5)	0 (0)	6 (37.5)	0 (0)	0.03 (11.1; 4)
Hypergammaglobulinemia (n=79)	19 (95)	44 (74.6)	4 (40)	12 (75)	0 (0)	0.009 (13.6; 4)
Decreased A/G ratio (n=68)	16 (80)	33 (55.9)	4 (40)	14 (87.5)	1 (100)	0.03 (10.81; 4)
Renal azotemia (n=2)	0 (0)	0 (0)	0 (0)	1 (6.3)	1 (100)	<0.001 (55.4; 4)
Increased creatinine (n=3)	0 (0)	0 (0)	0 (0)	2 (12.5)	1 (100)	<0.001 (46.4; 8)
Increased urea (n=6)	0 (0)	1 (1.7)	1 (10)	3 (18.8)	1 (100)	0.001 (27.8; 8)
Proteinuria (n=31)	1/2 (50)	4/58 (6.9)	10/10 (100)	15/16 (93.8)	1/1 (100)	<0.001 (64.5; 4)
Isosthenuria (n=10)	0/2 (0)	4/58 (6.9)	2/10 (20)	3/16 (18.8)	1/1 (100)	0.03 (10.7; 4)

Abbreviations: A/G ratio: albumin/globulin ratio; n: total number of dogs; SPE: serum protein electrophoresis.

A negative correlation between total leukocytes and band neutrophil concentrations with age was also observed, nevertheless, this finding may lack of clinical significance since young dogs tend to have increased white blood cells and band neutrophils numbers [45].

Hemostatic disorders such as epistaxis, hematuria, and hemorrhagic diarrhea have been reported with CanL [4,5,7,11]. Furthermore, these clinical signs have been associated with primary homeostasis defects (thrombocytopathy and/or vasculitis) and mucosal ulcerative lesions and appear to be unrelated to decreased platelet concentration [46]. In the present study, the frequency of thrombocytopenia was low (2.8%) in agreement with previous studies [11,13,41], but in contrast with other studies where the frequency ranged between 20-50% [5,7,47]. These controversial results could be explained by the findings reported previously where platelet numbers were reduced to a greater extent in those dogs with overt leishmaniosis where renal disease, bone marrow dysfunction, and inflammation are more frequently seen [48,49]. Also, thrombocytosis was infrequent in seropositive sick dogs (1.9%) as reported previously [11].

In concordance with previous reports, the most frequent clinicopathological alteration in our study was dysproteinemia usually characterized by the presence of hyperproteinemia secondary to hyperglobulinemia, specifically due to an increase in the gamma globulin and/or beta globulin proteins and less frequently alpha2 globulins, and a decreased A/G ratio [5,7,11,13,20,42,46,49]. In addition, our study showed that the most frequent serum electrophoretic pattern observed in the seropositive sick group was consistent with chronic antigenic stimulation. These findings are related to the exaggerated humoral response with a polyclonal proliferation of B lymphocytes and the consequent production of non-protective anti-*Leishmania* antibodies seen in those diseased dogs with leishmaniosis [6,50]. Moreover, in contrast with other studies, the frequency of hypoalbuminemia was low (11.3%) and mostly associated with increased alpha2 globulins suggesting a probable ongoing active inflammation [5]. However, hypoalbuminemia related to or exacerbated by the presence of proteinuria should also be considered. Also, the low frequency of liver and renal involvement observed in our study could also influence the lesser degree of hypoalbuminemia [5]. In addition, as expected, since an uncontrolled humoral response will reflect on proteins concentrations, a positive correlation between total proteins, globulins, alpha2 globulins, beta and gamma globulins and EU, and a negative correlation between albumin and A/G ratio and EU was found.

Renal disease is also a frequent feature in dogs diagnosed with leishmaniosis [51–53], being renal azotemia and proteinuria the most common laboratory abnormalities indicating renal involvement. Although a high prevalence of renal pathology is detected by histopathology [51,54,55], routine renal parameter alterations stating renal compromise are less frequently observed [4,5,11,20,52]. The kidney disease associated with

CanL is primarily of glomerular origin consequence of the deposition of circulating immune complexes at different levels of glomeruli structure [51,55]. Initially, renal involvement is observed by the presence of proteinuria without azotemia. As glomerular damage progresses, secondary tubulointerstitial nephritis and azotemia develop leading to end-stage renal failure or nephrotic syndrome, most striking cause of death in CanL [51,52,55]. Our study, in agreement with previous reports, shows renal azotemia as a rare clinicopathological finding while proteinuria without renal azotemia was the second most common laboratory abnormality in seropositive sick dogs at the time of diagnosis [11,20]. We also found a great proportion of dogs with inadequate USG, however, only a few were between isosthenuria levels, and other causes of polyuria/polydipsia were not ruled out. Therefore, a renal involvement was not confirmed in those seropositive sick dogs that presented with inadequate USG as the sole altered renal parameter. When the relationship between signalment, EU, and biochemical parameters was studied, a positive correlation was observed between UPC and antibody levels as previously reported in serum [11] and urine samples [56,57]. This result is expected since proteinuria is caused by immune-mediated glomerulonephritis [58]. Moreover, we found a positive correlation between age and UPC, most likely explained by a more deteriorated renal function in older dogs in addition to the fact that older dogs tend to have more renal and hematologic alterations [20], and a negative correlation between creatinine levels and age, probably associated with decreased muscle mass in older dogs.

Hepatocyte damage was uncommon as in agreement with other studies [4,5,11,49]. Furthermore, regarding liver parameters, a positive relationship was found between age and ALT levels and between EU and ALP levels. ALT is an unspecific marker of hepatocyte damage that could be increased with numerous groups of diseases frequently found in older dogs [27]. Thus, this observation could be the explanation for the trend of higher levels of ALT in older dogs. ALP is a marker of cholestasis in dogs. In addition, ALP can be affected by endogenous or exogenous cortisol levels [27]. Therefore, the observation of a stress response in our seropositive sick dogs could be the explanation for the relationship between ALP levels and EU.

LeishVet group proposed a classification of four clinical stages (from mild disease in stage I to very severe disease in stage IV) based on clinical signs, clinicopathological abnormalities and serological status [28]. This tool also suggests different treatment protocols and prognoses for each clinical stage and can be used in the clinical setting. In a previous study performed in Spain [29], a group of dogs were diagnosed with CanL, classified by LeishVet stage and followed-up during treatment. The majority of these dogs (86%) were classified as stage II and most of them (75%) were further sub-classified as stage IIa while stage IIb presented a lower proportion (25%) [29]. Furthermore, few dogs (14%) were classified in stage III [29]. Similarly, in the present study, apparently healthy *L. infantum*-seropositive sick dogs were classified by

LeishVet clinical stage and most of them were classified in stage IIa (55.1%) while stage IV had the lowest proportion of dogs (0.9%). These results were to be expected as the dogs did not present any clinical signs and most of them presented few clinicopathological abnormalities which is more common in lower stages of CanL (I and II) [28]. Furthermore, an important difference regarding serological status was found between *L. infantum*-seropositive healthy dogs (with subclinical infection and no clinical stage) and *L. infantum*-seropositive sick dogs (with clinical stage). Sick seropositive dogs presented higher endpoint EU than healthy seropositive dogs and a higher proportion of dogs with high to medium seropositive results. These results are in concordance with previous studies that reported that dogs with high antibody levels show more pronounce clinicopathological abnormalities and, thus, are classified in higher stages of leishmaniosis [29,59–61].

Interestingly, the risk of seropositivity to *L. infantum* has been associated with several factors such as age, breed, and the dog's environment, among others. The risk of seropositivity to *L. infantum* has been reported to increase with the dog age which seems to be related to repeated exposure to *Leishmania* [21,62–64], although a bimodal age distribution with one peak in young dogs (under 2 years old) and a second peak in older dogs (over 8 years old) has also been commonly reported [65]. Sex has also been reported to be a risk factor of seropositivity to *L. infantum* with male dogs presenting a higher risk of exposure to *Leishmania* infection than female dogs [21,64], although other studies did not detect differences between male and female dogs [62,66]. However, sex could be associated to other factors that could increase the probability of seropositivity to *L. infantum* such as the size of the dog and being used as a guard dog and living outdoors [25,67]. Environmental factors such as living outdoors or indoors have also been detected as huge risk factors of seropositivity to *L. infantum* [21,62,65,66]. In this study, no differences in age, sex, breed and weight were observed between *L. infantum*-seropositive healthy dogs (with subclinical infection and no clinical stage) and *L. infantum*-seropositive sick dogs (with clinical stage) which could indicate that, even if the characteristics of the dogs could be a risk factor for being *L. infantum*-seropositive, it does not seem to present a risk factor to disease development, worsening of clinicopathological abnormalities and ELISA results. However, as previously mentioned, several correlations with age and clinical data (ALT levels, UPC, creatinine levels) were observed and could be easily explained as older dogs tend to present clinicopathological abnormalities due to age-related diseases and that could be a risk factor and affect CanL development and worsening.

Conclusions

In conclusion, this report describes the signalment and clinical data of apparently healthy *L. infantum*-seropositive dogs. Furthermore, the most common clinicopathological abnormalities in apparently healthy *L. infantum*-seropositive sick dogs are also reported. Serum protein alterations were the most consistent finding in this group of dogs followed by proteinuria and lymphopenia. Meanwhile, other frequent alterations in dogs with leishmaniosis such as anemia were frequently observed. Moreover, a clear relationship was found between EU and hematological and biochemical alterations, with dogs with higher antibody levels having a tendency for a higher degree of laboratory alterations.

Abbreviations

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; CanL: Canine leishmaniosis; CBC: complete blood count; CI: confidence interval; conc: concentration; EDTA: ethylenediaminetetraacetic acid; EU: ELISA units; max: maximum; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; min: minimum; OD: optical density; PBS: phosphate buffered saline; RBC: red blood cell count; SPE: serum protein electrophoresis; UPC: urinary protein/creatinine ratio; USG: urinary specific gravity; WBC: leukocytes concentration.

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Consent to participate

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Consent was obtained from the owner or the tutor of the dog(s) to collect the samples and perform routine laboratory tests and ELISA.

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Chapter 8

A blinded, randomized and controlled multicenter clinical trial to assess the efficacy and safety of Leisguard® as an immunotherapeutic treatment for healthy dogs infected with *Leishmania infantum*

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Abstract

Background: Domperidone (Leisguard®) is an immunomodulatory drug that has demonstrated positive results in relation to *Leishmania* infection in dogs. Previous studies have investigated the use of domperidone as preventive in healthy dogs and for treatment of sick dogs with leishmaniosis. However, no studies have been published in healthy *L. infantum*-seropositive dogs. The aim of this study was to evaluate the clinical efficacy and safety of domperidone as immunotherapy in *Leishmania*-seropositive healthy dogs.

Methods: A total of 111 seropositive to *L. infantum* but otherwise healthy dogs were included in the study: 67 dogs were treated with domperidone (Leisguard®) at 0.5 mg/kg and 44 dogs received placebo, once daily for 4 consecutive weeks. Monthly treatments were repeated every 4 months until the end of the one-year follow-up period. Veterinary examinations were performed on days 0, 30, 120, 150, 240, 270 and 360. Samples of blood and urine were collected on days 0, 120, 240 and 360 for routine laboratory tests and quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies. Dogs that developed disease during the follow-up period were withdrawn from the study and classified as sick dogs. Furthermore, adverse drug reactions observed throughout the study were reported.

Results: Thirty dogs developed disease during the follow-up period: 13/67 (19.4%) in the group treated with domperidone and 17/44 (38.6%) in the placebo treated group. When dogs were classified by their initial in-house ELISA result (low positive [n=68] versus medium to high seropositive [n=43]), 11 dogs developed disease in the low seropositive group: 4/40 (9.1%) treated with domperidone and 7/24 (29.2%) treated with placebo, while 19 dogs developed disease in the medium to high seropositive group: 9/23 (39.1%) treated with domperidone and 10/20 (50%) treated with placebo. Thus, low seropositive dogs treated with domperidone were significantly less likely to develop disease when compared to low seropositive dogs treated with placebo (p=0.04). No differences were found between domperidone and placebo in medium to high seropositive dogs (p>0.05). Three dogs treated with domperidone presented self-limiting diarrhoea during the follow-up period.

Conclusions: Healthy dogs with low *L. infantum* antibody levels treated with domperidone (Leisguard®) were less likely to develop disease when compared to placebo treated dogs. Furthermore, Leisguard® presented a good safety profile.

Keywords: antibody level, canine, domperidone, leishmaniosis, placebo, Spain.

Background

Canine leishmaniosis (CanL) due to *Leishmania infantum* is prevalent in more than 80 countries worldwide [1,2]. The disease is considered a major zoonosis in Europe and its control and prevention constitute a major goal for veterinary and clinical health research and regulating agencies [3,4].

A broad range of immune responses and clinical manifestations have been described in canine *L. infantum* infection [5,6]. In fact, the development of clinical leishmaniosis is closely influenced by the immune response of the host which is very complex, still fairly unknown and determined not only by genetics but also by acquired factors [7,8]. The immune response requires a balance between inflammatory and regulatory responses to control *L. infantum* infection and avoid disease development [7,8]. For example, a dog that displays a protective cell-mediated immune response characterized by interferon gamma (IFN-γ) release that stimulates the activation of macrophages to produce nitric oxide (NO) and reactive oxygen species (ROS) for intracellular killing of amastigotes should be able to control *Leishmania* infection. In contrast, another dog that displays mainly a non-protective marked humoral immune response combined with absent or diminished cell-mediated immunity (CMI) will be susceptible to *Leishmania* infection, present a high parasite burden and finally clinical disease [7]. Furthermore, as the infection progresses towards disease, there is a decrease of T cell proliferation, IFN-γ production and a lack of macrophage activation resulting in a reduction of parasite elimination [8].

As the manifestations of leishmaniosis are closely influenced by the dog's immune response, there is a wide and variable range of different clinical presentations. The most common clinical signs of CanL due to *L. infantum* are skin lesions, weight loss and generalized lymphadenomegaly, among a large variety of other clinical conditions [6,9]. Furthermore, some laboratory findings such as hyperproteinemia, hyperglobulinemia, hypoalbuminemia, non-regenerative anemia and persistent proteinuria are also suggestive of CanL [2,6]. Four clinical stages of CanL have been designated based on clinical signs, clinicopathological abnormalities and serological status to classify dogs presenting CanL, and different treatment protocols and prognoses are suggested for each clinical stage from stage I (mild disease) to stage IV (very severe disease) [10].

The treatment administered in CanL is usually long-term, sometimes with no chance of discontinuation, and aims to reduce parasitic load [6]. Since there is no drug that can achieve a complete elimination of the parasite, a relapse of the disease would be expected [11]. The most common treatment consists on antimonials, which actively reduce parasitic load, together with allopurinol, which has a parasitostatic effect and, therefore, maintains parasitic load at low levels [2,6,11]. These drugs are not entirely safe as they present adverse effects; the most frequent of which are nephrotoxicity [12,13],

urolithiasis [14] or digestive disorders [15]. In addition, resistances to several of these drugs have also been documented such as resistances to antimonials [16] or allopurinol [17]. Considering the current knowledge that the immune system is the hallmark of the outcome of *Leishmania* infection and that the treatments used present adverse effects and resistances, the most promising approach would be the use of immunotherapy to improve the specific immune response against parasites [18].

Domperidone is a drug that has demonstrated positive results in relation to *Leishmania* infection in dogs [19–22] and mice [23] due to its immunomodulatory effects. The origin of the effects of domperidone is related to the release of serotonin that causes a reversible increase in blood levels of prolactin [24]. Prolactin has been classified as a pro-inflammatory lymphocyte-derived cytokine [25] and its increase induces a boost of CD4+ T lymphocytes, in addition to the release of cytokines such as interleukin (IL)-2, IL-12, IFN-γ and tumor necrotic factor alpha (TNF-α), producing activation of natural killer (NK) and macrophages, followed by a decrease in CD4+ T helper (Th)2 and tumor necrotic factor beta (TNF-β) [26–28].

The use of domperidone has been studied in healthy [22,29] and sick dogs with leishmaniosis [19–21]. A lower risk of developing clinical leishmaniosis in healthy seronegative dogs was observed when compared to dogs left untreated [22]. In dogs with clinical leishmaniosis, a reduction of clinical signs was observed in those that presented a mild disease [20] while a reduction of serum creatinine, globulins, gamma globulins, anti-*L. infantum* antibody titers and C-reactive protein was observed in dogs with leishmaniosis affected by chronic kidney disease (CKD) [19]. Moreover, dogs with clinical leishmaniosis that were treated with a combination of furazolidone and domperidone, showed a reduction of skin lesions [21]. However, no studies have yet been published in healthy *L. infantum*-seropositive dogs treated with domperidone.

As stated previously, a broad range of immune responses and clinical manifestations have been described in canine *L. infantum* infection and there is an important number of dogs which are seropositive and healthy [6]. For example, the seroprevalence of *L. infantum* in Spain has been reported to be around 10% [30–32], although the prevalence of dogs that develop the clinical disease is usually lower than 10% [2,33]. These healthy *L. infantum*-seropositive dogs are usually scientifically neglected and few recommendations have been published such as using repellents all year round, monitoring without treatment, short treatments with conventional anti-*Leishmania* drugs or immunotherapy [2,6,34]. Even though, there is still limited evidence for treatment outcomes for these dogs and the efficacy of these recommendations remain inconclusive [6].

Therefore, there is still limited information regarding the use, efficacy and safety of immunotherapy using domperidone in the clinical setting. The aim of this study was to

evaluate the clinical efficacy and safety of domperidone (Leisguard®) as immunotherapy in *Leishmania*-seropositive healthy dogs.

Methods

Clinical trial design

This was a blinded, randomized and controlled multicentre clinical trial with a follow-up of 1 year. The study started on September 2020 and the last sample was received on July 2022. The clinical trial was performed in several veterinary practices and dog shelters of various regions of Spain, a country with a reported average seroprevalence of *L. infantum* infection around 10% [30–32].

Treatments and randomized assignment

The clinical trial included two groups with different treatments. One group was treated with domperidone (Leisguard®) and named treated group (TG) while the other group was treated with a placebo and named control group (CG). Both domperidone and placebo were administered orally. The dose of domperidone was 0.5 mg/kg, or its equivalent, for placebo in volume, once daily, during 4 consecutive weeks. Treatment was repeated every 4 months until the end of the 1 year follow-up period. Both domperidone and placebo had to be administered mixed with food or administered directly in the mouth of the dog.

Domperidone and placebo had the same appearance and were labelled as treatment A or treatment B. Therefore, treatment administration was blinded. Veterinarians were instructed of which product (A or B) were going to administer to each dog, but neither the veterinarians nor the owners or caregivers of the dog(s) had knowledge about which product was being administered. Furthermore, the owners or caregivers of the dog(s) had to fill a data collection form to record both the daily treatment administration and any adverse drug reaction or lack of efficacy occurring during the study.

A randomized assignment of the treatment was also performed. Leisguard® and placebo were distributed in all sites of the clinical trial with a 2:1 ratio, so for each two dogs included in the TG, only one dog was included in the CG.

Sample size

In order to estimate differences between percentages of the two treatment groups (TG and CG), the necessary sample size was calculated based on the proportions of the parameter of interest in the groups, the confidence level and the power [35]. The sample size was calculated for unilateral tests, with a 2:1 proportion, a confidence level of

95%, an 80% of power and a potential dropout of a 20% [35]. The sample size required was 116 dogs in the TG and 58 dogs in the CG. Hence the total sample size required was 174 dogs.

Dog selection and inclusion/exclusion criteria

Dogs of different sexes (entire or neutered), breeds (pure breed or crossbreed), ages, weights and living situation (client-owned dogs or dogs from shelters) were able to be enrolled in the clinical trial. Female dogs known to be pregnant or lactating were not able to be enrolled.

The inclusion criteria included the following characteristics: (a) not have been previously diagnosed with clinical leishmaniosis, (b) present a recent seropositive result for the detection of *L. infantum* antibodies and (c) be healthy (do not show clinical signs or clinicopathological abnormalities compatible with leishmaniosis). Dogs were considered healthy when they did not present clinical signs and/or clinicopathological abnormalities based on a physical examination and complete blood count (CBC), biochemistry profile and urinalysis. CBC, biochemistry and urinalysis had to be within reference intervals. However, a slight variation outside the reference intervals (always no more than 5%) was evaluated individually and, then, it was assessed if the results were truly of clinical relevance or not to the patient.

The exclusion criteria included the following characteristics: (a) poor body condition such as dogs with very low weight (evident bony prominences, no palpable fat, loss of muscle mass), (b) have been previously treated with anti-*Leishmania* drugs (meglumine antimoniate, allopurinol, miltefosine…), immunomodulators (Leisguard®, Impromune®…) or vaccines against *Leishmania* (CaniLeish®, Letifend®), (c) have been recently treated (at least the last month) with drugs that could affect the outcome of the disease or the action of domperidone such as immunosuppressive drugs (corticosteroids, azathioprine, cyclosporine, tacrolimus), antibiotics (quinolones) and dopaminergic drugs (dopamine, dobutamine, cabergoline), and (d) incapacity of following a 1-year treatment or to comply with the follow-up visits.

Withdrawal criteria

Dogs had to be withdrawn of the study when at least one of the following situations occurred: (a) presence of an adverse drug reaction that compromised the ongoing treatment, (b) appearance of clinical signs and/or clinicopathological abnormalities of leishmaniosis, specifically when needing anti-*Leishmania* treatment, (c) need of other treatments that could interfere with the results of the clinical trial, when interfering in the outcome of the infection or the action of domperidone (quinolones, cabergoline, omeprazole, cimetidine, dopamine, dobutamine, corticosteroids…) and (d) females in pregnancy or lactation.

Study flowchart

A flowchart of the study is depicted in Table 8.1. Exclusion before enrolment was mainly due to presence of clinicopathological abnormalities or incapacity of following a 1-year treatment or to comply with the follow-up visits. Exclusion after day 0 visit was mainly due to incapacity of following a 1-year treatment or to comply with the follow-up visits.

Examination and sampling

The veterinary examinations and data collection were performed on days 0, 30, 120, 150, 240, 270 and 360.

An initial evaluation of the dog was performed on day 0 by the veterinarian to confirm that the dog was healthy and could be enrolled in the clinical trial. First, the clinical history of the dog was registered with information about signalment (breed, age, sex, reproductive status, environment, diet) and medical history (vaccination status, previous or current diseases, current medications). Then, a clinical evaluation was performed by the veterinarian. The information registered was: general appearance (mental status, attitude, body condition, hydration, body weight, temperature, and heart/pulse rate) and physical examination (description of abnormalities or lesions and presence of external parasites). The clinical evaluation was then repeated on days 30, 120, 150, 240, 270 and 360. A clinical evaluation was also performed in cases of early withdrawal and for any dog experiencing a serious adverse drug reaction. Dogs that showed any adverse drug reaction due to treatment or evidence of illness were closely monitored as needed throughout the study.

Samples of blood and urine were collected on days 0, 120, 240 and 360 for further laboratorial tests. Blood samples were collected by jugular or metatarsian venepuncture. Urine was obtained by free catch or cystocentesis. Once collected, all samples were refrigerated until shipment. Shipment was performed no later than 24-48 hours after collection of the samples.

On days 30, 150 and 270, the veterinarian confirmed treatment compliance and that the dog was still healthy after the administration of the treatment.

Routine laboratory tests

The investigated parameters are specified in Table 8.2. The hematology panel was performed with XN1000 SYSMEX (Sysmex España SL, Spain), the biochemistry panel and urinary protein creatinine ratio (UPC) were performed with VITROS 5600 ORTHO (Ortho clinical diagnostics, New Jersey, USA), the serum electrophoresis was performed with CAPILLARYS 3 SEBIA (Sebia, Hispania SA, Spain) and the urine panel

(except UPC) was performed with BECKMAN strips (Beckman Coulter, California, USA). Reference intervals of each parameter are also depicted in Table 8.2.

Quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies

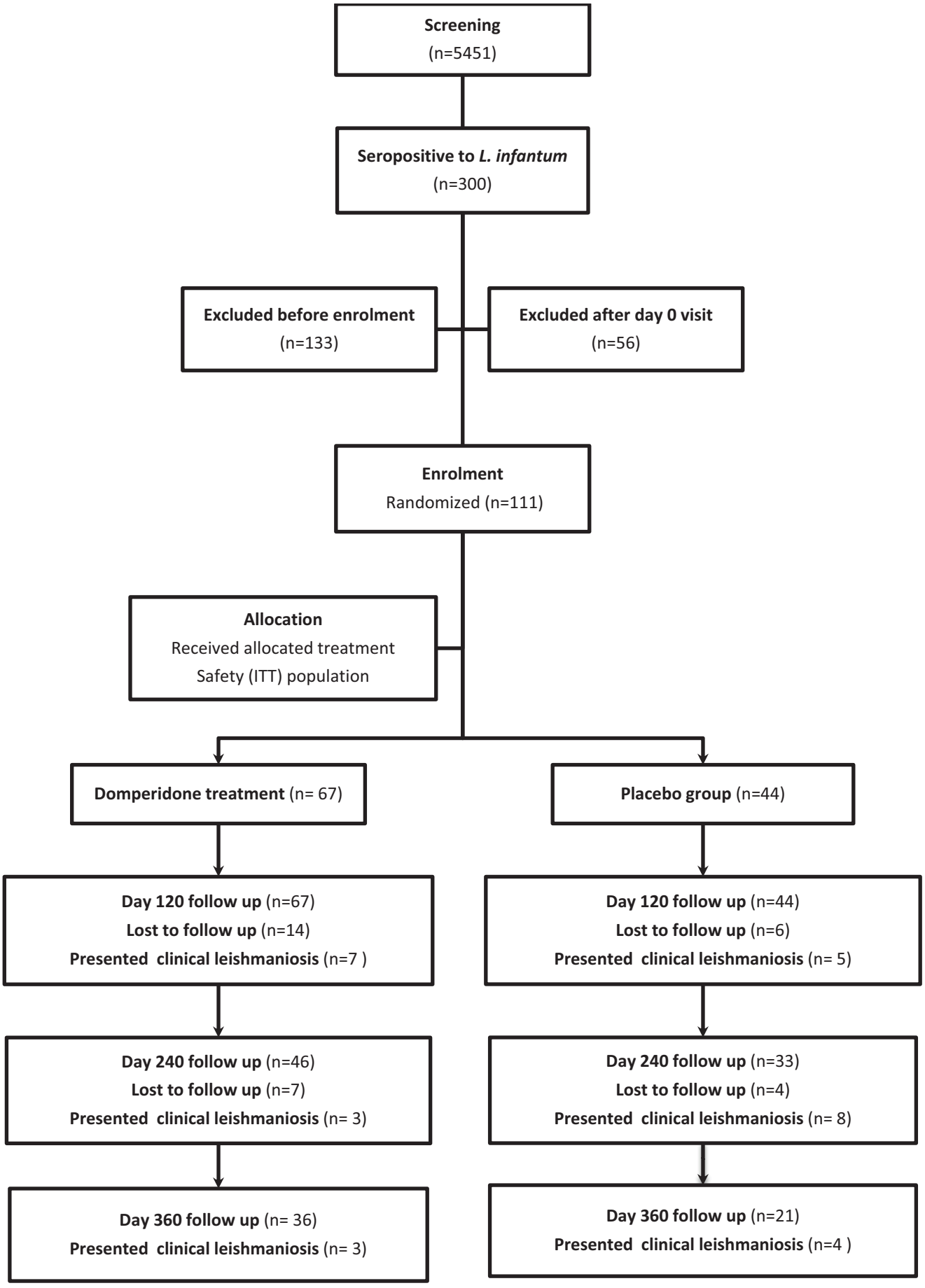
An in-house ELISA was performed on sera as previously described [36]. Briefly, samples were first diluted to 1:800 in phosphate buffered saline (PBS)-Tween with 1 % dry milk and then incubated 1 h at 37 ºC. Afterwards, the plate was washed three times with PBS–Tween and once with PBS. After the washes, peroxidase conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.16 ng/µL was added to the plate and incubated 1 h at 37 ºC. After incubation, washes were repeated as described above, and *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) were added to the plate. Finally, the reaction was stopped with 5M H2SO4. The results were read at 492 nm in a spectrophotometer machine (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China) and were defined as ELISA units (EU) in relation with a positive canine sera sample used as a calibrator set at 100 EU. The cut-off of the sera in-house ELISA was already determined at 35 EU using the ELISA results of 80 dogs from a non-endemic area as previously described [37]. Cut-off was stablished by the standard deviation (SD) method, consisting on multiplying the SD of the results by four and adding up the mean of the results obtained in the ELISA (mean + 4 SD). Furthermore, sera was classified as high positive when having a result equal or higher than 300 EU, medium positive when having a result equal or higher than 150 EU and lower than 300 EU, low positive when having a result equal or higher than 35 EU and lower than 150 EU, and negative when having a result lower than 35 EU.

All samples classified as medium or high positive were further studied using a two-fold serial dilution ELISA. Sera two-fold dilutions were started at 1:800 and continued for 7 to 11 further dilutions. The result was also quantified as EU related to a calibrator arbitrary set at 100 EU, with an optical density (OD) value of one at the 1:800 dilution. The mean values of the dilutions at which the OD were close to one were chosen for the calculation of the EU using the following formula: (Sample OD/Calibrator OD) x 100 x dilution factor.

Efficacy variables

Efficacy variables were classified in two groups: primary and secondary outcomes. The primary outcome focused on the development of the disease; thus, dogs were classified as healthy or sick. Dogs were considered healthy when they did not present clinical signs and/or clinicopathological abnormalities based on physical examination and hematology, biochemistry profile and urinalysis. Hematology, biochemistry, and urinalysis had to be within reference intervals as described in the inclusion criteria.

Table 8.1 Flowchart displaying the number of dogs screened, recruited, lost to follow-up and analysed.



Abbreviations: n: number of dogs.

Table 8.2 Parameters of routine blood and urine tests and reference intervals.

Hematology panel (units)	Reference intervals [38,39]	Biochemistry panel	Reference intervals [40,41]
RBC (10^6/µL)	5.1-7.6	Total protein (g/L)	54-71
Hemoglobin (g/dL)	12.4-19.2	Albumin (g/L)	26-33
Hematocrit (%)	35-52	Globulin (g/L)	27-44
MCV (fL)	60-77	A/G ratio	0.86-1.93
MCH (pg)	21.9-26.3	ALT (U/L)	21-102
MCHC (g/dL)	34.4-38.1	ALP (U/L)	20-156
WBC (10^9/L)	5.6-20.4	Creatinine (mg/dL)	0.5-1.5
Neutrophils conc (10^9/L)	2.9-13.6	Urea (mg/dL)	21.4-59.9
Lymphocytes conc (10^9/L)	1.1-5.3	Serum electrophoresis (g/L)	sero-albumin (24.4-49.6); alpha-1 globulin (1.7-4.5); alpha-2 globulin (3.8-10.2); beta globulin (8-18); gamma globulin (2.6-11.7)
Monocytes conc (10^9/L)	0.4-1.6	**Urine panel**	**Reference intervals [41]**
Eosinophils conc (10^9/L)	0.1-3.1	Urine strip*	
Basophils conc (/µL)	0-200	UPC	<0.5
Platelet conc (10^3/µL)	200-500	USG (g/L)	>1030
Evaluation of blood smear		Physical colour and appearance, microscopic appearance and sediment analysis	

*Urine strip included qualitative information about density, acetone, pH, proteins, blood, nitrites, glucose, urobilinogen, urobilin and leukocytes.

Abbreviations: A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine transaminase; conc: concentration; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells concentration; UPC: urinary protein creatinine ratio; USG: urinary specific gravity; WBC: leukocytes concentration.

The secondary outcome focused on each studied parameter in routine laboratory tests and quantitative in-house ELISA and its changes between days (0, 120, 240 and 360). Thus, a change was reported when the results of the parameters between days presented a significant increase or decrease. Furthermore, seroreversion (changing from a seropositive result to a seronegative) in endpoint in-house ELISA for *L. infantum* was also investigated between days (0, 120, 240 and 360).

Safety evaluation

Adverse drug reactions were used to evaluate the safety of the products. An adverse drug reaction was defined as any observation in the treated dog that was unfavourable, unintended and occurred after the administration of the product. The adverse drug reaction was immediately registered with a detailed description and, depending on the severity of the adverse drug reaction, the treatment could be interrupted. Dogs that showed adverse drug reactions were closely monitored as needed throughout the clinical trial and withdrawn if necessary. This information was recorded in the data collection form of both the veterinarian and the owner or caregiver of the dog.

Statistical analysis

The statistical analysis was performed using the package Stats for the software R i386 3.6.1 for Windows, using Fisher's exact test for qualitative variables and Mann-Whitney *U* test for quantitative variables to compare between treatment groups (TG *vs* CG). Log-rank test was performed to detect differences between the event curves of treatment groups (TG *vs* CG) with the studied event being the occurrence of disease development. The Shapiro–Wilk test was performed to detect normal distribution of quantitative variables. A p-value of < 0.05 was considered statistically significant. Graphs were plotted using Graphad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Signalment and clinical data

Characteristics of all dogs included in the clinical trial are depicted in Table 8.3. The most common breeds in the TG were Labrador retriever (7.5%) and Spanish Greyhound (4.5%) while German shepherd (6.8%), Beagle (4.5%) and Jagdterrier (4.5%) were the most common in the CG. No differences were found between treatment groups between breed, sex, age and weight (Table 8.3).

Table 8.3 Qualitative and quantitative characteristics of the dogs included in the study.

Qualitative characteristics		Total (n=111) % (95% CI)	TG (n=67) % (95% CI)	CG (n=44) % (95% CI)	*p*-value (Fisher's exact test)
Breed	**Crossbreed**	47.7 (38.2-57.4)	53.7 (41.1-66)	38.6 (24.4-54.5)	0.127
	Purebred	52.3 (42.6-61.8)	46.3 (34-58.9)	61.4 (45.5-75.6)	
Sex	**Female**	44.1 (34.7-53.8)	47.8 (35.4-60.3)	38.6 (24.4-54.5)	0.435
	Male	55.9 (46.1-65.3)	52.2 (39.7-64.6)	61.4 (45.5-75.6)	
ELISA interpretation at day 0	**High or medium positive**	38.7 (29.6-48.5)	34.3 (23.2-46.9)	45.5 (30.4-61.2)	0.319
	Low positive	61.3 (51.6-70.4)	65.7 (53.1-76.9)	54.6 (38.9-69.6)	
Quantitative characteristics		**Median (min-max)**	**Median (min-max)**	**Median (min-max)**	***p*-value (Mann-whitney *U* test)**
Age (years)		5 (1-14)	5 (1-14)	4 (1-13)	0.094
Weight (kg)		24 (6-55)	22 (6-50)	25 (10-55)	0.193
Endpoint ELISA (EU) at day 0		165 (40-3965)	155 (40-1954)	183 (55-3965)	0.320

Abbreviations: CG: control group; CI: confidence interval, EU: ELISA units; max: maximum; min: minimum; n: number of dogs; TG: treated group.

In-house ELISA results and interpretation of all dogs at initial day 0, and also classified by treatment group (TG and CG), are depicted in Table 8.3. There was a high percentage of low seropositive dogs included in the study (around 60%) while 40% were medium or high seropositive (Table 8.3). No differences were detected between treatment groups when comparing in-house ELISA results and their interpretation at day 0 (Table 8.3).

A total of 31 dogs (28%) were lost to follow-up during the study (Table 8.1). Of these dogs, 20 dogs (14 in the TG and six in the CG) were lost to follow-up after day 120, and 11 dogs (seven in the TG and four in the CG) were lost after day 240. These dogs were lost to follow-up mainly due to adoption, moving to another region and other causes such as pregnancy or sudden death by a car accident.

Efficacy variables

Primary outcome

Thirty dogs developed disease during the follow-up period (Table 8.4, Table 8.5). Thirteen (13/67; 19.4%) were from the TG while the other 17/44 (38.6%) were from the CG. A significant difference was observed (Fisher's Exact test: p=0.03, OR=2.62, CI=1.11-6.17) which indicated that the TG was less likely to present disease development compared to the CG. Most of the dogs that developed disease presented clinicopathological abnormalities while a minority presented also clinical signs. The specific clinical signs and clinicopathological abnormalities that the dogs developed are depicted in Table 8.6. Dogs were classified by LeishVet clinical staging: one dog of the CG was in stage I, 24 dogs (nine of the TG and 15 of the CG) were in stage IIa, three dogs of the TG were in stage IIb and two dogs (one of the TG and one of the CG) were in stage III [10]. The median of the endpoint ELISA performed at day of failure in the dogs that developed disease was 1629 EU with a minimum of 36 EU and a maximum of 6151 EU.

When the dogs were classified not only by their treatment group, but also by their initial in-house ELISA result (low positive *vs* medium to high seropositive), different outcomes were observed. In the low seropositive group (n=68), a total of eleven dogs developed disease being four of them in the TG (4/44, 9.1%) and seven in the CG (7/24, 29.2%). Thus, low seropositive dogs treated with domperidone were significantly less likely to develop disease when compared to low seropositive dogs treated with placebo (Fisher's Exact test: p=0.04, OR=4.12, CI=1.06-15-94). In the medium to high seropositive group (n=43), a total of 19 dogs developed disease being nine of them in the TG (9/23, 39.1%) and 10 in the CG (10/20, 50%). Thus, no differences were found between treatments in medium to high seropositive dogs (p>0.05).

Table 8.4 Signalment and clinical data of dogs with disease progression in the TG group.

Dog number	Treatment group	Sex	Age (years)	Breed	Endpoint ELISA result (EU)				Clinical signs	Clinicopathological findings
					Day 0	Day 120	Day 240	Day 360		
10	TG	Female	2.5	Spanish Greyhound	704	915	2664	-	None	Hyperproteinemia, hyperbetagammaglobulinemia, decreased A/G ratio, proteinuria
23		Female	2.5	Border collie	149	197	195	457	Skin lesions (dermatitis), weight loss, lymphadenomegaly	Hyperproteinemia, hyperbetagammaglobulinemia, decreased A/G ratio
25		Male	2	Crossbreed	942	4019	-	-	None	Hyperproteinemia, hyperbetagammaglobulinemia, decreased A/G ratio
29		Male	2	Crossbreed	1587	2623	-	-	Weight loss, alopecia	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
39		Male	5	Crossbreed	905	1722	-	-	None	Hyperproteinemia, hypergammaglobulinemia, proteinuria
40		Male	6	Crossbreed	255	342	-	-	None	Hyperproteinemia, hyperalfabetagammaglobulinemia, decreased A/G ratio
44		Male	4	Spanish hound	1774	1776	-	-	Skin lesions (exfoliative and ulcerative dermatitis), weight loss, alopecia	Hypergammaglobulinemia, decreased A/G ratio, proteinuria, prerenal azotemia
46		Male	3	Labrador retriever	295	793	2910	-	None	Hyperproteinemia, hyperalfagammaglobulinemia, decreased A/G ratio, anemia, proteinuria
48		Male	4	Warren hound	1181	5287	-	-	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
53		Female	11	Crossbreed	229	1569	1550	2939	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio, anemia
61		Female	5	Brittany spaniel	107	844	963	-	None	Hyperproteinemia, hyperalfabetagammaglobulinemia, decreased A/G ratio
68		Male	3	Crossbreed	165	408	1563	6151	Weight loss, lymphadenomegaly	Hyperproteinemia, hyperbetagammaglobulinemia, decreased A/G ratio, anemia
107		Male	5	Boxer	183	232	-	-	None	Hyperproteinemia, hyperbetaglobulinemia

Table 8.5 Signalment and clinical data of dogs with disease progression in the CG group.

Dog number	Treatment group	Sex	Age (years)	Breed	Endpoint ELISA result (EU)				Clinical signs	Clinicopathological findings
					Day 0	Day 120	Day 240	Day 360		
11	CG	Male	2	German shepherd	1720	4344	-	-	None	Hyperproteinemia, hypergammaglobulinemia
18		Male	2	German shepherd	88	23	19	36	None	Hyperproteinemia, hyperbetaglobulinemia, decreased A/G ratio
19		Male	13	Crossbreed	107	112	126	124	Skin lesions (dermatitis)	Hyperproteinemia, hypergammaglobulinemia
42		Female	3	Jagd terrier	247	1768	-	-	Lymphadenomegaly	Hyperproteinemia, hyperalfagammaglobulinemia, decreased A/G ratio, proteinuria, renal azotemia
63		Female	1	Spanish bulldog	132	113	77	-	None	Hyperproteinemia, hyperbetagammaglobulinemia, decreased A/G ratio, leukocytosis
64		Female	12	Warren hound	186	234	205	-	None	Hyperproteinemia, hyperalfabetagammaglobulinemia, decreased A/G ratio, anemia
73		Female	2	Crossbreed	503	2386	-	-	Skin lesions (exfoliative dermatitis), lymphadenomegaly, conjunctivitis	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
81		Male	1	Warren hound	57	70	-	-	None	Hyperproteinemia, hyperalfabetagammaglobulinemia, decreased A/G ratio
84		Male	7	Yorkshire terrier	680	946	1537	-	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
85		Male	2	Crossbreed	1694	3409	3517	-	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
87		Female	2	Weimaraner	258	1698	4415	-	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
89		Male	6	Labrador retriever	98	68	-	-	Skin lesions (dermatitis)	None
90		Female	7	Jagd terrier	852	1965	2279	-	None	Hyperproteinemia, hypergammaglobulinemia
104		Female	5	Dogo Argentino	171	258	669	-	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio
106		Female	6	Beagle	417	449	207	373	None	Hyperproteinemia, hypergammaglobulinemia, decreased A/G ratio, anemia
109		Male	3	Boxer	453	549	517	176	None	Anemia
110		Male	5	Crossbreed	521	545	583	-	Skin lesions (exfoliative dermatitis), weight loss	Hyperproteinemia, hypergammaglobulinemia

Abbreviations: CG: control group; EU: ELISA units; TG: treated group.

Table 8.6 Clinical signs and clinicopathological abnormalities of dogs that developed disease.

Clinical signs	Total (n=30) Number of cases (%)	TG (n=13) Number of cases (%)	CG (n=17) Number of cases (%)
Skin lesions (specifically exfoliative dermatitis)	6 (20)	2 (15.4)	4 (23.5)
Weight loss	5 (16.7)	4 (30.8)	1 (5.9)
Generalized lymphadenomegaly	4 (13.3)	2 (15.4)	2 (11.8)
Alopecia	2 (6.7)	2 (15.4)	0 (0)
Conjunctivitis	1 (3.3)	0 (0)	1 (5.9)
Clinicopathological abnormalities	**Total (n=30) Number of cases (%)**	**TG (n=13) Number of cases (%)**	**CG (n=17) Number of cases (%)**
Hyperproteinemia	27 (90)	12 (92.3)	15 (88.2)
Hyperglobulinemia	28 (93.3)	13 (100)	15 (88.2)
Hypergammaglobulinemia	26 (86.7)	12 (92.3)	14 (82.4)
Hyperbetaglobulinemia	11 (36.7)	7 (53.9)	4 (23.5)
Hyperalfaglobulinemia	6 (20)	3 (23.1)	3 (17.6)
Decreased A/G ratio	22 (73.3)	11 (84.6)	11 (64.7)
Mild normocytic normochromic non-regenerative anemia	6 (20)	3 (23.1)	3 (17.6)
Proteinuria	5 (16.7)	4 (30.8)	1 (5.9)
Prerenal or renal azotemia	2 (6.7)	1 (7.7)	1 (5.9)
Leukocytosis with mature neutrophilia	1 (3.3)	0 (0)	1 (5.9)

Abbreviations: A/G: albumin/globulin; CG: control group; TG: treated group.

When a log-rank test was performed (Fig 8.1), a significant difference between the disease development curves was observed between the TG and the CG (Day 360, log-rank test: X^2=4.03, *df*=1, *p*=0.04). The disease development curve in the TG group presented a proportion of 23.4% at day 360 while the CG presented a proportion of 45.6% at day 360 (Fig 8.1). When the dogs were classified not only by their treatment group, but also by their initial in-house ELISA result (low positive versus medium to high seropositive), different outcomes were also observed. In the low seropositive group, a significant difference between the disease development curves was observed between the TG and the CG (Day 360, log-rank test: X^2=4.67, *df*=1, *p*=0.03). In this case, the TG curve presented a proportion of 11.9% at day 360 while the CG curve presented a 35.4% at day 360 (Fig 8.2). In the medium to high seropositive group, no difference between the disease development curves was observed between the TG and the CG (*P*>0.05). In this case, the TG curve presented a proportion of 46.1% at day 360 and the CG event curve presented a 57.5% at day 360 (Fig 8.3).

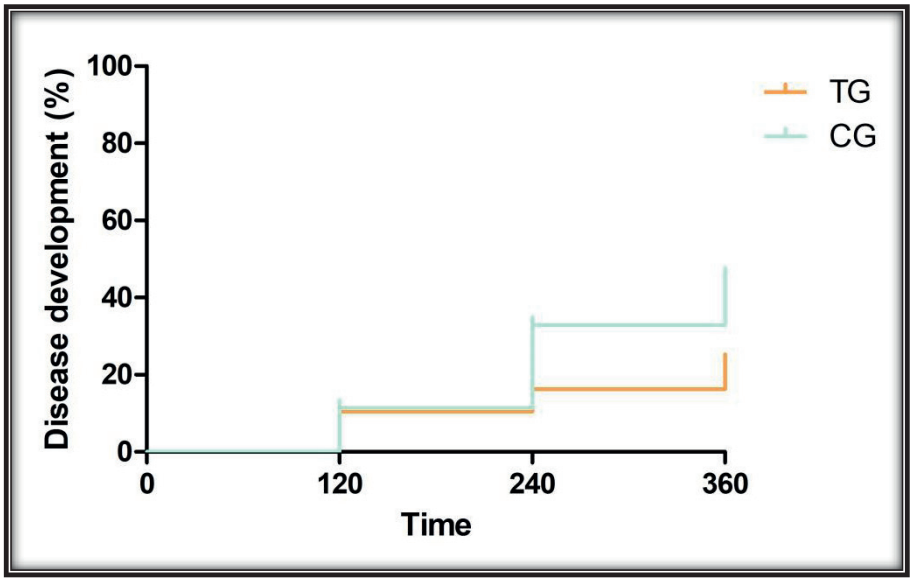


Fig 8.1 Disease development curves by treated groups of all dogs (Day 360, log-rank test: X^2=4.03, *df*=1, *p*=0.04). Abbreviations: CG: control group; TG: treated group.

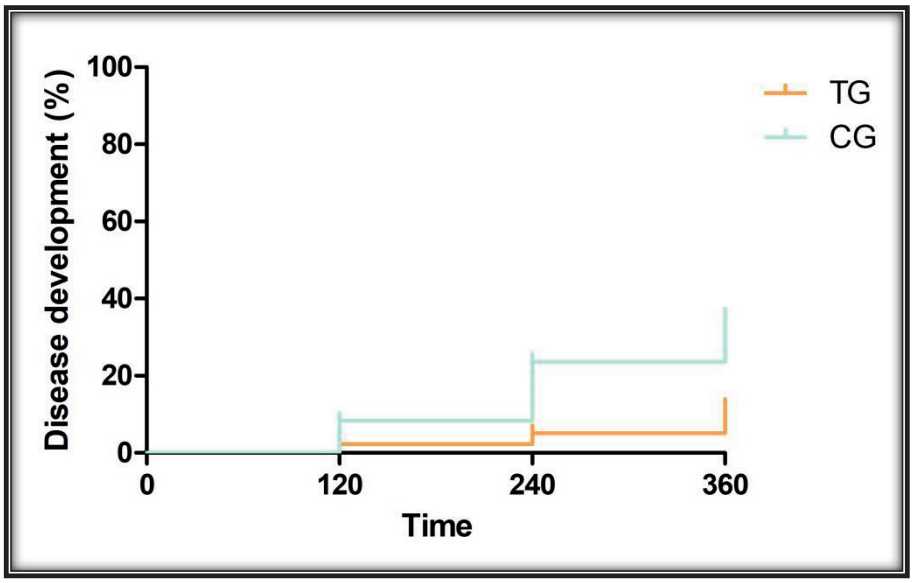


Fig 8.2 Disease development curves by treated groups of low seropositive dogs (Day 360, log-rank test: X^2=4.67, *df*=1, *p*=0.03). Abbreviations: CG: control group; TG: treated group.

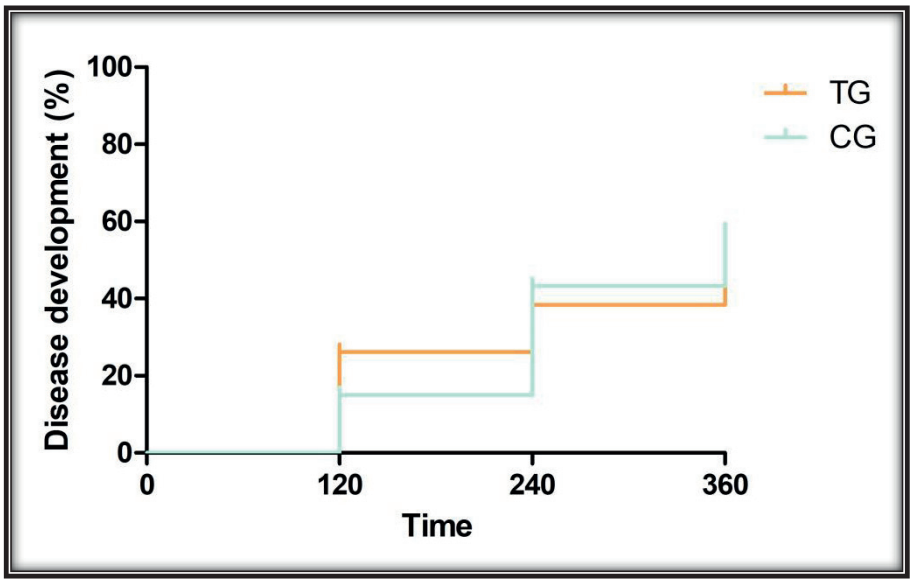


Fig 8.3 Disease development curves by treated groups of medium to high seropositive dogs (Day 360, log-rank test: X^2=0.05, *df*=1, *p*=0.83). Abbreviations: CG: control group; TG: treated group.

Secondary outcome

The in-house ELISA results of each day (0, 120, 240 and 360) are depicted in Table 8.7. No differences in in-house ELISA results were detected between the studied days (p>0.05). Information about in-house ELISA results of each day classified by treatment group is also depicted in Table 8.7. No differences in in-house ELISA results by day were found between treatment groups (p>0.05).

The in-house ELISA results of the dogs with disease development of each day (0, 120, 240 and 360) are depicted in Table 8.8. The in-house ELISA results were significantly higher at days 120 and 240 when compared to day 0 (Table 8.8).

Information about in-house ELISA results of dogs with disease development of each day classified by treatment group is also depicted in Table 8.8. In the TG, the in-house ELISA results were also significantly higher at days 120 and 240 when compared to day 0 while, in the CG, the in-house ELISA results were only significantly higher at day 120 when compared to day 0 (Table 8.8).

Safety variables

Only three dogs in the TG (5%) presented an adverse drug reaction during the study. The three dogs developed self-limiting diarrhea for one or two days after domperidone administration. This adverse drug reaction only occurred during the first administration of domperidone which was initiated at day 0. On subsequent administrations starting at days 120 and 240, no adverse drug reaction occurred. No adverse drug reactions were observed in dogs treated with placebo.

Discussion

The development of clinical leishmaniosis depends largely on the immune response of the host [7,8]. Thereby, treatments that can enhance the host's immune system could provide an alternative direction to combating the infection [18,42,43]. This is the first published clinical trial testing the clinical efficacy and safety of domperidone (Leisguard®) in healthy dogs seropositive to *L. infantum* infection.

In the present study, it was observed that dogs treated with domperidone were less likely to present disease development than dogs treated with placebo. These differences were highly significant in low seropositive dogs while, in medium to high seropositive dogs, disease development was similar in both groups. This was to be expected as high antibody levels have previously been associated with dissemination of the parasite and clinical disease [6,36]. Therefore, based on the results of this study, in healthy dogs with high antibody levels, treatment with domperidone alone would not be enough to avoid disease development.

Table 8.7 Endpoint ELISA results (EU) of the dogs at days 0, 120, 240 and 360.

Group (number of dogs)	Endpoint ELISA result (EU) at Day 0 Median (min-max)	Endpoint ELISA result (EU) at Day 120 Median (min-max)	Endpoint ELISA result (EU) at Day 240 Median (min-max)	Endpoint ELISA result (EU) at Day 360 Median (min-max)
Total (111)	165 (40-3965)	164 (18-5287)	171 (3-4415)	124 (3-6151)
TG (67)	155 (40-1954)	161 (18-5287)	139 (3-2910)	124 (3-6151)
CG (44)	183 (55-3965)	186 (22-4344)	205 (19-4415)	124 (34-1699)

Abbreviations: CG: control group; EU: ELISA units; max: maximum; min: minimum; TG: treated group.

Table 8.8 Endpoint ELISA results (EU) of the dogs with disease development at days 0, 120, 240 and 360.

Group (number of dogs)	Endpoint ELISA result (EU) at Day 0 Median (min-max)	Endpoint ELISA result (EU) at Day 120 Median (min-max)	Endpoint ELISA result (EU) at Day 240 Median (min-max)	Endpoint ELISA result (EU) at Day 360 Median (min-max)
Total (30)	277 (57-1774)	819 (23-5287)[a]	816 (19-4415)[b]	373 (36-6151)
TG (13)	295 (107-1774)	915 (197-5287)[c]	1556 (195-2910)[d]	2939 (457-6151)
CG (17)	258 (57-1720)	545 (23-4344)[e]	550 (19-4415)	150 (36-373)

Abbreviations: CG: control group; EU: ELISA units; max: maximum; min: minimum; TG: treated group.

[a]significantly higher when compared to day 0 (Wilcoxon signed-rank test : W:-423, $p<0.0001$)

[b]significantly higher when compared to day 0 (Wilcoxon signed-rank test: W:-133, $p=0.004$)

[c]significantly higher when compared to day 0 (Wilcoxon signed-rank test: W:-91, $p=0.0002$)

[d]significantly higher when compared to day 0 (Wilcoxon signed-rank test: W:-21, $p=0.031$)

[e]significantly higher when compared to day 0 (Wilcoxon signed-rank test: W:-121, $p=0.005$)

In the present study, most of the dogs that developed disease presented clinicopathological abnormalities such as hyperglobulinemia, hyperproteinemia and decrease A/G ratio while a minority presented also clinical signs such as skin lesions or weight loss. These clinicopathological findings and clinical signs have already been described in dogs with CanL [6,44]. The fact that the majority of the dogs only presented clinicopathological abnormalities with no clinical signs and, thus, was apparently healthy, highlights the importance of performing routine laboratory tests in apparently healthy *L. infantum*-seropositive dogs to detect disease development and progression which could shorten treatment duration and also improve disease prognosis [6,10]. Furthermore, even though renal azotemia typical of renal failure has been labelled as an uncommon laboratory finding in apparently healthy dogs [2,5,6], one dog presented renal azotemia in this study. In our case, the follow-up was every four months and this dog presented unexpected renal azotemia during this short period of time. These results not only highlight again the importance of performing routine laboratory tests in both blood and urine, but also the importance of a controlled follow-up that should be shorter than four months similar to the monitoring recommended in dogs under treatment for CanL [2,6].

Surprisingly, no differences were found in antibody levels during the follow-up period between dogs treated with domperidone and dogs treated with placebo. In a previous study [19], a reduction of anti-*L. infantum* antibody levels was observed in dogs with leishmaniosis affected by CKD and treated with domperidone. In this case, it is possible that dogs with clinical disease and very high seropositivity have a reduction of anti-*L. infantum* antibody levels when treated with domperidone while, in the present study, the majority of dogs were healthy and presented low antibody levels. However, a statistical increase of antibody levels in those dogs with disease progression was observed at days 120 and 240. These observations were to be expected as the increase of anti-*L. infantum* antibody levels is usually linked to disease progression in dogs with *L. infantum* infection [6,36].

Additionally, only three dogs treated with domperidone presented mild adverse drug reactions. The three dogs presented a self-limiting diarrhea for one or two days. This adverse drug reaction is already listed in the prospectus of the product [45]. Furthermore, it is detailed that this effect should disappear after the treatment is withdrawn [22,45,46], although in the present study the treatment with domperidone was not withdrawn, as the treatment was still administered for four consecutive weeks, and the diarrhea also disappeared. Therefore, the administration of domperidone in healthy seropositive dogs appears to be clinically safe.

Healthy *L. infantum*-seropositive dogs are usually scientifically neglected and there is no strong evidence of whether it is better to monitor them without treatment or treat them with conventional anti-*Leishmania* drugs or immunotherapy [2,6]. For example, a previous study that treated clinically healthy *Leishmania*-infected dogs with dietary nucleotides showed that the use of dietary nucleotides was safe and could be able to reduce the rate of disease progression, although it was also stated that further clinical trials with larger sample sizes and other drug combinations were needed to confirm these observations [47]. In the present study, domperidone was also able to reduce disease progression, specifically in low seropositive dogs, and, thus, could be used as treatment for those scientifically neglected dogs that are infected by *L. infantum*, but do not present clinical disease [6]. Furthermore, the advantages of domperidone compared to other products are that it is a treatment that can be administered orally and presents a very good safety profile.

Unfortunately, the sample size of dogs included in the study (111 dogs) was not the same as the one previously calculated and required (174 dogs). Sample size estimation is a critical step in planning a clinical trial as it may lead to rejection of an efficacious product, approval of an ineffective product and ethical issues related to product exposition to more subjects than necessary [35]. When a sample size is under-estimated (the sample size selected is less than what was required), the statistical analysis may result in non-significance, even though clinical significance exists [35]. In the present study, significant differences were observed between domperidone and placebo, even though the sample size was lower than required.

Further studies must investigate the use of domperidone in dogs with clinical leishmaniosis in combination with traditional therapies such as antimonials and allopurinol. The use of immunotherapy could shorten treatment duration which could reduce the incidence of adverse effects produced by traditional therapies, and also improve the prognosis of the dog [12,13,15,18].

Conclusions

This study shows that healthy dogs with low *L. infantum* antibody levels that were treated with domperidone were less likely to develop disease when compared to dogs treated with placebo. Therefore, domperidone appears to be a drug to be used in healthy dogs with low antibody levels in the clinical setting. Furthermore, domperidone presented a good safety profile.

Abbreviations

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine transaminase; CI: confidence interval; CG: control group; EU; elisa units; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; OD: optical density; OR: odds ratio; PBS: phosphate buffered saline; RBC: red blood cells concentration; SD: standard deviation; UPC: urinary protein creatinine ratio; TG: treated group; WBC: leukocytes concentration.

Informed consent

Study authorization was obtained from the Spanish authority, Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) with the authorization number 008/EPA-2383ESP.

All animal owners and shelter personnel signed an informed consent before enrolment of the dog in the study.

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Chapter 9

Discussion

New insights of prevention in *L. infantum* infection

The prevention and control of *L. infantum* infection in dogs is one of the most important practices to decrease transmission and, therefore, decrease the prevalence of canine *L. infantum* infection [1,2]. In *L. infantum* endemic countries such as Spain, the use of preventive measures is highly recommended in both seronegative dogs and seropositive (healthy or sick) dogs [1,2]. The recommendations include the use of repellents all year around (or during the sand fly season), vaccination against *L. infantum* and administration of domperidone [1,2]. Furthermore, periodic check-ups and serological tests are also recommended [1,2].

In this thesis, a study on the current use of the different available preventive measures against *L. infantum* infection in Spain was performed and is described in chapter 3. Information about the current use of preventive measures was not previously available as most of the previous studies about preventive measures focused on the veterinary recommendations instead of the real use of these products by the dog owners [3–7]. These studies [3–7] were performed using questionnaires sent to veterinarians and found out that most veterinarians recommended preventive measures against *L. infantum* to their clients as endorsed by the published guidelines [1,2]. However, the use of preventive measures by dog owners is known to depend not only on veterinary recommendations, but also on several other factors such as purchasing power and dog owner knowledge about CanL [8,9]. In the study described in chapter 3, information from 3762 dogs was gathered and more than 90% of the dogs confirmed application of at least one preventive measure with repellents being used in more than 80% of the dogs while vaccines and Leisguard® were used by less than 50%, which is similar to the veterinary recommendations that prioritize the use of repellents over vaccines or Leisguard® [5,6].

In chapter 3, the available repellent brands were also investigated. In previous studies [6,10], it was reported that the most frequently recommended brands of repellents were Seresto® (collar), Advantix® (spot-on) and Scalibor® (collar). The study described in chapter 3 showed similar results with the most used collar being Scalibor® while Advantix® was the most used spot-on. Interestingly, a study performed in north-eastern Spain [5] described a preference for recommending collars (98%) over spot-on (67%), in disagreement with the results described in chapter 3, in which no difference between the use of collar or spot-on was detected. Regarding the available vaccine brands, another study [6] reported a higher use of Letifend® than Canileish®. However, the study performed in this thesis (chapter 3) differs as a higher use of Canileish® was found when compared with Letifend®. The discrepancy between the study performed in this thesis and the previously mentioned study [6] is probably due to the fact that, in chapter 3, the data included was from 2012 to 2018. This period of time is important as Canileish® was available in the market since 2012 while Letifend® was only available

in the market since 2016 [1,11,12]. Regarding domperidone (Leisguard®), in the study performed in chapter 3, it was observed that domperidone was more frequently administered to smaller dogs, similarly detected in a previous study [10]. A good reason for this observation is that domperidone dose administration is linked to body weight, so large dogs need a higher daily dose than small dogs and, therefore, a higher expenditure [13]. Another reason could be related to the fact that small size dogs are more prone to adverse reactions after vaccination and, therefore, dog owners would be more likely to use other products to avoid vaccine adverse reactions [14,15].

Furthermore, as mentioned before, several other factors such as purchasing power and dog owner knowledge about CanL could impact the use of preventive measures against *L. infantum* infection [8,9]. For example, a direct association between socioeconomic status of the dog owner and CanL was previously documented [8]. Thus, owners with a low income could not afford some products which could affect the disease control and the survival of the dog [8]. Also, the presence of a backyard at the residence with a vegetation predominance was also associated with CanL [8] which could be a consequence of an environmental factor, but also of a lower use of preventive measures in periurban and rural areas as detected in chapter 3. In another study [9], several risk factors were associated with a higher number of dogs with leishmaniosis. In this study [9], rural areas, large dogs (usually used as guard dogs) and the lack of dog owner knowledge about CanL were associated to dogs with leishmaniosis. Coincidentally, in the study described in chapter 3, large dogs were also more frequently classified in the high-risk exposure group and living in rural or periurban areas than small dogs and, therefore, more likely to be infected by the parasite.

Key points:

- **More than 90% of the dogs applied at least one preventive measure against *L. infantum* infection in Spain.**
- **The most used preventive measures were repellents (more than 80%) followed by vaccines and domperidonde (Leisguard®) (less than 50%).**
- **The most used repellent brands were Scalibor® and Advantix®.**
- **Several factors such as living area and dog size might play an important role in the use of preventive measures that could affect the risk of *L. infantum* infection.**

Future studies:

- **The current use of preventive measures in other *L. infantum* endemic countries and in non-endemic countries such as Germany or United Kingdom.**

New insights of diagnosis in *L. infantum* infection

The early diagnosis of *L. infantum* infection is another important practice in the clinical setting as it can detect early disease development and help in the prevention and control of *L. infantum* infection in dogs [1,12]. The detection of disease development in the first stages (when the dog presents few clinical signs and laboratory findings) has a better prognosis and usually need shorter treatments [2]. The diagnosis of *L. infantum* infection often requires an integrated approach, including a clinicopathological examination and specific laboratory tests [2,16]. In addition, as previously mentioned, periodic check-ups and serological tests are also recommended in the clinical practice in *L. infantum* endemic countries [1,2].

In chapter 3, a study on the current use of serological screening tools in *L. infantum* infection in dogs in Spain was performed. Interestingly, rapid tests and ELISA were the preferred screening tools by clinicians as previously reported in other studies [3,5–7,10]. In the study described in chapter 3, rapid tests were used in the clinical setting by more than 50% of the cases while ELISA was used around a 30%. Other tests such as IFI were used by less than 10%. The high use of rapid tests was probably due to their fast results, low price and easy performance compared to other tests such as ELISA and IFI that usually need to be conducted by trained personnel in reference laboratories. However, ELISA was used more than IFI probably because IFI's interpretation is subjective and its result depends on the operator's experience and skill to interpret the test while ELISA is interpreted objectively using an ELISA reader to quantify the result [17]. Unfortunately, the results described in chapter 3 highlight an increasing problem in the clinical setting as qualitative rapid tests have a good specificity but are less sensitive than quantitative laboratory tests such as IFI and ELISA and, therefore, rapid tests are more likely to misdiagnose seropositive cases [2,12,18,19].

In chapter 5, an in-house ELISA for the detection of specific antibodies against *L. infantum* in canine OT was developed. The in-house ELISA was adapted from a technique that was currently being used in canine serum samples [20]. The use of OT instead of serum could improve the control and prevention of *L. infantum* as its collection is easy, cheap, non-invasive and painless, and could be performed by untrained personnel. Consequently, OT could be of use in specific cases, such as dogs that do not have easy access to veterinary clinics or aggressive dogs that can only be touched by its owner. Fortunately, the presence of several types of immunoglobulins (IgA, IgG and IgM) had already been investigated in saliva [21]. IgA is known to be secreted in the salivary glands by plasma cells while IgG and IgM are present in saliva as plasma-derived antibodies [21]. Furthermore, the presence of anti-*Leishmania* antibodies in canine oral fluid samples was already investigated in previous studies [22–25]. These studies showed great success at discriminating between seropositive and seronegative dogs [22–25], although, when specifically only IgA was evaluated, the

technique was not successful at correctly differentiating seropositive dogs from seronegative dogs [22–25]. Nonetheless, none of these studies evaluated the ability to detect anti-*Leishmania* antibodies by using a quantitative in-house ELISA technique in OT samples. In chapter 5, the agreement between the qualitative interpretation of serum and OT ELISA results was evaluated using two methods: (1) percent agreement and (2) agreement according to the kappa agreement statistic. A kappa agreement over 0.80 is needed to be able to validate a new test [26]. When the kappa agreement was interpreted for the total of the OT samples (n=407), a substantial agreement of 0.66 was found, which, unfortunately, was not sufficient to affirm that OT could be used to correctly differentiate between seropositive and seronegative dogs by means of an in-house ELISA. However, a high number of dogs in the study described in chapter 5 presented subclinical infection and low seropositive antibody levels, which was a likely explanation of why the agreement was lower than found in previous studies where the dog population studied was mostly of sick dogs [24,25]. Consequently, when the kappa agreement was obtained only for seronegative dogs and sick dogs with clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection, an almost perfect agreement of 0.84 was obtained, which was enough to affirm that OT could be used to correctly detect antibodies against *L. infantum* in dogs with clinical leishmaniosis or progressing towards disease by means of an in-house ELISA. Even so, there were samples that showed disagreements between the results of serum and OT ELISA. There were samples that showed a positive result in serum and a negative result in OT. The most plausible reason could be related to a lesser ability to detect seropositive dogs with low serum antibody levels and, therefore, very low OT antibody levels as when these dogs were excluded from the statistical analysis, the kappa agreement highly improved. Another reason could be a lack of homogenous OT sample collection, as even if untrained personnel can perform this procedure, it is difficult to perform correctly if the standardized protocol is not followed as described in chapter 5 [27]. As the samples in the study were collected by several veterinarians, even though a standardized protocol was recommended and agreed to, we could not confirm that all samples were always collected in a similar manner. On the other hand, there were also samples that showed a negative result in serum and a positive result in OT. One reason for this result could be related to sand flies which mainly feed on skin areas with very little hair, such as the face [19], which could lead to a local expression of parasite-specific immunoglobulins before the parasite disseminates systemically. Another reason could be an as-yet unknown cross-reactivity with another pathogen, such as oral bacteria, in some dogs with poor dental hygiene and dental disease, such as gingivitis, stomatitis and periodontal disease.

Furthermore, in chapter 4 and 5 of this thesis, serological surveys in Spain using different serological screening tools and samples were performed. In chapter 4, a serological survey of *L. infantum* infection in apparently healthy dogs was performed using a commercial ELISA (Leiscan®) while, in chapter 5, secondary to the development

of an OT ELISA, a serological survey was performed with a quantitative in-house ELISA in both serum and OT [20]. The seroprevalence of canine *L. infantum* infection in dogs in Spain has been previously investigated and, between 2011 and 2020, seroprevalence rates of around 10% have been described [28–30]. Interestingly, different results were observed in the studies performed in chapter 4 and 5. In chapter 4, the seroprevalence rate was lower than expected (6%) while, in chapter 5, the seroprevalence rate was higher (37%). However, the result in chapter 4 could be explained by the inclusion criteria as only apparently healthy dogs with no clinical signs were included in the study and, furthermore, dubious results were not considered as positive results. On the other hand, in chapter 5, the result could be related to the number of sick dogs included in the study that was over 10% of the dogs depending on the region investigated. In terms of specific Spanish areas, previous serological surveys in Spain have documented similar results to the ones described in chapter 4 and 5, detecting lower rates in the North of Spain and higher rates in the Southeast [28–30] which is also the nearest region to the Mediterranean. However, the results found in Islas Baleares in chapter 4 are lower than expected (7%) when compared to previous studies that found seroprevalence rates of around 20% [29–31]. This could be explained with the same reasons as the lower overall seroprevalence rate: only sampling apparently healthy dogs and not considering dubious results as positive. Furthermore, several risk factors related to seropositivity to *L. infantum* were observed. Adult and sick dogs presenting clinical signs and/or clinicopathological abnormalities had higher serological rates of *L. infantum* infection than young and apparently healthy dogs. A high rate should be expected in sick dogs that have been already diagnosed with leishmaniosis and still present clinical signs and/or clinicopathological abnormalities [2,19]. Regarding age, previous studies have described that puppies (under 1 year old) present a lower rate of *L. infantum* infection than dogs aged over 1 year old [28,30] and that the risk of *Leishmania* infection increases with increasing age [30].

In this thesis, other diagnostic techniques related to the immune response were also investigated. Specifically, a study of the effect of storage on NBT reduction test in dog blood samples was performed to confirm if this technique could be used in blood samples collected more than 24 h ago is described in chapter 6. NBT reduction test could be an interesting test to be performed in dogs with leishmaniosis to assess canine neutrophil function [32–35]. For example, dogs with leishmaniosis presenting a mild disease have a higher NBT reduction rate than healthy dogs [32] and, thus, the NBT reduction test could be used to detect improvement in these cases. In fact, any abnormalities or diseases that affect phagocytic activity in both inflammatory and bacterial processes could be studied using the NBT reduction rate. However, the NBT reduction test protocol demands sample processing within 2 to 6 h after blood collection [36], which can be difficult in the clinical setting. Even so, a previous study performed on human samples [37] described that storing blood samples for up to 8 h at 4ºC did not influence the test results. In the study described in chapter 6, the storing of

the dog blood samples at 4ºC was longer, varying from 24 to 72 h, and the results were similarly not influenced by the time lapse. This finding might enable the NBT reduction test to win a place among the standard laboratory tests available to the clinician to assess canine neutrophil function in sick dogs. Furthermore, dog characteristics such as breed, sex and clinical status did not seem to affect the NBT reduction rate during storage, although more information should be included to confirm these results.

Key points:

- **In the clinical setting, the most used screening tools were rapid tests (over 50% of the cases) and ELISA (over 30%).**
- **An in-house ELISA for the detection of specific antibodies against *L. infantum* in canine OT was developed with promising results.**
- **A lower *L. infantum* seroprevalence rate (6%) was observed in apparently healthy dogs in Spain.**
- **Factors such as age and clinical status can influence seropositivity to *L. infantum*.**
- **NBT reduction test could be performed up to 72h after dog blood collection without influencing the test results.**

Future studies:

- **The current use of screening tools in other *L. infantum* endemic countries and in non-endemic countries such as Germany or United Kingdom.**
- **To investigate different techniques to detect the OT quality to confirm correct collection of the sample before performing OT ELISA.**
- **To perform OT ELISA in dogs with poor dental hygiene and presenting dental diseases.**
- **To investigate seronegative dogs that tested positive in OT ELISA to detect seroconversion.**
- **To investigate antibody kinetics in seropositive dogs that tested negative in OT ELISA.**
- **To investigate other serological techniques that could be performed using OT instead of other more traditional samples.**
- **To investigate the effect of storage on NBT reduction test in samples from dogs with other diseases that could affect the number of activated neutrophils such as monocytic ehrlichiosis or diabetes mellitus.**
- **To evaluate if the NBT reduction test could be performed in dog blood samples stored longer than 72h.**
- **To investigate the clinical uselfulness of NBT reduction test in dogs with leishmaniosis and for predicting prognosis.**

New insights of immunotherapy in *L. infantum* infection

As mentioned previously, one of the most promising prophylactic and therapeutic approach against *L. infantum* infection should include the use of immunotherapy to enhance the specific immune response of the dog against the parasite [38]. Immunotherapy could be used in several ways related to *L. infantum* [38]. For example, domperidone, a dopamine D_2 receptor antagonist, has been used in seronegative dogs to prevent parasite infection [5,39] and in sick dogs with clinical signs of leishmaniosis to help reducing the clinical signs [5,40–42]. In seronegative dogs, domperidone is usually used as a preventive measure against *L. infantum* infection [5,39]. On the other hand, in sick dogs with clinical signs of leishmaniosis, domperidone is used with other products such as antimonials and allopurinol to control and reduce clinical signs of leishmaniosis together with a reduction of the anti-leishmanial antibody levels [40–42]. However, most of these studies mentioned above present some limitations such as lack of an appropriate control group [39–42], short follow-up periods [39] and small numbers of dogs studied [39,41,42]. Furthermore, no previous studies have investigated the use of domperidone in *Leishmania*-seropositive healthy dogs.

As there is little information about *Leishmania*-seropositive apparently healthy dogs, which are a clinically neglected group regarding monitoring and treatment of *L. infantum* [2], a descriptive study of the signalment, clinicopathological findings and serological status of *Leishmania*-seropositive apparently healthy dogs is described in chapter 7. Improving the knowledge about these dogs is highly important to control and prevent *L. infantum* infection in endemic countries and disease progression [1]. According to a longitudinal study [43], *Leishmania*-seropositive healthy dogs will develop clinical signs and clinicopathological abnormalities over time. The study presented in chapter 7 reported that an important proportion of apparently healthy dogs by physical examination can present several clinicopathological findings without evident clinical signs and, therefore, the disease could be diagnosed earlier if a full clinical examination and routine laboratory tests were performed after seropositivity to *L. infantum* was confirmed. Most of the dogs that presented clinicopathological findings were classified in LeishVet stage IIa [12] and the most consistent findings were plasma protein alterations including polyclonal hypergammaglobulinemia, hyperproteinemia and decreased A/G ratio, proteinuria and lymphopenia. Furthermore, the majority of healthy dogs without clinicopathological abnormalities presented low antibody levels against *L. infantum* antigen while most apparently healthy dogs with clinicopathological alterations presented medium to high antibody levels which is in concordance with previous studies that reported that dogs with high antibody levels show more pronounced clinicopathological abnormalities and, thus, are classified in higher stages of CanL [20,24,44]. In fact, also in chapter 7, dogs with higher antibody levels showed a tendency for a higher degree of laboratory alterations. Interestingly, age was also reported as a risk factor as it presented several correlations with clinical data such

as ALT and creatinine levels. It has already been reported that older dogs tend to present clinicopathological abnormalities due to age-related diseases and that could also be a risk factor and affect disease development and worsening [45,46].

In chapter 8, *Leishmania*-seropositive healthy dogs without clinicopathological abnormalities were further studied in a blinded, randomized and controlled multicentre clinical trial to assess the efficacy and safety of Leisguard® as an immunotherapeutic treatment to prevent disease development. As previously mentioned, the development of clinical leishmaniosis depends largely on the dog's immune response [47,48]. Thus, a treatment that can enhance the immune response of the dog could provide an alternative direction to combating the infection [38,49,50]. In this thesis, the first clinical trial testing the clinical efficacy and safety of domperidone (Leisguard®) in healthy dogs seropositive to *L. infantum* infection is presented in chapter 8. In this study, dogs treated with domperidone were less likely to present disease development than dogs treated with placebo. These differences were highly significant in low seropositive dogs while, in medium to high seropositive dogs, disease development was similar in both treatment groups. The reason for these results is probably related to the association between high antibody levels and dissemination of the parasite and clinical disease observed in previous studies [20]. Therefore, based on the results in chapter 8, treatment with domperidone alone would not be enough to avoid disease development in healthy dogs with high antibody levels. Furthermore, most of the dogs that developed disease presented clinicopathological abnormalities such as hyperglobulinemia and hyperproteinemia, while a minority presented also clinical signs such as skin lesions or weight loss. These clinicopathological findings and clinical signs have already been described in dogs with leishmaniosis [2,6]. The fact that the majority of the dogs only presented clinicopathological abnormalities with no clinical signs and, thus, were apparently healthy, highlights again the importance of performing routine laboratory tests in apparently healthy *L. infantum*-seropositive dogs to detect disease development which could improve disease prognosis and shorten treatment duration [2,12]. Surprisingly, no differences were found in antibody levels during the follow-up period between dogs treated with domperidone and dogs treated with placebo. In a previous study [41], a reduction of anti-*L. infantum* antibody levels was observed in dogs with leishmaniosis affected by CKD and treated with domperidone. It could be possible that dogs with clinical disease and very high seropositivity present a significant reduction of anti-*L. infantum* antibody levels when treated with domperidone while, in the study described in chapter 8, the majority of dogs were healthy and presented low antibody levels. Additionally, only three dogs treated with domperidone presented mild adverse drug reactions. The three dogs presented a self-limiting diarrhea for one or two days. This adverse drug reaction is already listed in the prospectus of the product [51]. Furthermore, it is detailed that this effect should disappear after the treatment is withdrawn [13,51,52], although in the present study the treatment with domperidone was not withdrawn, as the treatment

was still administered for four consecutive weeks, and the diarrhea also disappeared. Therefore, the administration of domperidone in healthy seropositive dogs appears to be clinically safe.

Key points:

- ***Leishmania*-seropositive apparently healthy dogs can present several clinicopathological findings that could indicate disease development.**
- **The majority of *Leishmania*-seropositive apparently healthy dogs without clinicopathological alterations presented low antibody levels against *L. infantum* antigen.**
- **The majority *Leishmania*-seropositive apparently healthy dogs with clinicopathological alterations presented medium to high antibody levels against *L. infantum* antigen.**
- **The majority *Leishmania*-seropositive apparently healthy dogs with clinicopathological alterations were classified in LeishVet stage IIa and the most consistent findings were plasma protein alterations, proteinuria and lymphopenia.**
- **Domperidone treatment was able to reduce disease development in *Leishmania*-seropositive healthy dogs.**
- **Domperidone treatment was highly effective in reducing disease development in low seropositive dogs.**
- **Administration of domperidone in healthy seropositive dogs was clinically safe.**

Future studies:

- **To investigate the immune response of *Leishmania*-seropositive apparently healthy dogs and its interaction with clinicopathological findings and disease development.**
- **To investigate the use of domperidone in dogs with clinical leishmaniosis in combination with traditional therapies such as antimonials and allopurinol.**

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Conclusions

1. The seroprevalence of *L. infantum* in apparently healthy dogs in Spain varied from almost no infection in the Northern areas to being over 10% in the Southeast close to the Mediterranean basin.
2. The majority of dogs (91.5%) used preventive measures against *L. infantum* infection in endemic regions being repellents the most used preventive measures while vaccines and Leisguard® were second-line options.
3. The most used serological screening tools in the clinical setting to detect specific *L. infantum* antibodies were rapid tests (56.7%) and ELISA tests (34.1%).
4. An in-house ELISA for the detection of specific antibodies against *L. infantum* in OT was developed with promising results in sick dogs with high antibody levels.
5. The NBT reduction test was validated and could be performed up to 72 h after collection of canine blood if correctly refrigerated at 4ºC.
6. *Leishmania*-seropositive apparently healthy dogs by physical examination can present several clinicopathological findings and, therefore, disease could be diagnosed earlier. Most of the sick dogs were classified in LeishVet stage IIa and the most consistent findings were plasma protein alterations, proteinuria and lymphopenia.
7. The majority of *Leishmania*-seropositive healthy dogs without clinicopathological abnormalities presented low antibody levels against *L. infantum* antigen while most apparently healthy dogs with clinicopathological alterations presented medium to high antibody levels.
8. The use of domperidone in healthy dogs proved to be effective against disease development, especially in dogs with low *L. infantum* antibody levels.
9. Domperidone presented a good safety profile in *Leishmania*-seropositive healthy dogs.

Addendum

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Immunotherapy in clinical canine leishmaniosis: a comparative update

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ABSTRACT

Leishmaniosis due to *Leishmania infantum* is a complex infection that can affect both humans and dogs, and present a wide range of clinical signs and clinicopathological abnormalities. The conventional treatment of this disease is challenging due to the fact that complete parasitological cure commonly does not occur. Furthermore, treatment of the disease with the conventionally used drugs has several shortcomings. These include the need for long-term treatment, side effects and the formation of drug resistance. Moreover, it is important to highlight that the host immune responses play a crucial role in the outcome of this infection. For this reason, the use of immunotherapy in clinical leishmaniosis to improve the result of treatment with the conventional anti-leishmanial drugs by enhancing the immune response is imperative. The aim of this review is to provide a comparative overview of the wide range of immunotherapeutical approaches and strategies for the treatment of *L. infantum* infection in animals focusing on dogs.

1. Introduction

Leishmanioses are important neglected tropical diseases in humans caused by different species of the protozoan genus *Leishmania* (Pace, 2014). *Leishmania infantum*, which causes a zoonotic disease with the dog as the main reservoir, is encountered most frequently in the Mediterranean basin, Middle East, Asia and South America (Baneth et al., 2008; Solano-Gallego et al., 2009). *Leishmania infantum* is transmitted through the bite of a female phlebotomine sand fly (Akhoundi et al., 2016; Petrella et al., 2015). The biological cycle is alternated between an amastigote form in a vertebrate host and the promastigote form in the gut of the sand fly vector (Akhoundi et al., 2016; Baneth et al., 2008; Solano-Gallego et al., 2009).

The manifestations of canine leishmaniosis (CanL) are closely associated with the host's immune responses (Hosein et al., 2017). The two extreme profiles representing the wide spectrum of immune responses are “resistant” hosts that display a protective lymphocyte T helper 1 (Th1)-cell mediated immune response, and “extremely susceptible” hosts displaying a marked humoral immune response combined with absent or diminished cell-mediated immunity (CMI) and a high parasite burden (Hosein et al., 2017).

The wide range of clinical manifestations found in *Leishmania* infection can vary from a total absence of clinical signs to a severe fatal clinical disease depending on the infecting species and the host immune response (Miró et al., 2008; Pace, 2014; Solano-Gallego et al., 2009). In CanL due to *L. infantum*, the most common clinical signs among a large variety of other clinical conditions are skin lesions, weight loss and generalized lymphadenomegaly (Baneth et al., 2008; Pennisi, 2015; Solano-Gallego et al., 2009).

The most common treatment for CanL includes the parenteral administration of antimonials, which combined with other drugs reduces the parasitic load (Miró et al., 2008; Solano-Gallego et al., 2011). Thus, the most frequent treatment is usually a combination of antimonials or miltefosine with allopurinol, which maintains the parasitic load at low levels (Reguera et al., 2016; Solano-Gallego et al., 2011, 2009). However, conventional anti-*Leishmania* drugs used in dogs can induce side effects such as nephrotoxicity, urolithiasis and digestive disorders (Ikeda-Garcia et al., 2007; Koutinas et al., 2001; Manna et al., 2008; Miró et al., 2009). In addition, drug resistance to antimonials (Carrió and Portús, 2002) or allopurinol (Yasur-Landau et al., 2017) has been described in dogs.

The aim of this review is to provide a comparative overview of the current approaches in the use of immunotherapy against leishmaniosis due to *L. infantum* in dogs with comparison to its use in experimental rodent and primate infections and human leishmaniasis.

2. Why is immunotherapy for clinical CanL important?

As previously mentioned, the outcome of infection by *Leishmania* depends largely on the host's immune response (Hosein et al., 2017;

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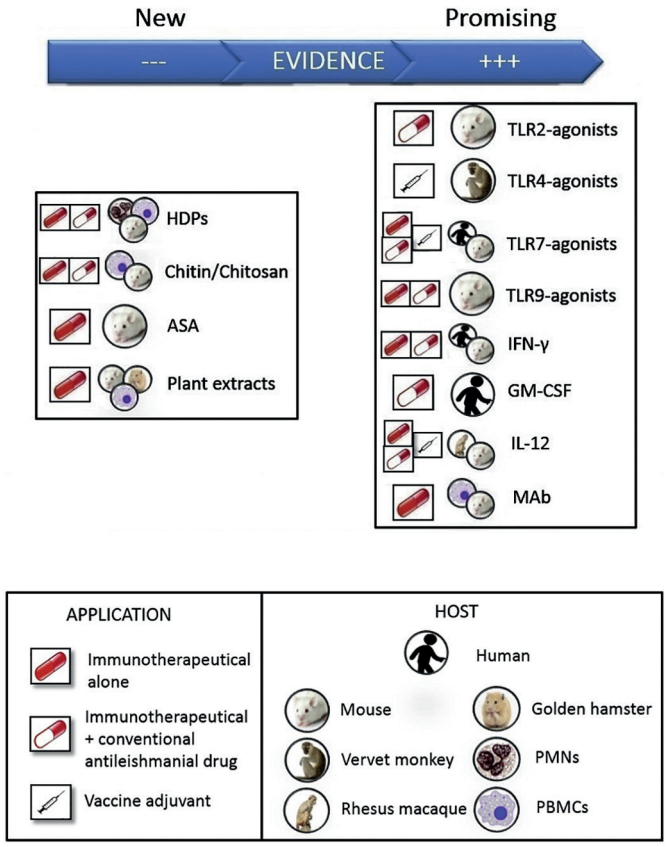

Fig. 1. Summary of compounds studied for their effect on the immune response against leishmaniosis in mice, primates and humans.
Abbreviations: ASA: Acetyl salicylic acid; GM-CSF: Granulocyte/macrophage colony stimulating factor; HDPs: Host defense peptides; Mab: Monoclonal antibody; PBMCs: Peripheral blood mononuclear cells; PMNs: Polymorphonuclear cells; TLRs: Toll-like receptors

Khadem and Uzonna, 2014; Paltrinieri et al., 2010). Thereby, treatment that can enhance the host's immune system could provide an alternative direction to combating the infection (Singh and Sundar, 2014; Taslimi et al., 2016).

The use of immunotherapy does not directly attack the pathogen, as other drugs would, but it modulates the host's immune response increasing its protection from the disease (Singh and Sundar, 2014). Following this idea, researches have been searching for different compounds that could improve the immune response against *Leishmania* infections (Fig. 1). Compounds such as dietary nucleotides and active hexose dietary compound (AHCC) (Impromune®), and domperidone (Leisguard®) have already been commercialized for usage in CanL. Dietary nucleotides appear to promote the phagocytic activity of macrophages and T lymphocytes in human infants (Carver et al., 1991; Navarro et al., 1999) and in experimentally infected rodents (Jyonouchi et al., 1996, 1994; Van Buren et al., 1985). AHCC has been reported to promote the activity of natural killer (NK) cells, proliferation of macrophages and differentiation of T lymphocytes to the Th1 cell subset in human and rodent peripheral blood mononuclear cells (PBMCs) (Lee et al., 2012; Aviles et al., 2008). Domperidone is a dopamine D2 receptor antagonist that can potentiate the immune response through modulating the effect of prolactin (Gómez-Ochoa et al., 2009; Lladró et al., 2017; Passos et al., 2014; Sabaté et al., 2014). Furthermore, several studies have evaluated alternative immunotherapeutical compounds not commercialized yet including several cytokines (Badaro et al., 1990; Santos et al., 2004), toll like receptor (TLR) agonists (Mutiso et al., 2012; Shakya et al., 2011) or chitosan (Hoseini et al., 2016), which yielded promising results (Fig. 2).

3. Commercially-marketed immunotherapy compounds

3.1. Domperidone (Leisguard®)

Domperidone is a dopamine D_2 receptor antagonist developed and synthesized in 1974 by Janssen Pharmaceutica (Beerse, Belgium) and patented in the U.S.A. in 1978.

Domperidone was approved for use for both prevention and treatment of CanL due to *L. infantum* by the Heads of Medicine Agencies (HMA) in 2011 (HMA, 2016). Specifically, it is indicated to reduce the risk of developing an active infection in seronegative healthy dogs as preventative measure and improving mild clinical disease, through the enhancement of the CMI response (HMA, 2016). This is due its capacity to potentiate the activity of phagocytic cells such as monocytes, macrophages and neutrophils, and potentially contributing to the establishment of a predominantly Th1 immune response (HMA, 2016). The origin of these effects is related to the release of serotonin in the hypophysis which causes a transitory increase in blood levels of prolactin (Gómez-Ochoa et al., 2009; Rovenský et al., 1995). Prolactin has been classified as a pro-inflammatory lymphocyte-derived cytokine (Hinterberger-Fischer, 2000). Hence, increasing the production of prolactin induces a boost of T $CD4^+$ lymphocytes, in addition to the release of cytokines such as IL-2 and IL-12, IFN-γ and TNF-α, producing an activation of NK cells and macrophages, followed by a decrease of $CD4^+$ Th2 cytokines and TNF-β (Di Carlo et al., 1993; Majumder et al., 2002; Richards et al., 1998). It is accepted that a predominantly Th1 immune response including IL-2, IL-12, IFN-γ and TNF-α is able to control leishmaniosis while susceptibility to the disease has been associated with IL-4 secretion and the Th2-type immune response (Alexander and Bryson, 2005; Costa et al., 2012; Hosein et al., 2017).

In a study performed by Gómez-Ochoa et al. (2012), healthy dogs that received a 30-day course of domperidone treatment showed a rapid increase of the percentage of activated neutrophils when compared with untreated dogs. In a previous study (Gómez-Ochoa et al., 2009), a clinical trial was performed with 98 dogs with mild clinical signs of leishmaniosis. In this study, domperidone was suggested as effective in controlling and reducing clinical signs of leishmaniosis in dogs together with a reduction of the anti-leishmanial antibody titer. In another study (Passos et al., 2014), treatment with furazolidone and domperidone was administered to twelve dogs naturally infected with *L. braziliensis* with good results related to the decrease of skin lesions associated with this infection. On the other hand, the control group of this same study (Passos et al., 2014) was not treated, thus, it was not possible to determine if the results were produced by domperidone, furazolidone or a combination of both.

However, all studies mentioned above present some limitations such as lack of an appropriate control group (Gómez-Ochoa et al., 2012, 2009; Passos et al., 2014), short follow-up periods (Gómez-Ochoa et al., 2012) and small numbers of dogs studied (Gómez-Ochoa et al., 2012; Passos et al., 2014). These limitations highlight the need for more complete studies to support the above-mentioned results.

Domperidone is marketed commercially in the clinical practice as treatment for CanL in several european countries with a high frequency of use in Spain, Portugal and Italy (Mattin et al., 2014). In a study performed in north-eastern Spain (Lladró et al., 2017), 7% of the clinics used domperidone alone or combined with allopurinol as a first-line treatment for CanL while 3.5% employed domperidone alone or combined with allopurinol as second-line treatment. Additionally, domperidone was the third most used compound as a preventive measure against leishmaniosis (50% of the investigated clinics) after topical insecticides (98%) and vaccination (67%) (Lladró et al., 2017). Importantly, some adverse effects associated with domperidone are sporadically observed in treated dogs and include mammary gland disorders, which disappear after treatment discontinuation, lethargy and digestive disorders (Agencia Española de Medicamentos y Productos Sanitarios, 2013; Lladró et al., 2017). Behavioral disorders

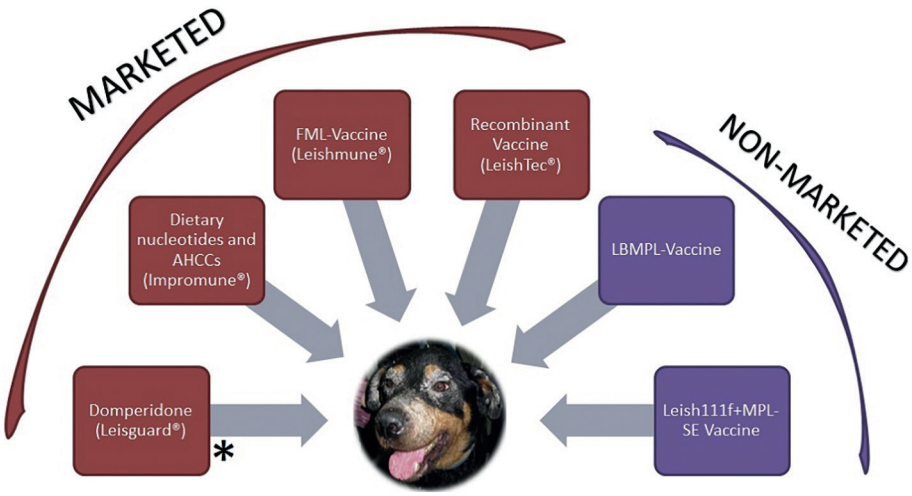

Fig. 2. Summary of the commercially available and non-available compounds and vaccines studied for the immunotherapy of clinical canine leishmaniosis. Abbreviations: AHCCs: Active hexose correlated compounds; FML: Fucose mannose ligand; LBMPL: *L. braziliensis* Promastigote protein + MPL; Leish111f: Recombinant protein of thiol-specific antioxidant (TSA), *Leishmania major* stress-inducible protein 1 (LmSTI1) and *Leishmania* elongation initiation factor (LeIF); MPL: Monophosphoryl lipid A; SE: Stable emulsion
*Domperidone is the only compound registered for treatment of sick dogs with mild CanL in some european countries (Spain, Portugal, Italy and Greece).

have also been documented rarely with domperidone treatment (Agencia Española de Medicamentos y Productos Sanitarios, 2013).

Domperidone is also used in human medicine as treatment of nausea and vomiting, but a restriction on the use of domperidone-containing medicines was issued in 2014 by the European Medicine Agency (EMA) (EMA, 2014) because of its multiple and dangerous side effects related to cardiopathies in humans. These side effects have not been studied in dogs yet, most likely because the recommended dose of domperidone falls within the safe range, but co-administration of this medication with other drugs with similar side effects or the use of products that could enhance the absorption of domperidone may induce effects similar to those observed in humans (Travi and Miró, 2018).

3.2. Dietary nucleotides and active hexose correlated compounds (AHCC) (Impromune®)

Dietary nucleotides are low molecular weight intracellular compounds which are naturally present in all types of food of plant and animal origin, although higher levels are found in meat, fresh seafood, seeds and dried legumes (Gil, 2002; Hess and Greenberg, 2012; Ulbricht et al., 2013). Dietary nucleotides have been widely studied and used to increase lipid metabolism, immune responses as well as the development and repair of tissue growth in human infants (Carver et al., 1991; Navarro et al., 1999) and rodents (Jyonouchi et al., 1996, 1994; Van Buren et al., 1985). Active hexose correlated compounds (AHCC) are alpha-glucan-rich dietary supplements extracted from mushrooms that has been reported to have antioxidant activity and induce improvement of the Th1 immune response associated with increment of NK cells, T cells, B cells and cytokines such as IL-12 and TNF-α in human PBMCs and rodents (Lee et al., 2012; Aviles et al., 2008).

A study performed with BALB/c mice investigated the effect of dietary nucleotides on the immune function (Xu et al., 2013) demonstrating that dietary nucleotides could enhance the innate and adaptive immune responses of mice through stimulation of Th cells and cytokines (Xu et al., 2013).

Several studies have investigated how dietary nucleotides could improve the treatment of CanL (Cortese et al., 2015; Segarra et al., 2018, 2017). Cortese and collaborators (2015) analyzed T cell populations including $CD3^+$ $CD4^+$ $Foxp3^+$ regulatory T cells (Treg), and $CD3^+$ $CD4^+$ IFN-γ^+ Th1 cells in the blood of dogs after treatment with an immune-modulating diet. A group of dogs treated with a standard anti-leishmanial drug treatment supplemented with an immune-modulating diet and a group of dogs with the same drug treatment but supplemented with a standard diet (Cortese et al., 2015) were studied. The results showed that dogs which received the immune-modulating diet presented an increase in Treg population and a decrease in Th1 inflammatory response in addition to a mild improvement in the decrease of clinical signs (Cortese et al., 2015). The effects of dietary nucleotides and AHCC were investigated in another clinical trial in dogs with CanL (Segarra et al., 2017). A standard treatment of meglumine antimoniate (Glucantime®) and allopurinol was administered to the first group while the second group received a combination of meglumine antimoniate and AHCC and dietary nucleotides (Segarra et al., 2017). The study results showed that both treatments presented similar efficacy and, therefore, this new treatment modality could be a good alternative for dogs with CanL suffering from adverse effects of allopurinol treatment such as urolithiasis and renal mineralization (Koutinas et al., 2001; Segarra et al., 2017). Despite these results, there is need for more studies on the treatment of CanL with dietary nucleotide due to the use of different diets used in the same group (Cortese et al., 2015; Segarra et al., 2017), short follow up period (Segarra et al., 2017), lack of a control group (Segarra et al., 2017) in the studies and the use of a non-standardized clinical scoring system (Segarra et al., 2017). In another study, clinically healthy *Leishmania*-infected dogs, most of which were seropositive, were also treated with dietary nucleotides and AHCC to prove the effect of the diet in delaying the progression of the disease (Segarra et al., 2018). The outcome of this study showed that the oral administration of dietary nucleotides and AHCC is safe and can reduce the rate of disease progression from a clinically healthy infected status into clinical disease, although it was also stated that additional clinical trials with other drug combinations and larger sample sizes are needed to confirm these observations (Segarra et al., 2018).

3.3. Therapeutic vaccines

Four vaccines have been marketed for CanL (Singh and Sundar, 2014; Solano-Gallego et al., 2017). Two of them are marketed commercially in Europe: CaniLeish® (Bongiorno et al., 2013; Moreno et al., 2014, 2012; Oliva et al., 2014) and Letifend® (Carcelén et al., 2009; Fernández Cotrina et al., 2018); while the other two were available in Brazil: Leishmune® (Borja-Cabrera et al., 2002; Marcondes et al., 2011) and Leish-Tec® (Grimaldi et al., 2017; Regina-Silva et al., 2016). Only the Leish-Tec® vaccine is currently available in Brazil while the Leishmune® vaccine has been taken off the market in Brazil.

Some of the canine vaccines, and other vaccines which are still in early stages of investigation, have been studied for use as treatment of clinical CanL. The major outcomes were similar in all studies: clinical improvement in treated dogs, which was more relevant in mild or moderate disease than in severe disease, and in some of them was also observed a reduction of the parasitic load. These studies are summarized in Table 1.

Table 1
Vaccines with proven immunotherapeutic activity against *L. infantum* in dogs.

Type of vaccine (Brand name)	Vaccine composition	Type of study	Number of dogs treated	Parasite species*	Outcome	Reference
FML-vaccine (Leishmune®)	FML + Riedel de Haen saponin	Multi-center, controlled, double-blind and randomized	31	*L. infantum*	Reduction of proportion of symptomatic dogs (from 100% to 38%) and deaths (from 54% to 12%)	(Borja-Cabrera et al., 2010)
FML-vaccine (Leishmune®)	FML + Riedel de Haen saponin	Single-center, controlled and randomized	12	*L. infantum* (experimental infection)	Improvement of the clinical profile and reduction of parasite load	(Santos et al., 2007)
FML-vaccine (Leishmune®)	FML + QuilA saponin	Single-center and open-label	5	*L. donovani* (experimental infection)	Reduction of clinical signs after complete vaccination (3/5). Died without symptoms (1/5). Died of disease (1/5)	(Borja-Cabrera et al., 2004)
FML-vaccine (Leishmune®)	FML + saponin R	Multi-center, controlled and open-label	21	*L. infantum*	Treated remained asymptomatic (19/21) or with mild clinical signs (2/21)	(Borja-Cabrera et al., 2004)
LaSap-vaccine	Total antigens of *L. amazonesis* + saponin	Single-center and open-label	8	*L. infantum*	Improvement of the clinical profile and reduction of parasitic load	(Viana et al., 2018)
LBMPL-vaccine	*L. braziliensis* promastigote protein+ MPL	Single-center, controlled and open-label	6	*L. infantum*	Normalization in RBC parameters, urea, creatinine, AST, ALP and bilirubin. Reduction of parasitic load and clinical signs (75%)	(Roatt et al., 2017)
Leish-111f + MPL-SE vaccine	Leish-111f (TSA + LmSTI1 + LeIF) + MPL-SE	Single-center, controlled and open-label	18	*L. infantum*	Improvement of the clinical profile (75% considered cured)	(Trigo et al., 2010)
Leish-111f + MPL-SE vaccine	Leish-111f (TSA + LmSTI1 + LeIF) + MPL-SE	Single-center, controlled, single-blind and randomized	15	*L. infantum*	Improvement of the clinical profile (better in moderate disease than in severe)	(Trigo et al., 2010)
(Leish-Tec®)	A2-based recombinant protein + saponin	Multi-center, controlled, double-blind and randomized	250	*L. infantum*	Reduction of rate of deaths without treatment	(Toepp et al., 2018)
rLdccys1-vaccine	Recombinant cysteine proteinase from *L. infantum* + *Propionibacterium acnes*	Single-center, controlled and open-label	10	*L. infantum*	Control of disease development and reduction of parasitic load	(Ferreira et al., 2014)

Abbreviations: A2: recombinant *L. infantum* and *L. donovani* amatigote-specific antigen; ALP: Alkaline phosphatase; AST: Aspartate transaminase; FML: Fucose mannose ligand; LeIF: *Leishmania* elongation initiation factor; Leish-111f: trifusion recombinant protein of TSA, LmSTI1 and LeIF; LmSTI1: *L. major* stress-inducible protein 1; MPL: Monophosphoryl lipid A; QuilA: saponin adjuvant produced with *Quilaja saponaria*; RBC: Red blood concentration; rLdccys1: recombinant cysteine proteinase from *L. infantum*; SE: Stable emulsion; TSA: Thiol-specific antioxidant.

* All infections except for those marked were natural infections.

4. Non-commercially available immunotherapy

4.1. Toll-like receptor (TLR) agonists

Toll-like receptors (TLRs) are type I transmembrane proteins which comprise one of the first defense lines against pathogens (Oda and Kitano, 2006). There are ten TLRs (TLR1–TLR10) described in dogs (Cuscó et al., 2014) as well as in humans, and 12 in mice (TLR1–9, TLR11–13) (Gay and Gangloff, 2007; Pasare and Medzhitov, 2004). TLRs are located in either the plasma or internal membranes of inflammatory cells including macrophages, dendritic cells (DC), NK cells and lymphocytes (T and B) as well as other types of cells such as keratinocytes. Their function is to bind conserved molecular structures found in large groups of pathogen-associated molecular patterns (PAMPs) and induce the secretion of inflammatory cytokines such as type-1 interferon (IFN), chemokines and co-stimulatory molecules (Medzhitov, 2001). TLR agonists are natural and synthetic PAMPs (Gnjatic et al., 2010) that bind to TLRs to activate signalling pathways to manage innate and acquired immune responses (Steinhagen et al., 2011). They amplify immune reactions against parasites by stimulating the production of pro-inflammatory cytokines playing an important role in controlling *Leishmania* infection (Ribeiro-Gomes et al., 2007). TLR agonists are promising compounds for prevention and immunotherapy in human leishmaniasis and CanL (Roatt et al., 2014). However, limited information is available on their potential treatment benefits to both species while the majority of research on this topic has been carried out in rodents or non-human primates.

4.1.1. TLR2 agonists

The immunotherapeutic potential use of the protein aggregate of magnesium–ammoniumphospholinoleate–palmitoleate anhydride (P-MAPA) was evaluated in dogs with leishmaniosis by Santiago et al. (2013). P-MAPA is a compound obtained from the fungus *Aspergillus oryzae* and it has been demonstrated to activate TLR2 in human embryonic kidney (HEK) cells (Fávaro et al., 2012). The clinical improvements observed in sick dogs with leishmaniosis treated with the immunomodulatory P-MAPA were accompanied with diminution of the skin parasite load, increased levels of IFN-γ and low IL-10 production after P-MAPA treatment (Santiago et al., 2013). PBMCs and macrophages from *Leishmania* infected dogs were also studied to investigate the immunomodulatory effect of P-MAPA (Melo et al., 2014). Macrophages from infected dogs treated with high concentrations of P-MAPA increased TLR2 expression when compared to controls. In addition, the concentration of reactive oxygen species (ROS) was increased in PBMCs from infected dogs suggesting the immunomodulator role of P-MAPA associated with restoring the immune balance (Melo et al., 2014).

The prophylactic action of Pam3Cys, a TLR2 agonist, in preventing pathogen infection and reducing their establishment was demonstrated using a murine model of *L. donovani* infection (Shakya et al., 2011). This study is reviewed in Table 2.

4.1.2. TLR4 agonists

The high potency with which TLR4 activates inflammatory pathways makes it an ideal target for therapeutic intervention and adjuvant development (Dowling and Mansell, 2016). Several studies have explored the use of a TLR4 agonist as adjuvant in vaccines against leishmaniosis (Carter et al., 2016; Coler et al., 2015; Duthie et al., 2016). Vaccinated vervet monkeys were challenged with virulent *L. donovani* parasites following intradermal inoculation of *L. donovani* sonicated antigen delivered with either alum, montanide ISA 720 (MISA) or the TLR4 agonist monophosphoryl lipid A (MPLA) (Mutiso et al., 2012). MPLA failed to induce increased IFN-γ production compared to the other two adjuvants (Mutiso et al., 2012). In a similar study described by Mutiso et al. (2012), a group of vervet monkeys treated with MPLA and *L. donovani* antigen showed significantly lower skin delayed-type hypersensitivity (DTH) to the sonicate antigen when

Table 2
Non-commercially available compounds which induce proven immune response activity against *L. donovani*.

Compound	Type of study	Experimental model	Drug route of administration	Immune response effect	Outcome	Reference
HDP[a] LL-37, E6, L-1018 and RI-1018	Single-center, controlled, open-label and *in vitro*	THP-1 human macrophages	Culture	Reduction of redox activity	Reduction of amastigote infection	(Marr et al., 2016)
Mab Anti-IL-10R	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of nitric oxide synthase activity and production of IL-12 and IFN-γ	Reduction parasite burden	(Murray et al., 2002)
Mab Anti-IL-10R and anti-GITR	Single-center, controlled, open-label and *in vivo*	C57BL/6 J mice	Intraperitoneal injection	Increase of production of IFN-γ and TNF-α	Reduction parasite burden	(Faleiro et al., 2016)
Plant extract *Grifola frondosa*	Single-center, controlled, open-label and *ex vivo*	BALB/c murine macrophages	Culture	Increase of production of IL-12, IL-1β, TNF-α. Downregulation of IL-10 and TGF-β. Increase of NO production	Inhibition amastigotes replication	(Sultana et al., 2018)
Plant extract *Sterculia villosa*	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of production of IL-12 and IFN-γ. Downregulation of IL-10 and TGF-β	Reduction parasite burden	(Das et al., 2017)
Plant extract *Withania somnifera*	Single-center, controlled, open-label and *in vivo*	Golden hamsters (*Mesocricetus auratus*)	Oral	Increase of production of IFN-γ and IL-12, inducible NO synthase mRNA transcript and suppressed levels of IL-4, IL-10 and TGF-β	Inhibition parasite multiplication	(Tripathi et al., 2017)
TLR2 agonist[b] Pam3Cys	Single-center, controlled, open-label and *in vivo*	BALB/c mice	Intraperitoneal injection	Increase of production of IFN-γ.	Reduction infection rate	(Shakya et al., 2011)

Abbreviations: anti-GITR: anti-glucocorticoid-induced TNF receptor related protein; HDP: Host defense peptide; MAb: Monoclonal antibody; NO: Nitric oxide; Pam3Cys: N-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-Cys-Ser-Lys4 hydrochloride; Redox: Reduction-oxidation.

[a] This compound was also studied in *L. major*.

[b] This compound was administered before and after *L. donovani* infection.

compared with treatment group exposed to alum as adjuvant with *L. donovani* antigen (Mutiso et al., 2012).

The potential use of TLR4 agonists in combination with leishmanial antigen was evaluated for immunotherapy of sick dogs with leishmaniosis (Roatt et al., 2017). Those sick dogs treated with both *L. braziliensis* antigen and a TLR4 agonist (MPLA) showed clinical improvement. Moreover, a reduction in the transmission of the *Leishmania* to sand flies evaluated by xenodiagnosis was observed. This study is also reviewed in table 1.

4.1.3. TLR7 agonists

Imiquimod (IMQ) is a TLR7 agonist that is currently approved as topical treatment of cutaneous leishmaniasis (CL) in humans (Sauder, 2003) and has also been utilized as a vaccine adjuvant in several studies of *Leishmania* infection (Emami et al., 2018; Zhang and Matlashewski, 2008). A successful case of topical use of imiquimod in CL due to *L. infantum* has been described in a 7 year old child from Balearic islands (Hervás et al., 2012). This patient's lesions were not improved by previous treatments received by the patient, including liposomal amphotericin (Hervás et al., 2012). The prophylactic and therapeutic use of topical resiquimod, a TLR7/8 agonist, were studied in a *L. infantum* visceral leishmaniasis (VL) murine model. Topical resiquimod was applied in conjunction with subcutaneous or intravenous inoculation of *L. infantum* originally isolated from a patient from north-eastern Brazil to vaccinate and challenge experimental animals. High levels of protection (> 90%) were achieved in vaccinated animals accompanied by resiquimod. Furthermore, BALB/c mice which were treated for 4 weeks with resiquimod after infection with virulent *L. infantum*, had a reduction in liver parasite burdens, demonstrating that resiquimod had beneficial immunomodulatory effects in experimental systemic, organ-infecting VL in mice (Craft et al., 2014). However, a pilot study using IMQ cream (Aldara®) as the only treatment for stage I-CanL papular dermatitis failed to cure lesions in dogs (Ordeix et al., 2018).

4.2. Cytokines

Cytokines have crucial roles in the control of infection as well as in the progress of disease manifestation. For this reason, many studies have focused on the use of cytokines as treatment for leishmaniosis. The mechanisms of action of the major cytokines involved in the disease outcome of leishmaniosis are those which influence the balance between the Th1 and Th2 cytokines. Various pro-inflammatory Th1 cytokines such as IL-12, IFN-γ, TNF-α and IL-2 have been identified as associated with the control of the disease. Contrarily, non-protective Th2 cytokines such as IL-4 and IL-10 have been related to susceptibility to the development of *Leishmania* infections (Costa et al., 2012).

The fact that IFN-γ activates macrophages to kill intracellular amastigotes and that it is mainly produced by antigen stimulated T lymphocytes has been repeatedly demonstrated in several animal species including mice (Bhattacharya et al., 2015), dogs (Hosein et al., 2017) and also in humans (Tripathi et al., 2007). However, recombinant human IFN-γ in conjunction with pentavalent antimonial (Glucantime®) therapy has been shown to induce an increase in treatment success and clinical cure; possibly due to the effect of IFN-γ induced macrophage activation (Badaro et al., 1990). Several studies in human patients from Brazil, Kenya, and India, have demonstrated that the use of IFN-γ therapy in VL accelerates the anti-parasitic effect of pentavalent antimonials compared with use of this cytokine alone (Badaro et al., 1990; Squires et al., 1993; Sundar et al., 1995; Sundar and Murray, 1995).

4.3. Monoclonal antibodies (MAbs)

The therapeutic monoclonal antibody (MAb) market has increased exponentially since the first MAb was commercialized in 1986. MAb products are currently approved for treatment of a large variety of diseases (Ecker et al., 2015). The advantages of therapeutic MAbs as a treatment usually include low toxicity, high specificity and versatility of activity (Jones, 2015).

IL-10 has been identified in the murine model as a potent suppressor of CMI during *Leishmania* infection (Kane and Mosser, 2001). A study of IL-10 antibody neutralization in cell cultures of splenic aspirates from human VL patients showed a decrease in the number of amastigotes concomitantly with an increased production of IFN-γ and TNF-α (Gautam et al., 2011). Moreover, anti-IL-10R MAbs used for the reduction of IL-10 levels to treat experimental *Leishmania* infection have been widely studied (Faleiro et al., 2016; Gautam et al., 2011; Murray et al., 2002). Those studies performed in murine models experimentally infected with *L. donovani* are summarized in Table 2.

However, the use of MAbs is not always beneficial. For example, the treatment of rheumatoid arthritis with MAbs in human clinical practice has been associated with an increased risk for VL (Bassetti et al., 2006; Guarneri et al., 2017; Khan et al., 2010). This is because the MAbs that were used for rheumatoid arthritis are TNF-α inhibitors and this cytokine is essential for granuloma formation and maintenance, which is an important defense mechanism against intracellular pathogens such as *Leishmania* spp. (Bassetti et al., 2006; Khan et al., 2010).

4.4. Host defense peptides (HDPs)

Host defense peptides (HDPs) are short peptides which can vary in length from 12 to 50 amino acids and have been detected in a wide range of animal, plant, fungal and bacterial species. HDPs are induced in response to specific stress situations such as inflammation or infection (Alba et al., 2012; Mansour et al., 2014; Steinstraesser et al., 2011). They also play a crucial role in the innate immunity and have a broad range of different activities that can vary from angiogenesis or cytokine induction to histamine release or chemotactic functions (Mansour et al., 2014; Steinstraesser et al., 2011). HDPs have been widely studied for their antimicrobial properties, such as topical treatment of wound infections for promoting healing (Alba et al., 2012; Mansour et al., 2014; Steinstraesser et al., 2011). However, the cost of manufacturing HDPs is currently too expensive to be applied in the clinical practice (Mansour et al., 2014).

In relation to *Leishmania* infection, different HDPs appear to have leishmanicidal activities through the activation of the immune defense. A study performed *in vitro* in promastigote cultures and in a mouse model *in vivo* (Erfe et al., 2012) investigated the efficacy of two peptides (RP-1 and AA-RP-1) against *Leishmania* infection. Both peptides had a significant antileishmanial effect against three different *Leishmania* species (*L. infantum*, *L. major* and *L. braziliensis*). RP-1 and AA-RP-1 triggered immediate effects on promastigotes while in the experimental infection, BALB/c mice presented a reduction in the *Leishmania* infection rate (Erfe et al., 2012). In another study performed in Canada (Marr et al., 2016), four peptides were investigated against *L. major* and *L. donovani*-infected THP-1 human macrophages (Table 2).

4.5. Plant extracts

Many different plant extracts have been studied for their leishmanicidal activity in promastigote cultures of *L. donovani* (Bhatnagar et al., 2017), *L. infantum* (Regueira-Neto et al., 2018), *L. braziliensis* (Regueira-Neto et al., 2018), *L. major* (Eskandari et al., 2016; Mirzaei et al., 2016) and *L. amazonensis* (Fadel et al., 2018). However, studies on antileishmanial plant extracts are still in early stages prior to verification of their efficacy and safety in animals. Three plant extract compounds have shown a potential to be beneficial against murine leishmaniosis due to *L. donovani* and they are summarized in Table 2.

4.6. Other compounds studied in other Leishmania species

Other compounds such as TLR 9 agonists, chitin and chitosan

nanoparticles and acetyl salicylic acid (ASA) have been investigated in *L. major* and *L. panamensis* infections mainly in rodent models and showed promising results. However, these compounds have not yet been applied as immunotherapeutical agents in *L. infantum* and *L. donovani* infections.

4.6.1. TLR9 agonists

TLR9 is an intracellular TLR involved in the recognition of un-methylated cytosine triphosphate deoxynucleotide followed by phosphodiester link with guanine triphosphate deoxynucleotide (CpG) oligonucleotides generally of bacterial and viral origin but also of self-DNA in immune-complexes. TLR9 is expressed by B cells, plasmacytoid DCs (pDCs) and also on some activated monocytes (Dowling and Mansell, 2016). Treatments with CpG alone or in combination with other products in experimental murine *L. major* (Raman et al., 2010; Zimmermann et al., 1998) and *L. panamensis* (Ehrlich et al., 2017) infections have been studied.

4.6.2. Chitin and chitosan nanoparticles

Chitin, a polymer formed by repeating units of β-(1–4)-poly-*N*-acetyl-D-glucosamine, is the second most abundant polysaccharide in nature (Lee et al., 2011). Its transformation to chitosan is produced through its deacetylation (Hoseini et al., 2016). Thus, the term chitosan is used for chitin with more than a 50% degree of deacetylation (Hoseini et al., 2016). Chitin preparations have powerful effects on immune responses and have been found to be nontoxic, non-allergenic, biodegradable and biocompatible (Lee et al., 2011; Muzzarelli, 1997). The immunomodulatory effects of chitin and/or chitosan have been investigated in *L. major*-infected murine macrophages (Dehghani et al., 2011) and experimental murine *L. major* infection (Ghotloo et al., 2015; Hoseini et al., 2016). The results of these studies showed that these microparticles induced the production of cytokines such as TNF-α, IFN-γ and IL-10 and reduced the size of skin lesions.

4.6.3. Acetyl salicylic acid (ASA)

Acetyl salicylic acid (ASA) is a nonsteroidal anti-inflammatory drug (NSAID) that can inhibit cyclooxygenase-derived prostaglandins, inflammatory reactions and platelet aggregation (Awtry and Loscalzo, 2000; Gilroy, 2005; Nahrevanian et al., 2012). ASA can induce the production of nitric oxide (NO) (Gilroy, 2005). Nahrevanian and collaborators (2012) investigated the oral administration of ASA after lesion appearance in *L. major*-infected BALB/c mice and found a decline in proliferation of amastigotes and reduction of lesion size together with an increase of NO in the blood of treated infected mice (Nahrevanian et al., 2012).

5. Conclusions

A wide range of different approaches have been investigated for treatment of CanL, whether alone, mostly in mild disease, or combined with conventional drugs in the more severely affected dogs. Published studies have mostly described promising results and have suggested that the use of some of these compounds in the clinical setting should be considered. Many of the compounds described here are still in initial pre-clinical trial phases and, therefore, more studies would be necessary to corroborate their clinical use. Hence, we conclude that more efforts need to be focused on studying these immunotherapeutic drugs and their potential use in the treatment of leishmaniosis in dogs and humans.

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Parasites & Vectors

RESEARCH Open Access

Use of preventive measures and serological screening tools for *Leishmania infantum* infection in dogs from Europe

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Abstract

Background: There are several screening tools for detecting *Leishmania infantum* infection in dogs and various preventive measures to protect against it. Some studies have investigated them, but not many have described their current use. The aim of this study was to investigate which preventive measures and serological screening tools for *L. infantum* infection were employed from 2012 to 2018 in dogs from different endemic European countries.

Methods: A set of electronic datasheets was completed for each dog from several veterinary centres. Classification of preventive measures included: (1) repellents, (2) vaccines and (3) immunomodulators. Classification of serological tests included the: (1) direct agglutination test (DAT), (2) enzyme-linked immunosorbent assay (ELISA), (3) indirect immunofluorescence (IFI), (4) rapid tests and (5) other assays. Dogs were also classified depending on their risk of exposure and living area.

Results: Information from 3762 dogs was gathered. Preventive measures were applied in 91.5% of dogs and the most frequently used were repellents (86.2%) followed by vaccines (39.8%) and Leisguard® (15.3%). The different types of repellents (collar and spot-on) were used similarly. A combination of a vaccine and repellents was preferred in the high-risk group while the low-risk preferred a combination of Leisguard® and a repellent (Chi-square test: $X^2 = 88.41$, $df = 10$, $P < 0.001$). Furthermore, all preventive measures were similarly used through the years except for repellents, which were predicted to have a small increase of use each year. Regarding serological screening tools, the most used were rapid and ELISA tests. Rapid tests, ELISA tests and DAT were used similarly through the years, but a significant change was found in the use of IFI and other assays whose use decreased a little each year.

Conclusions: Repellents were the preferred measure, while vaccines and Leisguard® were second-line options. Some dogs were not treated by any measures, which highlights the need for dog owner education. Moreover, there seems to be a preference for rapid tests in the clinical setting to detect specific *L. infantum* antibodies while ELISA or IFI are less often employed. This underlines an increasing problem, as qualitative rapid tests have a variable diagnostic performance limiting the adequate diagnosis of seropositive dogs in endemic areas.

Keywords: Leishmaniosis, Canine, Prevention, Screening diagnostic tools, Europe

Background

Canine leishmaniosis (CanL) caused by the protozoan *Leishmania infantum* is a zoonotic and endemic disease in the Mediterranean basin [1, 2]. This protozoan is transmitted by the bite of a female phlebotomine sand fly following a digenetic life cycle which consists of two different phases: (i) a promastigote phase, which is an

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extracellular and motile form that colonizes the middle gut of the sand fly, and (ii) an amastigote phase, which is an intracellular and non-motile form that colonizes macrophages of infected hosts [3, 4]. There are also other potential routes of transmission such as venereal [5, 6], transplacental [6, 7] and through blood transfusion [8, 9], which may play a marginal role compared to the vector transmission [10]. The dog (*Canis lupus familiaris*) is considered the main domestic reservoir for *L. infantum* infection in the Mediterranean basin [2, 10], while other mammals such as wild canids [11], rodents [12] and lagomorphs [13] may be able to maintain a wild life cycle.

The use of preventive measures against *L. infantum* infection has expanded over the last decades [14]. However, there are still two main ways to prevent this infection: (i) physical barriers and insecticides against the vector and (ii) immunoprophylaxis. Regarding the vector, it is recommended to avoid outdoor activities during dawn and dusk (when the vector is highly present), to use fine mesh nets in windows and to use topical insecticides such as synthetic pyrethroid-based compounds, which have both repellent and anti-feeding effects [1, 14, 15]. Topical insecticides are commercially available in different forms: impregnated collars, spot-on and sprays, each of which has different onset and maximum duration [3, 14]. Immunoprophylaxis can be divided into vaccines and immunomodulators. Domperidone (Leisguard®) is the only marketed immunomodulator for the prevention of CanL since 2012 [16]. Two commercial vaccines have been available for dogs in Europe: Canileish®, which was first launched in 2011 but is not marketed anymore (withdrawn from the market in 2021), and Letifend®, which was introduced commercially in 2016 and is currently the only available vaccine in Europe [3, 14, 17].

Moreover, CanL is a complex infection due to its variable clinical manifestations and a wide spectrum of clinical signs and laboratory findings, and several diagnostic techniques are available for its screening and diagnosis [17, 18]. Since a vaccine is available in Europe, serological screening is mandatory prior to vaccination of dogs [17]. In addition, annual screening of dogs is frequently performed in endemic areas to diagnose both dogs progressing towards disease and subclinical infections [10, 17]. The diagnostic methods used in the clinical setting include parasitological diagnosis (direct observation of the parasite), serological techniques (such as ELISA, IFI and rapid chromatographic immunoassay) and molecular techniques (PCR and quantitative PCR) [1, 17, 18].

Some studies have investigated the use of preventive measures in *L. infantum* endemic countries, although their focus was the efficacy and safety of those measures [16, 19, 20] or the veterinary recommendations for their use to dog owners [21–25]. In addition, the development and marketing of new preventive measures such as Letifend® may change the use of the already marketed products. Regarding serological screening tools, several studies have compared their sensitivity and specificity [18, 26, 27] or the use of different types of samples such as saliva [28]. However, the current use of the different preventive measures and serological screening tools available for *L. infantum* infection is relatively unknown. For all these reasons, the aim of this study was to investigate the most used serological screening tools and preventive measures against *L. infantum* infection in dogs from 2012 to 2018 and how their use changed through the years.

Methods

Veterinary clinics and cases

Veterinary clinics from Spain ($n=84$), Portugal ($n=3$), Italy ($n=17$) and Cyprus ($n=2$), which implemented at least two different preventive measures against *L. infantum* in dogs, were selected for a database search of clinical records by the authors from their contacts and client lists and were contacted to participate. Figure 1 shows the veterinary clinics that enrolled in the study including 67 from Spain, 3 from Portugal, 10 from Italy and 1 from Cyprus. These veterinary clinics provided information of dogs with the following inclusion criteria: (1) apparently healthy dogs and (2) a previous screening serological test for the detection of antibodies against *L. infantum* antigen before the initial use of the preventive measures.

Study design

Each veterinary clinic received a code to access a website with a set of electronic datasheets that allowed easy data entry. Once the datasheets were completed, their data were automatically uploaded to a common database from which the results were analysed.

The online questionnaire permitted gathering relevant clinical data about dog characteristics (sex, weight, age, breed, risk of exposure and living area) and types of serology tests and preventive measures used. Data of preventive measures were obtained from 2012 to 2017 while data of screening tools were collected from 2012 to 2018.

Case removal

After collection of cases, removal of inadequate cases was performed. A case was defined as inadequate when: (i) it did not comply with the previously established inclusion criteria or (ii) a duplicate case detected. When a duplicate case was detected, a thorough search was performed to confirm its duplicity as to not lose any information. Information about the same dog with two different preventive measures and non-overlapping timelines was not defined as a duplicate.

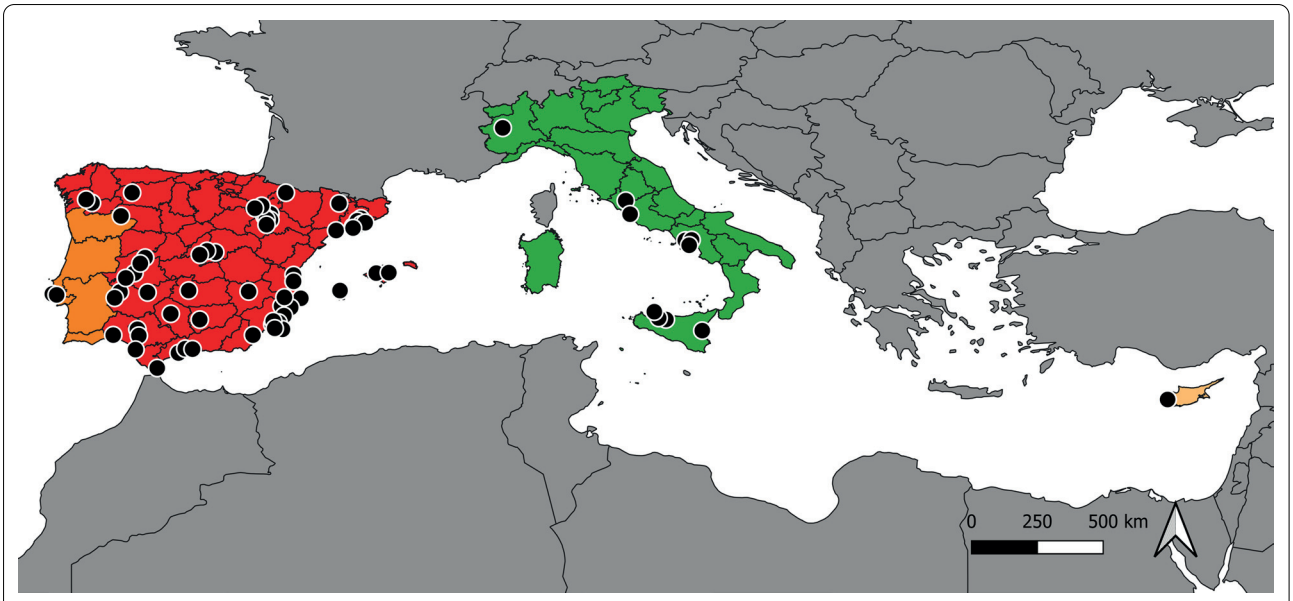

Fig. 1 Geographical distribution of all participating veterinary clinics from Europe. Spain is marked in red, Portugal in orange, Italy in green and Cyprus in yellow. Black dots represent each enrolled clinic in each country location

Preventive measures

Dogs were classified considering the combined use of preventive measures. Eleven groups were considered: (i) no preventive measures applied (NON), (ii) only repellents applied (REP), (iii) only Canileish® vaccine (CAN), (iv) only Letifend® vaccine (LET), (v) only Leisguard® (LEI), (vi) Canileish® vaccine + repellent (CAN + REP), (vii) Letifend® vaccine + repellent (LET + REP), (viii) Leisguard® + repellent (LEI + REP), (ix) Canileish® vaccine + Leisguard® (CAN + LEI), (x) Canileish® vaccine + Leisguard® + repellent (CAN + LEI + REP) and (xi) Letifend® vaccine + Leisguard® + repellent (LET + LEI + REP).

Another classification considered the individual use of each product. These four groups were defined as (i) repellent group, which included dogs that used repellent alone or in combination with other products (REP, CAN + REP, LET + REP, LEI + REP, CAN + LEI + REP and LET + LEI + REP), (ii) Canileish®, which included dogs that used Canileish® alone or in combination with other products (CAN, CAN + REP, CAN + LEI and CAN + LEI + REP), (iii) Letifend®, which included dogs that used Letifend® alone or in combination with other products (LET, LET + REP and LET + LEI + REP), and (iv) Leisguard®, which included dogs that used Leisguard® alone or in combination with other products (LEI, LEI + REP, CAN + LEI, CAN + LEI + REP and LET + LEI + REP).

Dogs that used repellent were classified in three different groups based on type of repellent employed: (i) collar, (ii) spot-on and (iii) collar + spot-on.

Classification of exposure risk and living area

Dogs were classified in two different groups depending on their exposure risk to *L. infantum* infection. High risk was considered when dogs lived outdoors or when dogs that despite living indoors went frequently for a walk in plot of land or forest areas at times when the vector was highly present, for example at dawn and dusk. Low risk classification included those dogs which lived indoors and went only for a walk in urban area or just at times when the vector was barely present.

Another classification depending on living area was also performed. Dogs were classified in three groups: urban area (living in cities or big towns with paved streets and small green areas), periurban area (city outskirts or towns surrounded by large green areas) and rural area (small towns or buildings built far away from human settlements like farms, usually agricultural areas and forests).

Screening tools

The screening tools were classified in five groups: (i) direct agglutination test (DAT), (ii) enzyme-linked immunosorbent assay (ELISA), (iii) indirect immunofluorescence (IFI), (iv) rapid tests and (5) other assays.

Additionally, a screening campaign by Ecuphar veterinaria SLU was performed in 2018 using Leiscan® and ELISA *in house* [29] to increase the number of enrolled dogs; therefore, a bias was to be expected.

Statistical analysis

A descriptive study of all collected data was performed. Quantitative variables (age, weight) were assessed using a non-parametric Mann-Whitney *U* test when two groups were compared (high and low risk) while the Kruskal-Wallis *H* test was used when three groups were compared (living area: urban, periurban or rural). Qualitative variables (sex, breed, preventive measures and serological screening tools) were assessed using a Chi-square test. A simple linear regression was calculated to predict the proportion of use for each preventative measure or serological test based on time (from 2012 to 2017 or from 2012 to 2018, respectively).

A P-value < 0.05 was considered statistically significant. The Shapiro-Wilk test was performed to detect normal distribution of quantitative variables. The statistical analysis was performed using the package Stats for the software R i386 3.5.1 for Windows. Maps were created using the Free and Open Source QGIS 3.10.4 for Windows. Graphics were plotted using Graphad Prism version 5.00 for Windows.

Results

Dog characteristics

Dogs from Spain (3603 dogs), Portugal (64 dogs), Italy (69 dogs) and Cyprus (26 dogs) were enrolled in this study with a total of 3762 dogs. Dog characteristics such as sex, age, weight, breed, risk of exposure and living area are displayed in Table 1. The most common breeds were Yorkshire terrier (7.1%), Labrador retriever (6.7%), German shepherd (6.2%), Maltese (3.9%), Boxer (3.8%), Golden retriever (3.7%) and French bulldog (3.5%).

No statistically significant differences were found between risk of exposure to the vector (low vs. high risk of exposure) when sex, age and breed were compared. A significant difference (Mann-Whitney test: $U = 1{,}876{,}996$, $Z = -13.46$, $n_1 = 2613$, $n_2 = 1125$, $P < 0.0001$) was noted when weight was compared between groups of risk of exposure to the vector. Large size dogs (21.9 ± 13.7 kg) were included in the high-risk group while small size dogs (15.7 ± 12.6 kg) were included in the low-risk group.

Quantitative and qualitative characteristics of dogs depending on their living area are listed in Table 2. No differences between groups were found when sex and breed were compared. In the case of age and weight, dogs living in rural areas were younger than dogs living in periurban or urban areas (Kruskal-Wallis *H* test: $X^2 = 10.73$, $df = 2$, $P = 0.005$) while dogs living in urban

Table 1 Qualitative and quantitative clinical characteristics of the dogs

Qualitative clinical characteristics	N	% (95% CI)
Sex		
Male	2006	53.4 (51.8–55)
Female	1753	46.6 (45–48.2)
Total	3759	
Breed		
Purebred	2711	72.3 (70.9–73.8)
Mixed breed	1037	27.7 (26.2–29.1)
Total	3748	
Risk of exposure		
High	2620	69.9 (68.4–71.4)
Low	1127	30.1 (28.6–31.6)
Total	3747	
Living area		
Urban area	1585	55.5 (53.6–57.3)
Periurban area	818	28.6 (27–30.3)
Rural area	455	15.9 (14.6–17.3)
Total	2858	

Quantitative clinical characteristics	*N*	Mean (± SD)	Minimum	Maximum
Age (years)	3755	7 (± 3.3)	0.5	18.5
Weight (kg)	3753	20 (± 13.7)	1.4	110

CI confidence intervals, *N* number of dogs, *SD* standard deviation

Table 2 Qualitative and quantitative clinical characteristics of the dogs depending on their living area

Qualitative clinical characteristics	Urban area ($N=1585$)		Periurban area ($N=818$)		Rural area ($N=455$)		*P*-value (Chi-square test)
	N	% (95% CI)	N	% (95% CI)	N	% (95% CI)	
Sex							
Male	842	53.1 (50.6–55.6)	461	56.4 (52.9–59.8)	241	53 (48.3–57.6)	0.284
Female	743	46.9 (44.4–49.4)	357	43.6 (40.2–47.1)	214	47 (42.4–51.7)	
Breed							
Purebred	1174	74.1 (71.8–76-2)	576	70.4 (67.2–73.5)	317	69.7 (65.2–73.9)	0.064
Mixed-breed	411	25.9 (23.8–28.2)	242	29.6 (26.5–32.8)	138	30.3 (26.1–34.8)	
Risk of exposure							$<0.001^{a*}$
High	925	58.4 (55.9–60.8)	676	82.6 (79.9–85.2)	436	95.8 (93.6–97.5)	
Low	660	41.6 (39.2–44-1)	142	17.4 (14.8–20.1)	19	4.2 (2.5–6.4)	
Quantitative clinical characteristics	*N*	Mean (± SD)	N	Mean (± SD)	N	Mean (± SD)	*P*-value (Kruskal-Wallis *H* test)
Age (years)	1585	7.2 (± 3.3)	817	7.1 (± 3.3)	455	6.6 (± 3.1)	0.005^{b*}
Weight (kg)	1585	17 (± 13.1)	818	23 (± 13.5)	455	23.9 (± 13.8)	$<0.0001^{c*}$

CI confidence intervals, *N* number of dogs, *SD* standard deviation

[a] $X^2=314.67$, $df=2$, $P<0.001$

[b] $X^2=10.73$, $df=2$, $P=0.005$

[c] $X^2=176.06$, $df=2$, $P<0.0001$

[*] *P*-value < 0.05 (statistically significant)

areas were smaller in size than dogs living in rural or periurban areas (Kruskal-Wallis *H* test: $X^2=176.06$, $df=2$, $P<0.0001$) (Table 2). Moreover, rural area dogs had a higher risk of exposure to *L. infantum* followed by periurban dogs and finally urban dogs (Chi-square test: $X^2=314.67$, $df=2$, $P<0.001$).

Preventive measures

General results

Preventive measures were applied for 3444 dogs (91.5%) of all the dogs enrolled. Younger dogs (6.9 ± 3.3 years) were more likely to be treated with preventive measures than older dogs (7.7 ± 3.5 years) (Mann-Whitney test: $U=614{,}890.5$, $Z=-3.79$, $n_1=317$, $n_2=3438$, $P=0.0002$). The individual use of each preventive measure in the 3444 dogs is plotted in Fig. 2. Repellents (alone or in combination with other products) were the most used preventive measure followed by vaccines (Canileish® or Letifend®) and Leisguard® (Fig. 2a). The different types of repellents (collar, spot-on or a combination of both) were used similarly (Fig. 2b) while, in the case of vaccines, Canileish® (60.8%) was more frequently used than Letifend® (39.2%) (Fig. 2c). No statistical differences were observed when the individual use of the different preventive measures depending on sex and breed were compared except for Canileish®, which was more often used in purebred dogs (Chi-square test: $X^2=9.26$, $df=1$, $P=0.002$) than in mixed-breed dogs. Regarding age, younger dogs were more likely to use repellent (Mann-Whitney test: $U=900{,}141.5$, $Z=-2.7$, $n_1=518$, $n_2=3237$, $P=0.007$), Letifend® (Mann-Whitney test: $U=1{,}084{,}731$, $Z=-6.42$, $n_1=3168$, $n_2=587$, $P<0.0001$) or Leisguard®

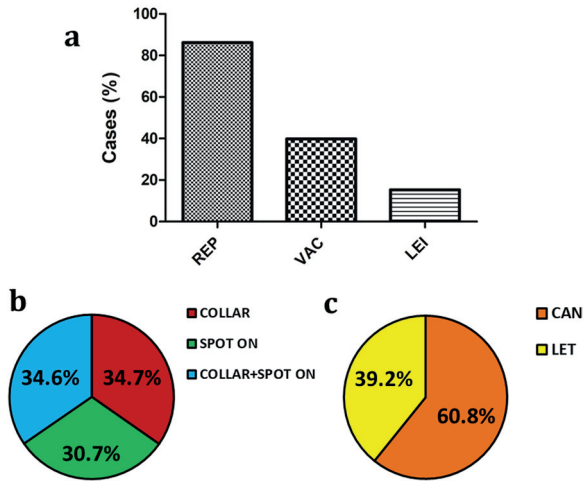

Fig. 2 Proportions of (**a**) the individual use of each preventive measure, (**b**) the type of repellent used and (**c**) the vaccine used. Preventive measures represented are repellent group (REP), which included dogs that used repellent alone or in combination with other products, vaccine group (VAC), which included dogs that used vaccine alone or in combination with other products, Leisguard® group (LEI), which included dogs that used Leisguard® alone or in combination with other products, Canileish® group (CAN) and Letifend® group (LET)

(Mann-Whitney test: $U=963{,}611.5$, $Z=-2.29$, $n_1=3184$, $n_2=571$, $P=0.02$) than older dogs. As for weight, larger dogs were more likely to use Canileish® (Mann-Whitney test: $U=1{,}213{,}325$, $Z=-2.72$, $n_1=2846$, $n_2=907$, $P=0.006$) while smaller dogs were more likely to use Leisguard® (Mann-Whitney test: $U=1{,}043{,}852.5$, $Z=-5.56$, $n_1=3180$, $n_2=573$, $P<0.0001$).

Figure 3 shows the combined preventive measures used in all the dogs. The most used preventive measure was repellent alone (Fig. 3). When comparing the proportions of sex, CAN + LEI and CAN + LEI + REP presented the highest proportion of females (58.6%) while REP presented the highest proportion of males (55.9%) (Chi-square test: $X^2=4.78$, $df=1$, $P=0.029$), but no other differences were found between the other groups (Table 3). Regarding breed, only CAN + REP was found to have a significantly higher proportion of purebred dogs (77%) when compared to the other preventive measures (44.4%) (Chi-square test: $X^2=16.53$, $df=6$, $P=0.011$) (Table 3). When comparing their age, LEI was found to be the oldest group (Table 3). Regarding weight, LEI + REP and LEI were the groups with smaller dogs and significantly different when compared to the other groups (Kruskal-Wallis H test: $X^2=45.82$, $df=10$, $P<0.0001$) (Table 3).

Additional file 1: Fig. S1 shows the use of the different marketed brands of each type of repellent: collar (Additional file 1: Fig. S1a) and spot-on (Additional file 1: Fig. S1b). The most used products were the Scalibor® collar (70%) and the Advantix® spot-on (61%). Significant differences were found regarding breed, age and weight. In detail, purebred dogs used more frequently a combination of both collar and spot-on, while mixed-breed dogs used collars alone more frequently (Chi-square test: $X^2=8.03$, $df=2$, $P=0.018$). Dogs using collars alone were younger (6.8 years) than dogs using spot-on alone (7.1 years) (Kruskal-Wallis H test: $X^2=6.27$, $df=2$, $P=0.044$) while dogs using spot-on alone were smaller in size (14.5 kg) than dogs using collars alone (22.2 kg) or a combination of collar and spot-on (22.5 kg) (Kruskal-Wallis H test: $X^2=299.11$, $df=2$, $P<0.0001$).

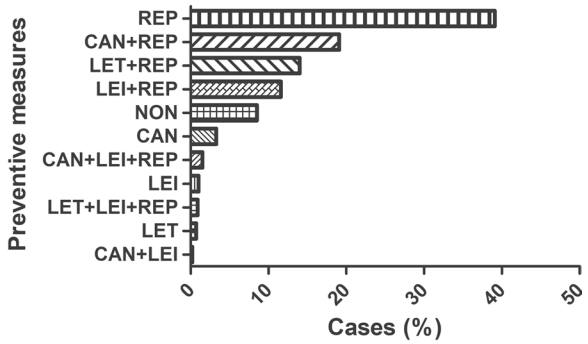

Fig. 3 Proportions of preventive measures used against *L. infantum* in all dogs studied. Preventive measures represented are only repellents applied (REP), Canileish® vaccine + repellent (CAN + REP), Letifend® vaccine + repellent (LET + REP), Leisguard® + repellent (LEI + REP), no preventive measures applied (NON), only Canileish® vaccine applied (CAN), Canileish® vaccine + Leisguard® + repellent (CAN + LEI + REP), only Leisguard® applied (LEI), Letifend® vaccine + Leisguard® + repellent (LET + LEI + REP), only Letifend® vaccine applied (LET) and Canileish® vaccine + Leisguard® (CAN + LEI)

Preventive measures by risk of exposure

The use of preventive measures against *L. infantum* was similar when risk of exposure was compared (91.3% high-risk group and 92.1% low-risk group). Letifend® was used more frequently in the high-risk group (Chi-square test: $X^2=107.02$, $df=1$, $P<0.001$) while Leisguard® was used more often in the low-risk group (Chi-square test: $X^2=54.69$, $df=1$, $P<0.001$). Regarding the type of repellents used, the high-risk group had a higher rate of using both types of repellents together (collar and spot-on) while the low-risk group had a higher rate of using collar or spot-on alone (Chi-square test: $X^2=92.80$, $df=2$, $P<0.001$).

Most of the preventive measures were more frequently used in the high-risk group except for LEI + REP and LEI, which were similarly used in both groups. In fact, LEI + REP and LEI were found to have a significantly higher proportion of use in the low-risk of exposure group than other preventive measures (Chi-square test: $X^2=88.41$, $df=10$, $P<0.0001$) (Table 3). On the other hand, LET + REP was found to have the highest proportion of use in the high-risk group and was significantly different when compared to the other groups (Table 3).

Preventive measures by living area

Preventive measures were applied differently depending on the living area showing a higher rate of use in urban area (93.2%) followed by periurban (91.6%) and rural (87.9%) areas (Chi-square test: $X^2=13.34$, $df=2$, $P=0.001$). The use of collar, spot-on and a combination of both was also compared between urban, periruban and rural areas and significant differences were found (Chi-square test: $X^2=194.23$, $df=4$, $P<0.001$) with a higher use of collar alone in rural and periruban areas while a combination of both collar and spot-on was preferred in urban areas (Fig. 4).

Furthermore, REP was the preventive measure used at the most similar frequency in all areas with 47.5% of use in the urban followed by 30.4% in the periurban and 22.1% in the rural areas. CAN + REP and LET + REP

Table 3 Qualitative and quantitative clinical characteristics of the dogs depending on the preventive measures used

Preventive measures	Sex (%, 95% CI)		Breed (%, 95% CI)		Age (years, mean ± SD)	Weight (kg, mean ± SD)	Risk of exposure (%, 95% CI)	
	Male	Female	Purebred	Mixed-breed			High	Low
NON (*N* = 318)	50 (44.4–55.6)	50 (44.4–55.6)	69.1 (63.7–74.1)	30.9 (25.9–36.3)	7.1 (± 3.5)	20 (± 14.3)	71.9 (66.6–76.8)	28.1 (23.2–33.4)
REP (*N* = 1468)	55.9 (53.3–58.5)	44.1 (41.5–46.7)	71.8 (69.4–74.1)	28.2 (25.9–30.6)	7 (± 3.4)	18.8 (± 13.2)	66.4 (63.9–68.8)	33.6 (31.2–36.1)
CAN (*N* = 125)	52 (42.9–61)	48 (39–57.1)	75 (66.4–82.3)	25 (17.7–33.6)	6.5 (± 2.8)	21.8 (± 14.6)	69.4 (60.4–77.3)	30.6 (22.7–39.6)
LET (*N* = 28)	53.6 (33.9–72.5)	46.4 (27.5–66.1)	71.4 (51.3–86.8)	28.6 (13.2–48.7)	4.4 (± 3.7)	19 (± 10.1)	60.7 (40.6–78.5)	39.3 (21.5–59.4)
LEI (*N* = 39)	41 (25.6–57.9)	59 (42.1–74.4)	61.5 (44.6–76.6)	38.5 (23.4–55.4)	8.8 (± 3.3)	11.9 (± 14.1)	43.6 (27.8–60.4)	56.4 (39.6–72.2)
CAN + REP (*N* = 719)	53.8 (50.1–57.5)	46.2 (42.5–49.9)	77 (73.7–80)	23 (20–26.3)	6.3 (± 3.1)	18 (± 14.7)	72.4 (69–75.6)	27.6 (24.4–31)
LET + REP (*N* = 527)	51.4 (47.1–55.8)	48.6 (44.2–52.9)	71.9 (67.9–75.7)	28.1 (24.3–32.1)	5.8 (± 3.3)	19 (± 13.1)	89.9 (87.1–92.4)	10.1 (7.6–13)
LEI + REP (*N* = 436)	52.5 (47.7–57.3)	47.5 (42.7–52.3)	70 (65.5–74.3)	30 (25.7–34.5)	6.1 (± 3.2)	12 (± 13.5)	53.8 (49–58.6)	46.2 (41.4–51)
CAN + LEI (*N* = 9)	22.2 (2.8–60)	77.8 (40–97.2)	44.4 (13.7–78.8)	55.6 (21.2–86.3)	4.4 (± 3.5)	10 (± 13.2)	88.9 (51.8–99.7)	11.1 (0–48.3)
CAN + LEI + REP (*N* = 57)	41.4 (28.6–55.1)	58.6 (44.9–71.4)	75 (61.6–85.6)	25 (14.4–38.4)	6 (± 3)	17.8 (± 15.7)	71.9 (58.5–83)	28.1 (17–41.5)
LET + LEI + REP (*N* = 32)	53.1 (34.7–70.9)	46.9 (29.1–65.3)	68.8 (50–83.9)	31.2 (16.1–50)	6 (± 1.2)	22.5 (± 16.8)	78.1 (60–90.7)	21.9 (9.3–40)
P-value	$P < 0.0001$*[a]		$P < 0.0001$*[b]		$P < 0.0001$*[c]	$P < 0.0001$*[d]	$P < 0.0001$*[e]	

CAN only Canileish® vaccine, *CAN* + *LEI* Canileish® vaccine + Leisguard®, *CAN* + *LEI* + *REP* Canileish® vaccine + Leisguard® + repellent, *CAN* + *REP* Canileish® vaccine + repellent, *CI* Confidence intervals, *LEI* only Leisguard®, *LEI* + *REP* Leisguard® + repellent, *LET* only Letifend® vaccine, *LET* + *LEI* + *REP* Letifend® vaccine + Leisguard® + repellent, *LET* + *REP* Letifend® vaccine + repellent, *N* number of dogs, *NON* no preventive measures applied, *REP* only repellents applied, *SD*: standard deviation

[a] Chi-square test: $X^2 = 39.63$, $df = 10$

[b] Chi-square test: $X^2 = 38.72$, $df = 10$

[c] Kruskal-Wallis *H* test: $X^2 = 84.15$, $df = 10$

[d] Kruskal-Wallis *H* test: $X^2 = 45.82$, $df = 10$

[e] Chi-square test: $X^2 = 88.41$, $df = 10$

* *P*-value < 0.05 (statistically significant)

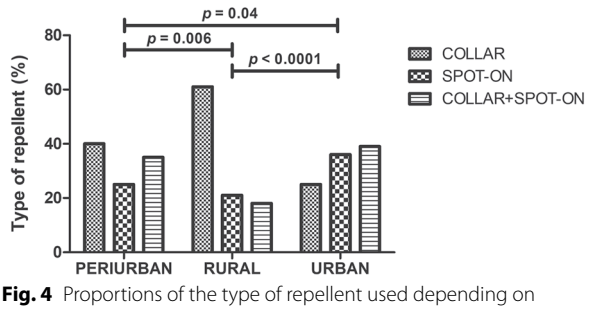

Fig. 4 Proportions of the type of repellent used depending on the living area. Statistical significance was found in the following comparisons: Periurban vs. rural (Chi-square test: $X^2 = 10.01$, $df = 2$, $P = 0.006$) and urban areas (Chi-square test: $X^2 = 6.07$, $df = 2$, $P = 0.04$) and rural vs. urban areas (Chi-square test: $X^2 = 26.75$, $df = 2$, $P < 0.0001$)

were significantly more used in urban areas with 64% and a 78% frequency, respectively (Chi-square test: $X^2 = 170$, $df = 20$, $P < 0.0001$). Moreover, LET + REP was significantly more used in urban areas than CAN + REP (Chi-square test: $X^2 = 30.35$, $df = 2$, $P < 0.001$).

Preventive measures trends

The use of the different products from 2012 to 2017 is plotted in Fig. 5. Repellents were the most used always by > 80% of the dogs studied (Fig. 5). A significant regression was only found in the use of repellents with an R^2 of 0.75 (Fig. 5). The predicted use of repellents was equal to -3252.31 + 1.66 of percentage of the use of repellents

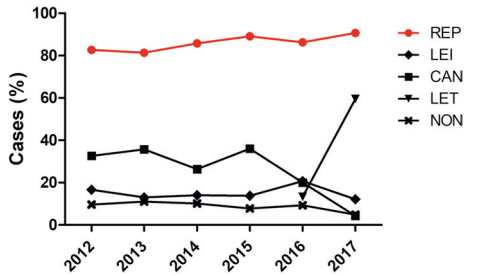

Fig. 5 Proportions of the use of the different products through the years studied (2012–2017). Preventive measures represented are repellent group (REP), which included dogs that used repellent alone or in combination with other products, Leisguard® group (LEI), which included dogs that used Leisguard® alone or in combination with other products, Canileish® group (CAN), which included dogs that used Canileish® alone or in combination with other products, Letifend® group (LET), which included dogs that used Letifend® alone or in combination with other products, and no preventive measures applied (NON). Data in red present a significant regression: REP ($F(1,4) = 12.15$, $P = 0.0252$)

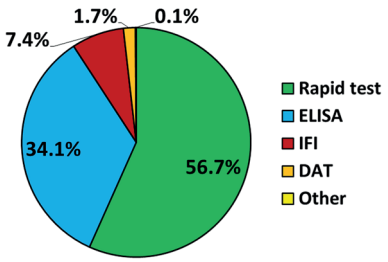

Fig. 6 Proportions of the different types of serological screening tests. Screening tools represented are the direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IFI), rapid tests and other assays

when time was measured in years, so the percentage of use of repellents increased 1.66% for each year.

Serological screening tools

General results

The different types of serological screening tests employed are shown in Fig. 6 while the different brands of serological screening tests are shown in Additional file 2: Fig. S2. Rapid tests were the most used (SNAP-Idexx) followed by ELISA tests (Leiscan®). IFI and DAT were used in < 10% of the cases (Fig. 6, Additional file 2: Fig. S2).

Screening tools trends

The use of the different types of serological screening tests from 2012 to 2018 is displayed in Fig. 7. Rapid tests followed by ELISA were the most frequently used techniques (Fig. 7). A significant regression was found on the

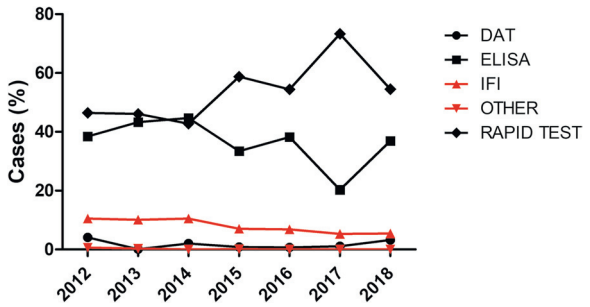

Fig. 7 Proportions of the use of the different types of serological screening tests through the years studied (2012–2018). Screening tools represented are direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IFI), rapid tests and other assays. Data in red present a significant regression: IFI ($F(1,5) = 35.08$, $P = 0.002$) and other ($F(1,5) = 9.23$, $P = 0.0288$)

use of IFI tests and other tests with an R^2 of 0.88 and 0.65, respectively. The predicted use of IFI tests was equal to 2066.12—1.02 of percentage of the use of IFI tests when time is measured in years, so the percentage of use of IFI tests decreased 1.02% for each year. The predicted use of other tests was equal to 172.86–0.09 of percentage of the use of other tests when time was measured in years, so the percentage of use of other tests decreased 0.09% for each year.

Discussion

Previous studies have investigated the veterinary recommendations for the use of preventive measures to dog owners in Spain and other European countries and found out that most veterinarians recommend preventive measures against *L. infantum* to their clients [21–25]. These recommendations can be linked directly to the results of the present study as at least one preventive measure was applied in > 90% of the dogs. Furthermore, veterinary recommendations seem to prioritize the use of repellents over vaccines or Leisguard® [22, 23], which is also highlighted by the results of the present study where a repellent was used in > 80% of the dogs while vaccines and Leisguard® were used by < 50% throughout all years studied. As expected, these recommendations are in line with the published guidelines [14], which endorse the use of repellents in both endemic and fringe areas, while vaccines and Leisguard® are described as optional.

Regarding repellent brands, a previous study [23] reported that the most frequently recommended were Seresto®, Advantix® and Scalibor®. Both the present study and an additional study [19] showed similar results with the most used collar being Scalibor® while Advantix® was the most used spot-on. Interestingly, a

study performed in north-eastern Spain [22] described a preference for recommending collars (98% of the veterinarians recommended collars to their clients) over spot-on (67% of the veterinarians recommended spot-on), in disagreement with the present results in which there was no difference between the use of collar or spot-on, although the reason for these results could be related to the higher use of collars in periurban and rural areas compared to urban areas found in this study. Regarding vaccines, Montoya et al. [23] reported a higher use of Letifend® than Canileish®. However, the present study differs as a higher use of Canileish® was found when compared with Letifend®. This discrepancy is due to the fact that data on dogs were included from 2012 when Canileish® was still on the market and Letifend® was not marketed yet [3, 14, 17].

Interestingly, Leisguard® was more frequently administered to smaller dogs [19], as observed in this study. One of the reasons for this result is that the Leisguard® dose administration is linked to body weight so large dogs need a high daily dose and therefore a higher expenditure than when being used for small dogs [16]. Another explanation is the fact that small size dogs are more prone to adverse effects after vaccination [30, 31].

An association between socioeconomic status of the dog owner and CanL has been previously documented [32]. Owners with a low income cannot afford some products and that may affect the disease control and even the nutrition and survival of the dog [32]. The presence of a backyard at the residence with a predominance of land and/or vegetation was also associated with CanL [32], which could be a consequence of not only an environmental factor but also of the smaller use of preventive measures in periurban and rural areas as described in the present study, among other factors. Another study from Brazil [33] went further and associated CanL with not just rural areas (small farms) but also the larger size of the dogs (usually used as guard dogs) and lack of owner knowledge about CanL. Coincidentally, in this study, larger dogs were more frequently classified in the high-risk exposure group and living in rural or periurban areas, which could explain its association with CanL.

The use of screening tools was also widespread as stated previously by other studies [19, 22–25]. Concerning serological tests, rapid tests and ELISA seem to be preferred by clinicians in the present study as previously reported [19, 22–25]. Rapid tests (56.7%) are being used more in the clinical setting probably because of their fast results, low price and easy performance, while other types of tests such ELISA (34.1%) and IFI (7.4%) are employed less because of increased time of performance and mainly because they need to be conducted in laboratories by trained personnel. However, ELISA is used more than IFI because IFI's interpretation is subjective and its result depends on the operator's experience and skill to interpret the test while ELISA is interpreted objectively using an ELISA reader to quantify the result [26]. These results highlight an increasing problem in the clinical setting as qualitative rapid tests have a good specificity but are less sensitive than quantitative laboratory tests such as IFI and ELISA and therefore rapid tests can misdiagnose seropositive cases [10, 17, 18, 34]. It is important to remark that rapid tests have a low sensitivity in detecting apparently healthy seropositive dogs [26]. This fact is extremely concerning when testing apparently healthy infected dogs as further investigations will not be performed and therefore infection will not be detected.

The limitations of the study are that, even as the study was expected to collect information from different countries, a limited number of dogs from Portugal, Italy and Cyprus were included, so the information received was mainly from Spain. Furthermore, just a small sample of the vast dog population of Spain (> 7.5 million registered dogs) [35] was included and the use of preventive measures might be overestimated.

Conclusions

In conclusion, dog owners in Spain follow the veterinarian's recommendations for the use of preventive measures against *L. infantum* infection as endorsed by the published guidelines. Repellents were the preferred measure, while vaccines and Leisguard® were second-line options. However, there are still dogs that do not use preventive measures in endemic regions. Regarding serological screening tools, there seems to be a preference for the use of rapid tests in the clinical setting to detect specific *L. infantum* antibodies while other types of tests such ELISA and IFI are less often employed. The results of this study reinforce the need to sensitize owners about the importance of protecting dogs against the parasite and clinicians about the limitations that qualitative serological techniques can present in the diagnosis of seropositive animals in endemic areas.

Abbreviations

CAN: Only Canileish® vaccine; CanL: Canine leishmaniosis; CAN + LEI: Canileish® vaccine + Leisguard®; CAN + LEI + REP: Canileish® vaccine + Leisguard® + repellent; CAN + REP: Canileish® vaccine + repellent; DAT: Direct agglutination test; ELISA: Enzyme-linked immunosorbent assay; IFI: Indirect immunofluorescence; LEI: Only Leisguard®; LEI + REP: Leisguard® + repellent; LET: Only Letifend® vaccine; LET + LEI + REP: Letifend® vaccine + Leisguard® + repellent; LET + REP: Letifend® vaccine + repellent; NON: No preventive measures applied; PCR: Polymerase chain reaction; REP: Only repellents applied.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05251-5.

Additional file 1: Figure S1. Proportions of **a**) the use of collar marketed brands and **b**) the use of spot-on marketed brands in all dogs studied.

Additional file 2: Figure S2. Proportions of the different brands of serological screening tests.

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Authors' contributions
JH, LSG and CM prepared study protocols and datasheets. MB, JH, CM, CA and LSG contacted veterinary clinics to participate in the study. CA also collected cases for the study. MB performed case removal, statistical analysis and prepared tables and figures. MB and LSG contributed with data analysis and interpretation. MB wrote the manuscript. LSG revised the manuscript. All authors read and approved the final manuscript.

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

Declarations

Ethics approval and consent to participate
No ethics approval was needed for this study as only retrospective information was collected. Signed informed consent about information used was obtained from veterinarians.

Consent for publication
Not applicable.

Competing interests
JH and CM have financial competing interests as they receive a salary from Ecuphar veterinaria SLU which markets both Leisguard® and Leiscan®.

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RESEARCH

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Detection of specific antibodies against *Leishmania infantum* in canine serum and oral transudate using an in-house ELISA

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Abstract

Background: Canine leishmaniosis caused by the protozoan *Leishmania infantum* is a complex infection due to its variable clinical signs and laboratory findings. Therefore, a broad range of techniques is available for diagnosis. Testing for specific antibodies in serum is the most commonly used technique, although the testing of other body fluids, such as oral transudate (OT), can be an alternative as its collection is non-invasive and testing can be performed by untrained personnel. The aim of this study was to assess and compare the detection of *L. infantum*-specific antibodies in paired samples of serum and OT collected from apparently healthy dogs and dogs with clinical leishmaniosis using an in-house enyzme-linked immunosorbent assay (ELISA).

Methods: Serum and OT were collected from 407 dogs, which varied in breed, sex, age, lifestyle and clinical status, by many practicing veterinarians in Spain. The main geographical areas of sampling included Barcelona ($n=110$), Mallorca ($n=94$), Cadiz ($n=54$) and Asturias ($n=47$). The majority of infected dogs were apparently healthy (89.9%) while 41 presented clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection and subsequently diagnosed with leishmaniosis (10.1%). An in-house ELISA was performed to quantify the anti-*Leishmania* antibodies in serum and OT.

Results: The *L. infantum* infection rate determined by the in-house ELISA was 37.1% in serum samples and 32.7% in OT samples. Serum and OT ELISA results showed a positive correlation (Spearman's correlation coefficient $r_s=0.6687$, $P<0.0001$). The percent agreement between the serum and OT ELISA results was 84%, while agreement according to Cohen's kappa statistic (κ) was substantial (0.66) when all samples were analyzed. The highest percent agreement (92.1%) between both tests was found in dogs from low endemicity regions and from sick dogs, with both groups presenting almost perfect agreement according to Cohen's κ agreement test (0.84). Few seronegative dogs ($n=23$) tested positive by the OT ELISA. The agreement between serum and OT went from almost perfect to moderate when the geographical distribution and clinical status were analyzed.

Conclusions: The results of this study demonstrated an almost perfect to moderate agreement between OT and serum samples tested using the in-house ELISA. These results are particularly promising in sick dogs with high antibody levels while the results seem less optimal in apparently healthy dogs with low antibody levels.

Keywords: Leishmaniosis, Dog, Oral transudate, Serology, Diagnosis, Spain

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Background

Canine leishmaniosis (CanL), a zoonotic and endemic protozoan disease caused by *Leishmania infantum*, is endemic in the Mediterranean basin [1, 2]. Transmission is mostly through the bite of a female phlebotomine sand fly following a digenetic life-cycle which consists of two different phases: an extracellular and mobile promastigote in the sand fly, and an intracellular and non-motile amastigote in the mammalian host [3]. Other confirmed transmission routes, such as venereal [4, 5] and transplacental [5, 6] transmission and through blood transfusion, also occur [7, 8]. The dog is considered to be the main domestic reservoir for *L. infantum* infection in the Mediterranean basin [2, 9], while other mammals may be able to maintain a wild-life cycle [10–12].

The seroprevalence of *L. infantum*-infected healthy dogs in western Europe was 23% between 1971 and 2006 [13]. In Spain, the seroprevalence has been reported to be around 10%, although it can vary from 0 to 57% depending on the region [14]. Moreover, the prevalence of dogs that develop the clinical disease is usually lower than 10% [15, 16]. CanL is a complex infection due to its variable clinical manifestations and wide spectrum of clinical signs and laboratory findings [9, 17, 18]. One factor underlying this variability is the dog's immune response, which requires a balance between inflammatory and regulatory responses to control *L. infantum* infection [19]. For example, neutrophils and macrophages play distinctive roles in the dog's initial immune ability to control the infection or to allow progression towards disease. Both neutrophils and macrophages phagocytize the parasite which can lead either to the elimination of the parasite through the production of reactive oxygen species (ROS), or to the survival of parasites within macrophages, leading to parasite persistence and dissemination [19]. T lymphocytes also play an integral role in preventing parasite growth and disease development as these T cells produce interferon gamma (IFN-γ) among other cytokines, such as tumor necrosis factor alpha, interleukin-2 or chemokines, which results in the differentiation, recruitment and activation of macrophages. However, as the infection progresses towards disease, there is a decrease in T cell proliferation and IFN-γ production and a lack of macrophage activation, resulting in a reduction of parasite elimination [19]. Many other factors can also affect the development of the disease, such as age, sex, host genetics, among others. To date, however, the mechanisms responsible for the dog's resistance or susceptibility are still unknown [15, 17].

Due to this complexity, CanL diagnosis often requires an integrated approach, including a clinicopathological examination and specific laboratory tests [9, 15, 18]. A full clinical history, thorough physical examination and several routine diagnostic tests, such as a complete blood count, biochemical profile, urinalysis and serum electrophoresis, are necessary when there is a suspicion of CanL [15, 18]. In addition, several diagnostic techniques are available that enable a definitive diagnosis of *L. infantum* infection, such as parasitological diagnosis (direct observation of the parasite), serological techniques (such as the enzyme-linked immunosorbent assay [ELISA] and indirect fluorescent antibody test) and molecular studies (such as quantitative PCR) [1, 17, 18, 20]. Parasitological methods and molecular studies can detect the presence of the parasite, by direct observation or detection of DNA, respectively, while serological techniques detect serum anti-*Leishmania* antibodies. The diagnostic techniques must be used with full knowledge of the basis of each test and its limitations, as well as how to correctly interpret the results [15, 17, 18].

Interestingly, these diagnostic techniques can be performed using different types of samples, such as blood, serum, urine and other infected tissues [15, 21–23]. The use of alternative samples, such as oral transudate (OT), hair or conjunctival swabs, has also been studied, with interesting results [24–27]. Immunoglobin A (IgA) can be found in OT as it is secreted in the salivary glands by plasma cells, along with immunoglobin G (IgG) and immunoglobin M (IgM), both of which are derived from plasma [28]. Specific antibodies against *L. infantum* have been previously detected in saliva samples of infected sick dogs only by means of a time-resolved immunofluorometric assay (TR-IFMA) [24, 29–31]. However, to the authors' best knowledge, the detection of antibodies against *L. infantum* by ELISA in OT from apparently healthy dogs has not been previously documented. The advantages of using OT instead of serum include a non-invasive, cheap and painless collection of the sample, which can also be performed by untrained personnel.

The aim of this study was to assess and compare the detection of *L. infantum*-specific antibodies in paired samples of serum and OT from apparently healthy dogs and from dogs with clinical leishmaniosis, using an in-house ELISA.

Methods

Dogs

A minimum sample size of 310 dogs was calculated [32] using an expected seroprevalence of *L. infantum* infection of 10% [14] and a power of 80%. Both serum and OT samples from 407 dogs varying in breed, sex, age, lifestyle and clinical status were collected between January of 2018 and June of 2021 by several veterinarians practicing in different areas of Spain (Fig. 1), a country endemic for CanL [14]. Dogs were chosen randomly from veterinary clinics, dog shelters and groups

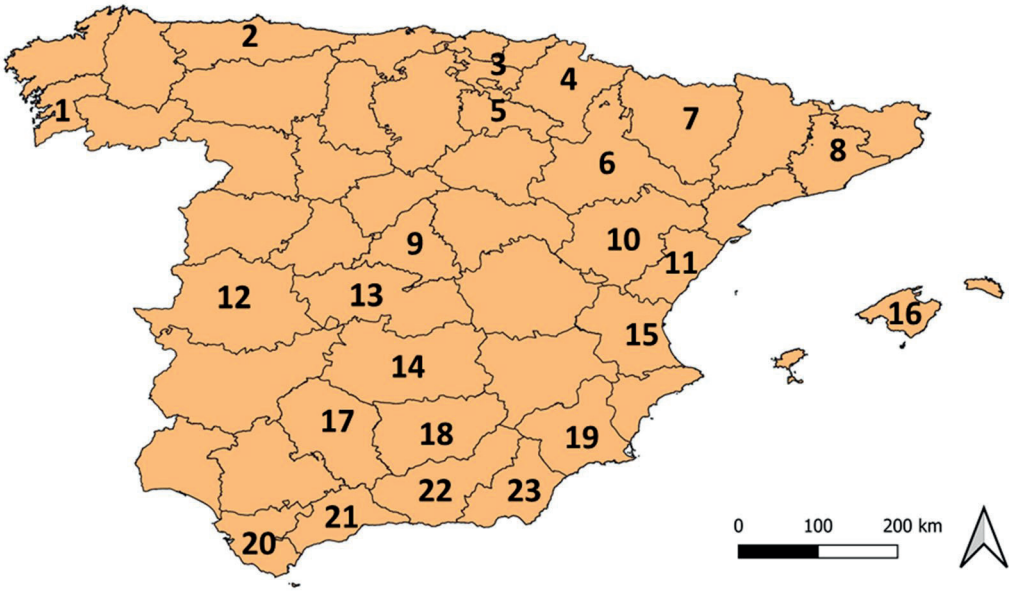

Fig. 1 Geographical distribution of dogs sampled in Spain: *1* Pontevedra ($n=5$), *2* Asturias ($n=47$), *3* Álava ($n=3$), *4* Navarra ($n=3$), *5* La Rioja ($n=1$), *6* Zaragoza ($n=10$), *7* Huesca ($n=1$), *8* Barcelona ($n=110$), *9* Madrid ($n=8$), *10* Teruel ($n=3$), *11* Castellón ($n=19$), *12* Cáceres ($n=3$), *13* Toledo ($n=1$), *14* Ciudad Real ($n=6$), *15* Valencia ($n=15$), *16* Mallorca ($n=94$), *17* Córdoba ($n=6$), *18* Jaén ($n=2$), *19* Murcia ($n=10$), *20* Cádiz ($n=54$), *21* Málaga ($n=4$), *22* Granada ($n=1$), *22* Almería ($n=1$)

of hunting dogs. The clinical data recorded included the signalment and clinical status of all dogs (Table 1). None of the dogs were vaccinated against CanL. Dogs were considered young if they were aged $\leq$ 1.5 years, while dogs aged > 1.5 years were considered to be adult. Dog characteristics, such as sex, age, breed and clinical status, and the significant differences between dogs are shown in Table 1.

The main sampling areas included Barcelona ($n=110$ dogs), Mallorca ($n=94$), Cádiz ($n=54$) and Asturias ($n=47$) (Table 1). In the additional sampling areas, fewer than 20 dogs were sampled per area, with a total of 102 dogs (Fig. 1). Dogs were also classified according to their clinical status. The majority of dogs were apparently healthy (89.9%) while 41 presented clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection and were diagnosed with leishmaniosis (10.1%) [9] (Table 1). Most dogs were sampled at the time of diagnosis and had not previously been treated with anti-*Leishmania* drugs, with the exception of three dogs that had been recently treated with allopurinol. Dogs from Asturias, an area with very low endemicity [14, 33], were classified as negative controls, while samples from sick dogs that were diagnosed with leishmaniosis were classified as positive controls.

Sampling

Blood samples were obtained by jugular or cephalic venepuncture and later centrifuged (Heraeus Labofuge 400R Centrifuge; Thermo Fisher Scientific, Waltham, MA, USA) at 789 *g* for 10 min to obtain serum.

OTs were collected by foam swabs (Ecouvillon PP; Dominique Dutscher, Bernolsheim, France) impregnated with hypertonic saline (NaCl 7.5%; B. Braun Melsungen AG, Melsungen, Germany) mainly as described previously [34] but with some modifications. The swabs were kept in the dog's mouth between the gum and the inner mucosa of the upper or lower lip for around 2 min and later centrifuged (Eppendorf Centrifuge 5418; Merck KGaA, Darmstadt, Germany) at 16,000 *g* for 1 min. After that, OTs were collected.

All samples, including both serum samples and OTs, were identified and stored at – 80 °C until further use.

Quantitative in-house ELISA for the detection of *L. infantum*-specific antibodies

Serum ELISA

The in-house ELISA was performed on serum samples of all dogs studied as previously described [21]. Briefly, samples were diluted to 1:800 in phosphate buffered saline (PBS) Tween with 1% dry milk and incubated at 37 °C for 1 h, following which they were washed three

Table 1 Signalment and geographical distribution of dogs enrolled in the study

Geographical distribution (number of dogs)	Sex (%, number of dogs)		Breed (%, number of dogs)		Most common breeds (%, number of dogs)	Age (%, number of dogs)[a]		Age, median (years, min–max)[a, e]	Clinical status (%, number of dogs)	
	Female[b]	Male[b]	Purebred[c]	Crossbreed[c]		Young[d]	Adult[d]		Healthy[f]	Sick[f]
Asturias (47)	51.1%, 24	48.9%, 23	89.4%, 42	10.6%, 5	English setter (17%, 8) and Mastiff (10.6%, 5)	8.9%, 4	91.1%, 41	5.5%, 0.5–12	100%, 47	0%, 0
Barcelona (110)	46.4%, 51	53.6%, 59	20.9%, 23	79.1%, 87	German Shepherd (4.5%, 5) and Labrador Retriever (3.6%, 4)	25.5%, 24	74.5%, 70	4%, 0.3–12	87.3%, 96	12.7%, 14
Cádiz (54)	44.4%, 24	55.6%, 30	29.6%, 16	70.4%, 38	Spanish sighthound (11.1%, 6)	17.5%, 7	82.5%, 33	3.5%, 0.5–16	100%, 54	0%, 0
Mallorca (94)	68.1%, 64	31.9%, 30	80.9%, 18	19.1%, 76	Ibizan Hound (54.3%, 51), Mallorca Shepherd dog (5.3%, 5) and Andalusian wine-cellar rat-hunting dog (5.3%, 5)	38%, 35	62%, 57	3%, 0.5–14	92.6%, 87	7.4%, 7
Total of provinces of origin (407)	51.4%, 209	48.6%, 198	46.7%, 190	53.3%, 217	Ibizan Hound (12.8%, 52), German Shepherd (3.9%, 16) and Mastiff (3.4%, 14)	22.8%, 79	77.2%, 267	4%, 0.3–16	89.9%, 366	10.1%, 41

max maximum, *min* minimum

[a] Age was not recorded in 2 dogs from Asturias, 16 dogs from Barcelona, 14 dogs from Cádiz, 2 dogs from Mallorca and 27 dogs from other Spanish regions

[b] Mallorca had a higher rate of female dogs (Chi-square test: $X^2 = 11.7$, $df = 3$, $P = 0.008$)

[c] Asturias and Mallorca had a higher rate of purebred dogs (Chi-square test: $X^2 = 110.9$, $df = 3$, $P < 0.001$)

[d] Mallorca had significantly more young dogs than Asturias (Fisher's exact test: $P < 0.0001$) and Cádiz (Fisher's exact test: $P = 0.025$), while Asturias had significantly more adult dogs than Barcelona (Fisher's exact test: $P = 0.024$)

[e] Dogs from Mallorca were significantly younger than those from Asturias (Mann–Whitney test: $U = 2740$, $n_1 = 45$, $n_2 = 92$, $P = 0.002$) and Barcelona (Mann–Whitney test: $U = 5106$, $n_1 = 94$, $n_2 = 92$, $P = 0.032$)

[f] Barcelona and Mallorca had some sick dogs (Chi-square test: $X^2 = 13.4$, $df = 3$, $P = 0.004$) while all dogs in Asturias and Cádiz were apparently healthy

times (3 min each wash) with PBS-Tween and once (1 min) with PBS. The samples were then incubated for 1 h at 37 °C with peroxidase-conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.16 ng/µl. After incubation, the plates were washed three times with PBS-Tween followed by an additional wash with PBS. Then, *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) were added to the plates and the reaction was finally stopped with 5 M H_2SO_4. The results were read at 492 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China) and were defined as ELISA units (EU) in relation with a positive canine serum sample used as a calibrator set at 100 EU. The cut-off of the serum in-house ELISA was already determined to be 35 EU using the ELISA results of 80 dogs from a non-endemic area, as previously described [35]. Cut-off was established by the standard deviation (SD) method, consisting of multiplying the SD of the results by four and adding up the mean of the results obtained by the ELISA (mean + 4 SD). Serum was classified as high positive when the result was ≥ 300 EU, medium positive when the result was ≥ 150 EU and < 300 EU, low positive when the result was ≥ 35 EU and < 150 EU and negative when the result was < 35 EU [35].

Oral transudate ELISA

The in-house ELISA was performed on OTs of all dogs studied as previously described [21] with some modifications. OT samples were diluted to 1:5 in PBS-Tween with 1% dry milk and incubated at 37 °C for 1 h. Washes were performed as described for the serum samples, and peroxidase conjugated Protein A (Peroxidase Conjugate Protein A; Merck KGaA) at a concentration of 0.5 ng/µl was added and then incubated at 37 °C for 1 h. Washes were repeated and *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD; Merck KGaA) were added to the samples. The reaction was stopped with 5 M H_2SO_4. As described for the serum samples, the results were read in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd.) at 492 nm and were quantified as EU relative to a positive canine OT sample used as a calibrator set at 100 EU. The cut-off of the OT in-house ELISA was established using the ELISA results of 30 non-infected healthy Beagles. With the values of these 30 dogs, the SD was calculated and multiplied by 4, and then added up to the mean of all the results (mean + 4 SD), resulting in a cut-off value of 28 EU. The OTs were then classified as positive when the result was ≥ than 28 EU and negative when it was < 28 EU.

Statistical analysis

A descriptive analysis of all collected data was performed. Qualitative variables (sex [female/male], breed [purebred/mixed breed], age [young/adult] and ELISA results [positive/negative]) were assessed with a Fisher's exact test when only two groups were compared and with a Chi-square test when there were more than two groups. Quantitative variables (age, EU) were assessed using a non-parametric Mann–Whitney U-test when two groups were compared (clinical status: apparently healthy/sick), and the Kruskal–Wallis H-test was used when more than two groups were compared (geographical distribution). Spearman's correlation test was carried out to detect a relationship between ELISA quantitative results of the serum and OT.

The agreement between the interpretation of the results of serum and OT ELISAs was calculated by percent agreement and by Cohen's kappa statistic (κ) for agreement (kappa agreement test). When evaluating kappa agreement, the agreement was considered to be slight when it ranged from 0.00 to 0.20, fair when at range 0.21–0.40, moderate at range 0.41–0.60, substantial at range 0.61–0.80 and almost perfect at range 0.81–1.00 [36].

A *P*-value of < 0.05 was considered to be statistically significant. The Shapiro–Wilk test was performed to detect normal distribution of quantitative variables. Areas where < 20 dogs were sampled were excluded from the geographical distribution analysis. The statistical analysis was performed using the package Stats for R software version i386 3.6.1 for Windows. Cohen's κ statistic for agreement was calculated using free on-line GraphPad software (https://www.graphpad.com/quickcalcs/kappa1/). Graphs were plotted using Graphad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Serum ELISA results

The rate of *L. infantum* infection determined by serum ELISA and the serological status of dogs (negative, low positive, medium positive or high positive) are shown in Table 2. The infection rate was significantly higher in adult dogs than in young dogs (42.7 vs 21.5%; Fisher's exact test: $P = 0.001$), and lower in apparently healthy dogs than in sick dogs (29.5 vs 100%; Fisher's exact test: $P < 0.0001$) (Table 2). No significant differences were observed between dogs of different sex and breed (Table 2). When dogs from different geographical locations were compared, a significantly lower rate of infection was found in Asturias when compared to the other

Table 2 Rate of *L. infantum* infection, percent agreement and Cohen's kappa agreement between enzyme-linked immunosorbent assay results for serum and oral transudate samples

Classification (number of dogs)	Number of positive dogs (%)		Percent agreement (%)	Cohen's κ agreement (interpretation)	95% CI of Cohen's κ agreement
	Serum ELISA	OT ELISA			
Total of dogs (407)	149 (36.6)	133 (32.7)	345 (84.8)	0.66 (substantial agreement)	0.59–0.74
Sex					
Female (209)	78 (37.3)	71 (34)	174 (83.3)	0.64 (substantial agreement)	0.53–0.75
Male (198)	71 (35.9)	62 (31.3)	171 (86.4)	0.7 (substantial agreement)	0.59–0.8
Age[a]					
Young (79)	17 (21.5)[b]	15 (19)[c]	69 (87.3)	0.61 (substantial agreement)	0.39–0.83
Adult (267)	114 (42.7)[b]	103 (38.6)[c]	224 (83.9)	0.67 (substantial agreement)	0.58–0.76
Breed					
Purebred (190)	63 (33.2)	63 (33.2)	158 (83.2)	0.62 (substantial agreement)	0.5–0.74
Mixed breed (217)	86 (39.6)	70 (32.3)	187 (86.2)	0.7 (substantial agreement)	0.6–0.8
Geographical distribution					
Asturias (47)	0 (0)[d]	3 (6.4)[e]	44 (93.6)	-[h]	-[h]
Barcelona (110)	30 (27.3)[d]	23 (20.9)[e]	99 (90)	0.73 (substantial agreement)	0.58–0.88
Cádiz (54)	9 (16.7)[d]	7 (13)[e]	48 (88.9)	0.56 (moderate agreement)	0.25–0.87
Mallorca (94)	33 (35.1)[d]	28 (29.8)[e]	74 (79.6)	0.54 (moderate agreement)	0.36–0.72
Clinical status					
Sick (41)	41 (100)[f]	37 (90.2)[g]	37 (90.2)	-[h]	-[h]
Apparently healthy (366)	108 (29.5)[f]	96 (26.2)[g]	308 (84.2)	0.61 (substantial agreement)	0.52–0.7
Negative control (Asturias) and positive control dogs (Sick) (88)	41 (46.6)	40 (45.5)	81 (92.1)	0.84 (almost perfect agreement)	0.73–0.95
Serological status					
High positive (26)	26 (100)	26 (100)	26 (100)	-[h]	-[h]
Medium positive (40)	40 (100)	34 (85)	34 (85)	-[h]	-[h]
Low positive (83)	83 (100)	50 (60.2)	50 (60.2)	-[h]	-[h]
Negative (258)	0 (0)	23 (8.9)	235 (91.1)	-[h]	-[h]
Negative control (Asturias) and high and medium positive dogs (113)	66 (58.4)	63 (55.8)	104 (92)	0.84 (almost perfect agreement)	0.74–0.94

CI confidence interval, *ELISA* enzyme-linked immunosorbent assay, *OT* oral transudate

[a] Age was not recorded in 61 dogs

[b] Fisher's Exact test: $P = 0.001$

[c] Fisher's Exact test: $P = 0.001$

[d] Chi-square test: $\chi^2 = 23.7$, $df = 3$, $P < 0.001$

[e] Chi-square test: $\chi^2 = 12.8$, $df = 3$, $P = 0.004$

[f] Fisher's Exact test: $P < 0.0001$

[g] Fisher's Exact test: $P < 0.0001$

[h] Cohen's kappa (κ) agreement could not be calculated in the Asturias, the seropositive sick dogs and the serological status groups because of the lack of positivity to both tests or the lack of negativity to both tests

locations (Chi-square test: $\chi^2 = 23.7$, $df = 3$, $P < 0.001$) (Table 2).

Regarding the quantitative ELISA results shown in Table 3, adult and sick dogs presented significantly higher median EU values than young and apparently healthy dogs, respectively (Fig. 2; Mann–Whitney test: $U = 12{,}389$, $n_1 = 267$, $n_2 = 79$, $P = 0.018$; Mann–Whitney test: $U = 829$, $n_1 = 366$, $n_2 = 41$, $P < 0.0001$). No significant differences were observed when different sexes and breeds were compared (Table 3). When groups from different geographical locations were compared (Table 3; Fig. 3a), Asturias (3.7 EU) presented a significantly lower median value than Barcelona (11.4 EU), Cádiz (6.3 EU) and Mallorca (25.3 EU) (Kruskal–Wallis H-test: $\chi^2 = 99.2$, $df = 3$, $P < 0.0001$) while Barcelona and Mallorca had significantly higher median values than Cádiz.

Table 3 Median values of serum and OT EU according to the degree of reactivity to sera ELISA

Classification of dogs (number of dogs)	Median of serum EU (min–max)[a]	Median of OT EU (min–max)[a]
Total of dogs (407)	17.7 (0–300)	14.9 (0–300)
Sex		
Female (209)	22.3 (0–300)	13.8 (0–300)
Male (198)	15.9 (0–300)	15.8 (0–300)
Age[b]		
Young (79)	11.0 (1.8–300)[c]	9.9 (0–250.5)[f]
Adult (267)	22.3 (0–300)[c]	18.1 (0–300)[f]
Breed		
Purebred (190)	16.9 (0–300)	16.0 (0–300)
Mixed breed (217)	18.2 (0–300)	13.6 (0–300)
Geographical location		
Asturias (47)	3.7 (0–7.4)[d]	8.6 (0.2–39.9)[g]
Barcelona (110)	11.4 (2.7–300)[d]	12.0 (0.2–300)[g]
Cádiz (54)	6.3 (0–300)[d]	4.1 (0–300)[g]
Mallorca (94)	25.3 (3.2–300)[d]	14.7 (2.2–166.5)[g]
Clinical status		
Sick (41)	300.0 (39.3–300)[e]	111.7 (11.6–300)[h]
Apparently healthy (366)	12.8 (0–300)[e]	12.9 (0–300)[h]
Serological status		
Negative (258)	7.0 (0–34.7)	9.7 (0–76.4)
Low positive (83)	59.2 (35–142.9)	38.1 (0–166.5)
Medium positive (40)	210.4 (150.4–291.8)	80.4 (0–300)
High positive (26)	300.0 (300)	160.9 (28.5–300)
Total positives (149)	132.8 (35–300)	59.2 (0–300)

EU ELISA units, *OT* oral transudate, *max* maximum, *min* minimum

[a] Samples with a value of 300 EU may actually be higher as the spectrophotometer is only able to read up to 3 units of optical density

[b] Age was not recorded in 61 dogs

[c] Mann–Whitney test: $U = 12{,}389$, $n_1 = 267$, $n_2 = 79$, $P = 0.018$

[d] Kruskal–Wallis *H*-test: $\chi^2 = 99.2$, $df = 3$, $P < 0.0001$

[e] Mann–Whitney test: $U = 829$, $n_1 = 366$, $n_2 = 41$, $P < 0.0001$

[f] Mann–Whitney test: $U = 12{,}863$, $n_1 = 267$, $n_2 = 79$, $P = 0.003$

[g] Kruskal–Wallis *H*-test: $\chi^2 = 38.7$, $df = 3$, $P < 0.0001$

[h] Mann–Whitney test: $U = 1461$, $n_1 = 366$, $n_2 = 41$, $P < 0.0001$

Oral transudate ELISA results

The rate of *L. infantum* infection determined on OT ELISA is shown in Table 2. Similar to the results for the serum samples, the rate of OT sample positivity was also significantly higher in adult (Fisher's exact test: $P = 0.001$) and sick dogs (Fisher's exact test: $P < 0.0001$) (38.6%) when compared to young dogs (19%) while it was lower in apparently healthy dogs than in sick dogs (26.2% vs 90.2%) (Table 2). No significant differences were observed in terms of sex and breed (Table 2). When comparisons were made between groups of dogs from different geographic locations, a significantly lower rate of infection was still found for dogs from Asturias compared to those from other locations (Chi-square test: $\chi^2 = 12.8$, $df = 3$, $P = 0.004$) (Table 2).

Regarding the quantitative ELISA results shown in Table 3, as found in the serum results, adult and sick dogs presented a significantly higher mean EU value than young and apparently healthy dogs, respectively (Mann–Whitney test: $U = 12{,}863$, $n_1 = 267$, $n_2 = 79$, $P = 0.003$; Mann–Whitney test: $U = 1461$, $n_1 = 366$, $n_2 = 41$, $P < 0.0001$) (Fig. 2b). No significant differences were observed between different sex and breed (Table 3). When groups of dogs from different geographical location were compared (Table 3; Fig. 3b), Asturias (8.6 EU) and Cádiz (4.1 EU) presented a significantly lower mean EU value than Barcelona (12 EU) and Mallorca (14.7 EU) (Kruskal–Wallis H-test: $\chi^2 = 38.7$, $df = 3$, $P < 0.0001$).

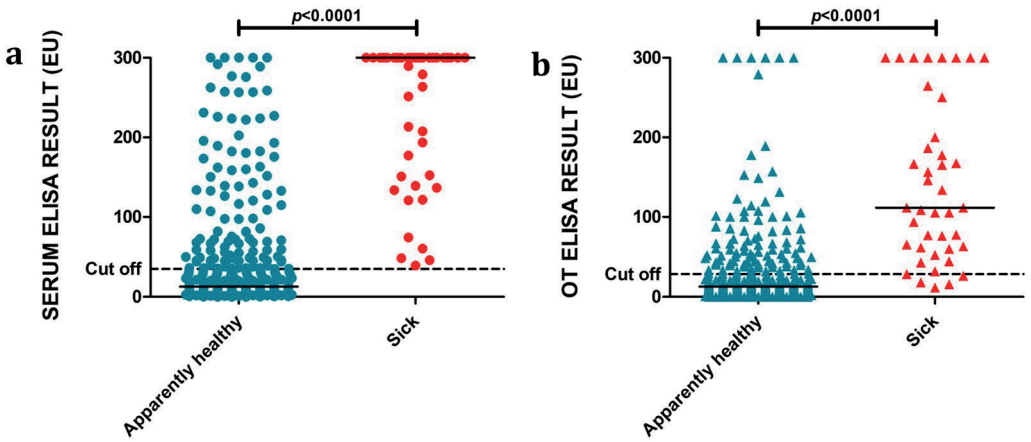

Fig. 2 Antibody levels against *L. infantum* (EU) as determined by the in-house ELISA performed on serum (**a**) and OT (**b**) samples collected from dogs classified according to clinical status (apparently healthy vs sick). Horizontal solid black lines indicate the median. Horizontal black dashed lines indicate the cut-off: 35 EU in serum ELISA and 28 EU in OT ELISA. *ELISA* enzyme-linked immunosorbent assay, *EU* ELISA units, *OT* oral transudate

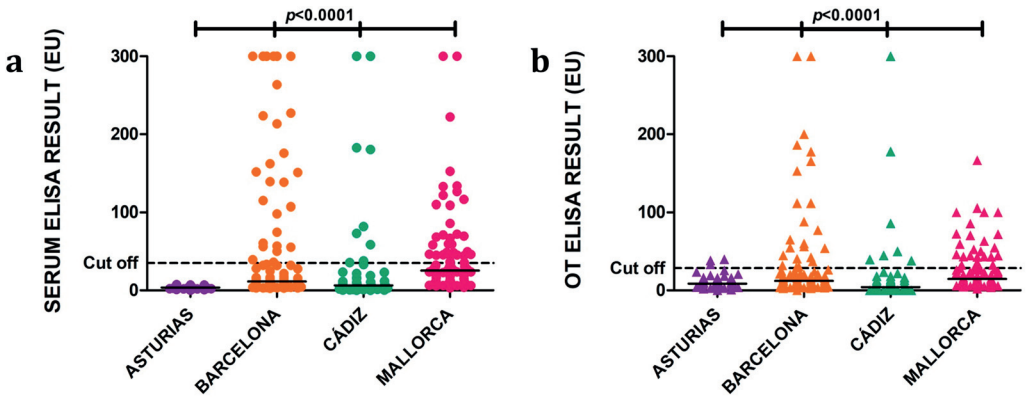

Fig. 3 Antibody levels against *L. infantum* (EU) by the in-house ELISA performed on serum (**a**) and OT (**b**) samples collected from dogs classified according to geographical distribution. Horizontal solid black lines indicate the median. Horizontal black dashed lines indicate the cut-off: 35 EU in serum ELISA and 28 EU in OT ELISA. *ELISA* enzyme-linked immunosorbent assay, *EU* ELISA units, *OT* oral transudate

Correlation and comparison between ELISA results for serum and OT samples

A positive correlation was established between the results of the in-house ELISA for the serum and OT samples (Spearman's correlation coefficient $r_s = 0.6687$, $P < 0.0001$) when all samples were studied (Fig. 4). The positive correlation improved when only Asturias dogs (negative control) and sick dogs (positive control) were investigated (Spearman's correlation coefficient $r_s = 0.7479$, $P < 0.0001$) and also when only Asturias seronegative dogs and high and medium seropositive dogs were studied (Spearman's correlation coefficient $r_s = 0.7585$, $P < 0.0001$). On the other hand, when only low seropositive dogs were investigated, the positive correlation was lower (Spearman's correlation coefficient $r_s = 0.3079$, $P = 0.005$).

Of the total of 407 dogs, 235 (57.7%) were negative by both serum and OT ELISA while 110 (27%) were positive to both tests. In contrast, there was disagreement regarding the remaining 62 dogs (15.3%). Six medium seropositive and 33 low seropositive dogs (9.6%) with a median of 55.3 EU (ranging from 35 to 288.9 EU) were negative by OT ELISA with a median of 12.4 EU (ranging from 0 to 27.2 EU) while 23 seronegative dogs (5.7%) with a median of 16.7 EU (ranging from 0.9 to 30.8 EU) were positive by OT ELISA with a median of 43.4 EU (ranging from 29.4 to 76.4 EU) (Fig. 5). The percentage agreement and Cohen's kappa agreement between serum and OT ELISA results was substantial

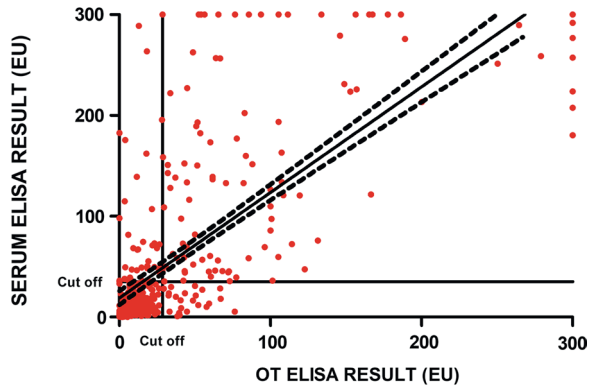

Fig. 4 Spearman's correlation coefficient (r_s) for the serum and OT ELISA results ($r_s[407] = 0.6687$, $P < 0.0001$). Red filled circles indicate the individual results for each sampled dog. The horizontal solid black line indicates the cut-off: 35 EU in serum ELISA (*Y*-axis) and 28 EU in OT ELISA (*X*-axis). *ELISA* enzyme-linked immunosorbent assay, *EU* ELISA units, *OT* oral transudate

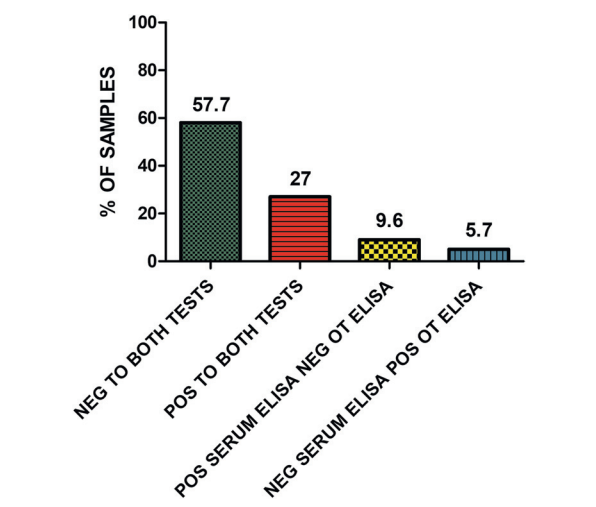

Fig. 5 Proportion of positive and negative samples based on the results of both the serum and OT ELISAs. *Neg* Negative, *Pos* positive

(0.66) when studying the whole group while it went from almost perfect to moderate depending on the classification studied (Table 2).

Comparison of the EU values for the serum and OT samples according to degrees of reactivity is shown in Table 3. When comparing the OT EU, antibody levels were found to be significantly higher in OT samples with a high or medium positive EU value for the serum ELISA than in those with a low positive serum ELISA (Kruskal–Wallis H-test: $\chi^2 = 43.2$, $df = 2$, $P < 0.0001$).

Discussion

A quantitative in-house ELISA technique [21] was adapted in the present study to detect specific anti-*Leishmania* antibodies in OT canine samples and to assess the diagnostic performance of this ELISA. This ELISA is currently performed on serum samples to detect specific immunoglobulins as it has been proven that most dogs infected with an active disease show high levels of different isotypes of antibodies [9, 18, 37]. The presence of several types of immunoglobulins has also been studied in saliva [28]. IgA has been proven to be present in saliva as it is secreted in the salivary glands by plasma cells, and plasma-derived antibodies have been found, such as IgG and IgM [28]. Specific canine anti-*Leishmania* antibodies have also been documented in oral fluid samples by using a TR-IFMA [24, 29–31], which is a technique that has shown a broader range of detection of antibodies in serum than ELISA. These studies showed great success at discriminating between seropositive and seronegative dogs with no overlapping in terms of evaluating IgG2 [24, 29–31]. However, the authors of these studies were not successful at correctly differentiating seropositive dogs from seronegative based on IgA evaluation [24, 29–31]. These studies provided the first evidence of the potential of oral fluid for the quantification of anti-*Leishmania* IgG2 to diagnose CanL [24, 29–31]. Nonetheless, no studies have evaluated the ability to detect anti-*Leishmania* antibodies by using a quantitative in-house ELISA technique in OT samples until now. Additionally, the first study performed on oral fluid samples for the diagnosis of CanL was carried out on a very homogeneous group of dogs, using dogs with advanced clinical leishmaniosis and high antibody levels [24], while in the present study, dogs with subclinical infection and low antibody titers were also included.

In the present study, the agreement between the qualitative interpretation of serum and OT ELISA results was evaluated using two methods: (1) percent agreement and (2) agreement according to the kappa agreement statistic. The percent agreement is easy to calculate and can be interpreted directly, but it does not take into account the agreements made by chance [38]. On the other hand, Cohen's kappa agreement statistic is a statistical value useful for assessing inter-rater or intra-rater reliability and takes into consideration the possibility of chance [38]. A Cohen's kappa agreement of > 0.80 is needed to be able to validate a new test [38]. When Cohen's kappa agreement was interpreted for the 407 dogs, a substantial agreement of 0.66 was found. As stated earlier in this text, this agreement is not sufficient to affirm that OT can be used to correctly differentiate between seropositive and seronegative dogs by means of an in-house ELISA. However, a high number of dogs in this study presented

subclinical infection and low seropositive antibody levels, which is a likely explanation of why the agreement was lower than found in previous studies where the dog populations studied were mostly sick dogs with advanced clinical leishmaniosis [24, 31]. When Cohen's kappa agreement was obtained only for seronegative dogs from Asturias (a low endemicity area) and for sick dogs with clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection, an almost perfect agreement of 0.84 was obtained. The same result (0.84) was found when Cohen's kappa agreement was obtained for seronegative dogs from Asturias and seropositive dogs with high or medium levels of antibody levels. These findings agree with those reported in previous studies [24, 31] and highlight the usefulness of detecting antibodies against *L. infantum* in OT in dogs with clinical leishmaniosis or progressing towards disease.

When the percent agreement was evaluated, an agreement of 84.8% was found. The remaining samples from 15.2% (62) dogs showed disagreements between the serum and OT ELISA. Included in these samples that disagreed, 39 were from seropositive dogs (39/62 dogs; 62.9%) that were negative by the OT ELISA. There are several reasons that could explain this disagreement in results from the OT and serum ELISA. First, there may be a lesser ability to detect seropositive dogs with a low serum antibody, as detected when comparing the Cohen's κ agreement statistic described above. This seems to be the most plausible reason as when only seronegative dogs from Asturias and sick dogs with clinical signs and/or clinicopathological abnormalities compatible with *L. infantum* infection were studied, the percent agreement increased to 92.1%. A similar result, i.e. 92%, was obtained when the results from only seronegative dogs from Asturias and seropositive dogs with high or medium levels of antibody levels were considered. This result was to be expected as the sick group presented a higher proportion of high serum antibody levels compared to the apparently healthy group which had a higher proportion of low antibody levels. Another explanation could be a lack of homogenous OT sample collection, as even if untrained personnel can perform this procedure, it is difficult to perform correctly if the standardized protocol is not followed as described [39]. For example, if the impregnated swabs were not kept in the mouth of the dog for at least 2 min, insufficient OT could have been absorbed. As the samples in this study were collected by several veterinarians, even though a standardized protocol was recommended and agreed to, we could not confirm that all samples were always collected in a similar manner. On the other hand, of these 62 disagreements, 23 seronegative dogs (23/62 dogs; 37.1%) turned out to be positive in the OT ELISA. These results were unexpected. One possible explanation is that sand flies mainly feed on skin areas with very little hair, such as the face [15], which could lead to a local expression of parasite-specific immunoglobulins before the parasite disseminates systemically. A second possibility is that there may be an as-yet unknown cross-reactivity with another pathogen, such as oral bacteria, in some dogs with poor dental hygiene and dental disease, such as gingivitis, stomatitis and periodontal disease. Further studies on the diagnostic performance of the OT ELISA are needed to evaluate this hypothesis.

When taking locations of origin into consideration, the percent agreement was higher in Asturias (93.6%), followed by Barcelona (90%), Cádiz (88.9%) and Mallorca (79.6%). In comparison, Cohen's kappa agreement was substantial in Barcelona (0.73), followed by Cádiz with a moderate agreement (0.56) and Mallorca, also with a moderate agreement (0.54).

Despite the OT showing a lower diagnostic value than serum according to the quantitative in-house ELISA used in this study, a good percentage of success was obtained for the OT samples. In addition, OT sample collection is easy, cheap, non-invasive and painless; consequently, OT could be of use in specific cases, such as dogs who do not have easy access to veterinary clinics, dogs that need a continued follow-up or aggressive dogs that can only be touched by its owner.

Further studies are needed to increase the reliability of the results of the present study. First, an investigation of the OT quality must be performed to confirm the correct collection of the samples before performing OT ELISA. In addition, a group of dogs with poor dental hygiene and presenting dental diseases could be added to the study population to assess the possibility of poor dental health being a factor of false positivity by OT ELISA. Also, it would be also of interest to perform a longitudinal study of those dogs that were seronegative yet tested positive by OT ELISA, as well as those dogs that tested negative for the OT ELISA yet tested positive by the serum ELISA, to describe antibody kinetics. Finally, other techniques using OT could also be developed and improved. Even ELISA as a serological test has some limitations in terms of the detection of infection as it can detect antibodies elicited by *Leishmania* vaccines in dogs [17].

The seroprevalence of canine *L. infantum* infection was around 10% [14, 33, 40] between 2011 and 2020 in Spain, which is lower than the seroprevalence detected in the present study (36.6%). In terms of specific Spanish areas, Asturias has always presented one of the lowest seroprevalence rates [14, 33, 40], usually around 1%, while the rates from Cádiz and Mallorca are usually higher than 15% [14]. These results resemble those found in the present study, with low rates in Asturias (0%) and high

rates in Cádiz (16.7%) and Mallorca (35.1%). Regarding the results found in Barcelona (27.3%), a previous study performed in 27 sick and 20 clinically healthy dogs in 2006 [41] documented a 65% seroprevalence of *L. infantum* in Barcelona, but no other studies in this area have been carried out in the last decade. However, seroprevalence rates of around 13% were detected in other areas of Catalonia [33, 40]. Interestingly, the seroprevalence rates detected in this study seem to be slightly higher than those described in previous studies [14, 33, 40, 41]. This could be related to the number of sick dogs included in the Barcelona (12.7%) and Mallorca (7.4%) groups. The incidence rate of human leishmaniosis in Spain was 0.62 cases per 100,000 inhabitants between 2005 and 2017, with cases mainly distributed throughout the Mediterranean region [42]. However, asymptomatic infections are also common in humans in Spain and Mediterranean basin countries as recently reviewed elsewhere [43].

We also detected higher serological rates of *L. infantum* infection in both adult and sick dogs. A high rate should be expected in sick dogs that have been already diagnosed with leishmaniosis and still present clinical signs and/or clinicopathological abnormalities [9, 15]. In terms of age of dogs, previous studies have found that puppies (< 1 year old) have a lower rate of *L. infantum* infection than dogs aged > 1 year old [33, 40] and that the risk of *Leishmania* infection increases with increasing age [40].

Conclusions

In conclusion, the present study demonstrates an almost perfect to moderate agreement between OT and serum samples using a quantitative in-house ELISA for *Leishmania* antibodies. These results are promising for the detection of infection in sick dogs with high antibody levels while they seem to be less optimal in apparently healthy dogs with low antibody levels. Further studies could improve OT serology and its reliability and value as a future diagnostic technique for *L. infantum* infection when compared with other diagnostic methods for CanL.

Abbreviations

CanL: Canine leishmaniosis; ELISA: Enzyme-linked immunosorbent assay; EU: ELISA units; IFN-γ: Interferon gamma; IgA: Immunoglobin A; IgG: Immunoglobin G; IgM: Immunoglobin M; OT: Oral transudate; PBS: Phosphate buffered saline; ROS: Reactive oxygen species; SD: Standard deviation; TR-IFMA: Time-resolved immunofluorometric assay.

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Authors' contributions

MB, AAF, MMA and LSG collected serum and OT samples. MB and JV performed laboratory work (ELISAs). MB performed statistical analysis and prepared the figures. MB and LSG contributed with data analysis and interpretation. JV wrote the first draft of the manuscript. MB and LSG wrote the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethical review and approval were waived for the study, due to non-invasive procedures (OT sampling) and use of residual samples (serum). A signed consent form was obtained from the owner or the person in charge of the dog(s).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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