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Autor

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UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

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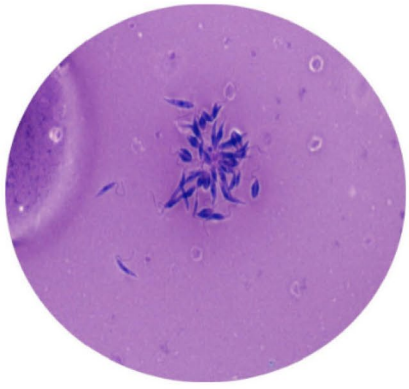
TESIS DOCTORAL

JACOBO GINER AUDIVERT



2023

DIRECTORES:
MAITE VERDE ARRIBAS
SERGIO VILLANUEVA SAZ

Tesis Doctoral

ESTUDIO DE LA INFECCIÓN POR *Leishmania infantum* EN MUSTÉLIDOS.

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Medicina y Sanidad Animal

Dra. MAITE VERDE ARRIBAS, Catedrática del Departamento de Patología Animal de la Facultad de Veterinaria de Zaragoza Dr. SERGIO VILLANUEVA SAZ, Profesor Ayudante Doctor del Departamento de Patología Animal de la Facultad de Veterinaria de Zaragoza, como Directores,

C E R T I F I C A N:

Que JACOBO GINER AUDIVERT ha realizado bajo su dirección los trabajos correspondientes a su Tesis Doctoral titulada: "Estudio de la infección por *Leishmania infantum* en mustélidos", que se ajusta al Proyecto de Tesis presentado y que cumple las condiciones exigidas para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizan la presentación de esta Tesis por compendio de publicaciones para que pueda ser juzgada por el Tribunal correspondiente.

Y para que conste, firman el presente certificado

En Zaragoza, a 28 de junio de 2023

Dra. Maite Verde Arribas

Dr. Sergio Villanueva Saz

Esta **Tesis Doctoral**, presentada por Jacobo Giner Audivert, de acuerdo con el informe correspondiente, autorizado por los directores de Tesis y el Órgano Responsable del Programa de Doctorado, se presenta como un compendio de trabajos previamente publicados. La unidad temática de todos ellos está directamente relacionada con la infección por *Leishmania infantum* en mustélidos, por lo que queda justificada su presentación por compendio de publicaciones.

Las referencias completas de los artículos que constituyen el cuerpo de la tesis son los siguientes (Publicaciones en revistas incluidas en "Journal of Citation Reports (JCR)"):

Artículo 1 (artículo original, Revista Indexada en JCR (Q1), segundo autor):

Alcover MM, **Giner J**, Rabasedas J, Roca-Geronés X, Verde M, Fernández A, Riera C, Fisa R, Villanueva-Saz S. First epidemiological survey of *Leishmania infantum* in the domestic ferret (*Mustela putorius furo*) in a canine leishmaniosis endemic area using serology and PCR. Parasit Vectors 2022 Oct 17;15(1):372. doi: 10.1186/s13071-022-05517-y.

Factor Impacto: 4,052
Área temática: Parasitología

Artículo 2 (artículo original, Revista Indexada en JCR (Q2), primer autor):

Giner J, Villanueva-Saz S, Fernández A, Gómez MA, Podra M, Lizarraga P, Lacasta D, Ruiz H, Del Carmen Aranda M, de Los Ángeles Jimenez M, Hernández R, Yzuel A, Verde M. Detection of Anti-*Leishmania infantum* Antibodies in Wild European and American Mink (*Mustela lutreola* and *Neovison vison*) from Northern Spain, 2014-20. *J Wildl Dis* 2022 Jan 1;58(1):198-204. doi: 10.7589/JWD-D-21-00027.

Factor Impacto: 1,626
Área temática: Ciencias Veterinarias

Artículo 3 (artículo original, Revista Indexada en JCR (Q1), segundo autor):

Villanueva-Saz S, **Giner J**, Verde M, Yzuel A, Ruiz H, Lacasta D, Riera C, Fisa R, Alcover MM, Fernández A. Antibodies to *Leishmania* in naturally exposed domestic ferrets (*Mustela putorius furo*) in Spain. *Vet Parasitol* 2021 Aug;296:109492. doi: 10.1016/j.vetpar.2021.109492.

Factor Impacto: 2,821
Área temática: Ciencias Veterinarias

Artículo 4 (artículo de revisión, segundo autor):

Villanueva-Saz S, **Giner J**, Marteles D, Verde M, Yzuel A, Riera C, Fisa R, Alcover M, Fernández A. Leishmaniosis caused by *Leishmania infantum* in ferrets: Update review. *Vet Anim Sci* 2021 Dec 27;15:100229. doi: 10.1016/j.vas.2021.100229.

Factor Impacto (JCI): 0,82
Área temática: Ciencias Veterinarias

Artículo 5 (artículo original, primer autor) **:**

Giner J, Villanueva-Saz S, Alcover MM, Riera C, Fisa R, Basurco A, Yzuel A, Trotta M, Fani C, Verde MT, Fernández A. Treatment and follow-up of a domestic ferret (*Mustela putorius furo*) with clinical leishmaniosis caused by *Leishmania infantum*. *Vet Parasitol Reg Stud Reports* 2020 Jul;21:100423. doi: 10.1016/j.vprsr.2020.100423.

Factor Impacto (JCI): 0,55
Área temática: Parasitología

Artículo 6 (artículo original, primer autor)**:**

Giner J, Villanueva-Saz S, Alcover MM, Riera C, Fisa R, Verde M, Fernández A, Yzuel A. Clinical leishmaniosis in a domestic ferret (*Mustela putorius furo*) treated with miltefosine plus allopurinol: Serological and clinical follow-up. *Vet Parasitol Reg Stud Reports* 2021 Jul;25:100607. doi: 10.1016/j.vprsr.2021.100607.

Factor Impacto (JCI): 0,63
Área temática: Parasitología

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Esta tesis doctoral ha sido posible debido al empeño continuado de entreverar mi espíritu científico entre la vida laboral y la personal, con el esfuerzo que ello conlleva por parte de la gente que me rodea, principalmente mi familia, que me ha apoyado en todo momento.

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Índice:

1. Revisión bibliográfica:

Una enfermedad zoonótica es una enfermedad que puede transmitirse entre animales y seres humanos; y puede ser provocada por virus, bacterias, parásitos, hongos y otros patógenos, siendo más del 60% de los patógenos humanos de origen zoonótico (Rahman et al., 2020). En el mundo actual y particularmente en nuestra sociedad, el contacto entre humanos y mascotas es una constante, habiendo cada día más hogares que optan por adquirir un animal de compañía (Hassani et al., 2020). Por ello, en términos de salud pública, es importante identificar, prevenir y controlar los posibles agentes con potencial zoonótico que puedan afectar a la salud de la población (Kahn, 2006). La mayoría de las enfermedades infecciosas zoonóticas están asociadas a parásitos transmitidos por los animales de compañía a los seres humanos. La toxoplasmosis, la leishmaniosis, la giardiasis, la equinococosis, la dirofilariosis y la toxocariosis son las enfermedades zoonóticas parasitarias más comunes (Baneth et al., 2016).

La leishmaniosis o leishmaniasis (*) es una enfermedad parasitaria zoonótica transmitida por vectores y producida por más de 20 especies de protozoos del género *Leishmania* (Clase Kinetoplasta, Familia Trypanosomatidae), siendo *Leishmania infantum* (*L. infantum*) el responsable de la enfermedad en Europa y su zimodema más común es el MON-1 (Baneth et al., 2016).

La transmisión de la leishmaniosis se produce durante la alimentación de las hembras de flebótomo de los géneros *Phlebotomus* (Europa, Asía y África) y *Lutzomyia* (América), al ingerir las formas intracelulares del parásito protozoario, denominados amastigotes, durante la ingesta al succionar sangre de los animales infectados (Cecílio et al., 2022). Un estudio entomológico realizado recientemente en España revela que las especies de flebótomos en nuestro país son: *Phlebotomus perniciosus* (*P. perniciosus*) (83.13%), *Sergentomyia minuta* (*S. minuta*) (9.47%), *P. ariasi* (5.62%), *P. sergenti* (0.89%) y *P. papatasi* (0.89%) (Galvez et al., 2020).

(*) El término leishmaniosis se acepta para describir la enfermedad en los perros, mientras que la leishmaniasis se utiliza con más frecuencia para hablar de la enfermedad en humanos. Sin embargo, ambos términos son utilizados indistintamente y en ocasiones han sido objeto de controversia por lo que el uso actual se basa más en la tradición educativa o preferencia personal que aplicar las normas (Miró et al., 2018).

Según un estudio reciente realizado en el entorno de los casos de leishmaniasis humana en España, *S. minuta* es la especie más prevalente, pero carece de una alta capacidad de transmisión de *L. infantum,* a diferencia de *P. perniciosus*, el cual posee un elevado potencial de transmisión y se considera el segundo vector más abundante en España (Díaz-Sáez et al., 2022). Esta especie*, P. perniciosus*,parece adaptarse tanto a interiores, incluidos los hogares urbanos, como a biotopos domésticos al aire libre (Díaz-Sáez et al., 2022). Además de los vectores responsables de la transmisión, se han descrito otras vías de transmisión, aunque de forma muy ocasional, como la vía percutánea, la vía vertical y la transfusión sanguínea (Alvar, 2012; Solano-Gallego, 2013).

El flebótomo se infecta al ingerir sangre con formas amastigote del parásito, contenidas en sangre de mamíferos. Posteriormente, el parásito sigue su desarrollo y se transforma en promastigote, comenzando a dividirse por fisión binaria, aumentando su número y migrando hacia la faringe y boca del flebótomo para ser inyectadas en la siguiente picadura. Después de ésta, los promastigotes entran en contacto con la circulación sanguínea del vertebrado y son fagocitadas por células del sistema retículo endotelial, para transformarse en amastigotes y comenzar a multiplicarse también por fisión binaria, hasta que rompen la célula y quedan libres en la circulación siendo fagocitadas de nuevo y repitiéndose el ciclo en el hospedador vertebrado hasta que son ingeridas por un nuevo flebotomo (Pereira et al., 2002; Bates, 2018; Clos et al., 2022).

La infección afecta principalmente a humanos (*Homo sapiens*) y perros (*Canis familiaris*), siendo estos últimos considerados como el principal reservorio de la infección (Gomes et al., 2020; Martí-Carreras et al., 2022). No obstante, un número cada vez más numeroso de estudios sugiere que la leishmaniosis puede afectar también a otros mamíferos y aves, suponiendo una preocupación para la salud pública por el posible papel epidemiológico en la enfermedad y para la medicina veterinaria como enfermedad emergente en otras especies (Cardoso et al., 2021).

Puesto que es posible que la transmisión a humanos se pueda producir también desde reservorios no identificados anteriormente (Palatnik-de-Sousa et al, 2011), se debería aplicar el concepto "Una sola salud (*One health*)", prestando un mayor énfasis, tanto en el conocimiento del ciclo de vida del parásito, como en su transmisión y patogenicidad para lograr una correcta prevención de la misma.

En relación con la presentación clínica de la enfermedad en el hombre, se describen seis presentaciones clínicas que dependerá, principalmente, de la especie de *Leishmania* responsable y de la respuesta inmunitaria de la persona (Rojas-Jaimes et al., 2019), y son definidas por la localización del parásito en los tejidos infectados: visceral, leishmaniasis dérmica post-kala-azar, cutánea, cutánea difusa, mucocutánea y leishmaniasis mucosa (Akhoundi et al., 2017). Entre ellas destacan principalmente:

- Leishmaniasis cutánea, que es la infección primaria y la forma más común y que se caracteriza por la aparición de una o más lesiones ulcerosas, no dolorosas, en zonas expuestas del cuerpo.
- Leishmaniasis muco-cutánea, con afectación de mucosas por diseminación de la forma cutánea.
- Leishmaniasis visceral o kala-azar, que afecta a órganos internos. Es la forma más grave y los principales síntomas son: fiebre irregular prolongada, esplenomegalia y pérdida de peso asociada o no a hepatomegalia moderada, adenopatías en regiones inguinal y cervical principalmente, leucopenia, anemia y/o trombocitopenia (McGwire et al., 2014; Akhoundi et al., 2017).
- Leishmaniasis dérmica post-kala-azar. Se denomina así a la leishmaniosis que se desarrolla en un subgrupo de pacientes que fueron tratados con éxito frente a esta enfermedad y que permanecen asintomáticos durante meses o años; pero que desarrollan una proliferación fulminante y progresiva de parásitos dentro de la dermis dando lugar a lesiones maculares, maculopapulares o nodulares difusas (McGwire et al., 2014).

En el perro, y en menor medida en el gato, *L. infantum* puede provocar, según la localización del parásito en los tejidos infectados (Solano-Gallego et al., 2011;

Gharbi et al., 2015; Abramo et al., 2021; Pereira et al., 2021; Garcia-Torres et al., 2022; Baneth et al., 2022), dos tipos de procesos:

- Enfermedad cutánea con lesiones dérmicas como dermatitis papular, dermatitis exfoliativa, úlceras, nódulos y/o onicogrifosis.
- Enfermedad visceral con síntomas como anorexia, pérdida de peso, fiebre, diarrea y epistaxis; y también otras lesiones causadas por inmunocomplejos, tales como vasculitis, artritis, uveítis o glomerulonefritis; y alteraciones clínico-patológicas como anemia no regenerativa e hiperproteinemia.

No obstante, la mayoría de los animales infectados permanecen asintomáticos.

El primer caso de infección por *L. infantum* en perros fue descrito en Túnez en 1908 (Nicolle et al., 1908), mientras que la primera descripción de leishmaniosis felina se realizó en Argelia en 1912 (Sergent et al., 1912). Recientemente también se ha diagnosticado en España en 2020 (Giner et al., 2020) la primera infección natural por *L. infantum* en otro animal de compañía, el hurón doméstico (*Mustela putorius furo*), con manifestaciones clínicas cutáneas.

La identificación de los animales infectados requiere de la utilización de pruebas de confirmación de la infección, pudiendo realizarse a través de diferentes procedimientos disponibles como son las pruebas parasitológicas, las pruebas moleculares y finalmente el diagnóstico serológico (Morales-Yuste et al., 2022):

a) Diagnóstico parasitológico:

El diagnóstico parasitológico puede realizarse mediante la observación de los amastigotes, a partir la aspiración con aguja fina y posterior visualización microscópica, en la citología obtenida con el material aspirado de un linfonodo superficial, una lesión cutánea, bazo o aspirado de médula ósea (Baneth et al., 2022). No obstante, también se ha descrito la presencia de amastigotes tras frotis de aspirado de masas nodulares de localización atípica como lengua, testículos y masas orales o nasales (Paltrinieri et al., 2016).

Otra técnica parasitológica para el diagnóstico de la leishmaniosis es el estudio histopatológico de biopsias de tejidos, que suele ir acompañada de una tinción de inmunohistoquímica específica para la detección del parásito, puesto que no siempre se observa y detecta fácilmente el parásito en el estudio histopatológico. Por ello se requiere de la inmunohistoquímica para confirmar la presencia de los amastigotes en las preparaciones histológicas) (Maia et al., 2008; Solano-Gallego et al., 2011; Morales-Yuste et al., 2022).

Finalmente, otra técnica parasitológica disponible, aunque su utilización en la práctica clínica diaria es menor, sería el aislamiento y posterior identificación mediante la utilización de medios de cultivo específicos como pueden ser el medio bifasico Novy- McNeal-Nicolle (NNN) o el medio líquido Schneider (Castelli et al., 2014; Ladopoulos et al., 2015; Santos et al., 2018). En el caso de la utilización de medios de cultivo se requerirán instalaciones de bioseguridad adecuadas ya que existe la posibilidad de contaminación microbiológica si la obtención de la muestra biológica no se ha realizado con las debidas condiciones asépticas. Por último, es importante tener en cuenta que, aunque el aislamiento y cultivo del parásito tiene una especificidad del 100 %, su sensibilidad es muy baja, ya que requiere una alta carga parasitaria (Maia et al., 2008).

b) Diagnóstico molecular:

A nivel molecular, las pruebas se basan en la reacción en cadena de la polimerasa (PCR), habiendo tres diferentes tipos de técnicas: la PCR convencional, la PCR anidada y finalmente la PCR cuantitativa en tiempo real (Castelli et al., 2021). Las dos primeras solo informan de un resultado dicotómico (positivo/negativo), mientras que la PCR en tiempo real, es más rápida de realizar y además, en caso de un resultado positivo, informa de la carga parasitaria presente en la muestra analizada (Tupperwar et al., 2008; Galluzzi et al., 2018; Morales-Yuste et al., 2022).

En el perro y en el gato, las muestras biológicas de elección para el diagnóstico molecular de esta enfermedad serían las procedentes de aspirados de nódulos linfáticos, especialmente procedentes de animales con linfoadenomegalia; así como las procedentes de aspirados de médula ósea. La toma de muestras en estos tejidos requiere de un proceso más invasivo y debe reservarse para casos especiales

como es la sospecha de la infección en un animal sin signos clínicos (Solano-Gallego et al., 2011; Pennisi et al., 2013).

No obstante, con estos métodos se pueden analizar gran cantidad de muestras para detectar ácido desoxirribonucleico (ADN) de *L. infantum* incluyendo muestras de pelo, piel, sangre, bazo, conjuntiva y orina (Solano-Gallego et al., 2007; Lombardo et al., 2012; Belinchón-Lorenzo et al., 2013; Mohammadiha et al., 2013; Pessoa-E-Silva et al., 2016; Benassi et al., 2017; Dantas-Torres et al., 2017; Ortega et al., 2017; Cantos-Barreda et al., 2020). Además de las muestras procedentes de linfonodos y médula ósea, se consideran como muestras más sensibles las muestras procedentes de bazo, piel y conjuntiva; mientras que las muestras de sangre, pelo y orina pueden utilizarse, pero la sensibilidad diagnóstica es menor (Solano-Gallego et al., 2011).

La PCR cuantitativa permite determinar la carga parasitaria en el mismo tejido a lo largo del tiempo, información que resulta muy útil en el seguimiento de la enfermedad durante el tratamiento (Pennisi et al., 2005; Manna et al., 2008). Por último, es importante destacar que la información proporcionada por la PCR no debe separarse de los datos obtenidos de las evaluaciones clínico-patológicas y serológicas (Solano-Gallego et al., 2011).

c) Diagnóstico serológico:

La respuesta humoral específica en la leishmaniosis canina es, en general, muy intensa, con altos niveles de inmunoglobulinas específicas que permiten el diagnóstico serológico. Además, la seroconversión ocurre a los pocos meses de la infección (Morales-Yuste et al., 2022) con una media de 5 meses (rango 1-22 meses) para infecciones naturales y de 3 meses (rango 1-6 meses) para infecciones experimentales (Moreno & Alvar, 2002).

La serología es la técnica diagnóstica de elección en la clínica diaria puesto que es poco invasiva y permite la detección de anticuerpos específicos en animales de compañía a partir de las 4-12 semanas post infección; si bien en las infecciones subclínicas este periodo de detección puede alargarse varios años (Moreno et al., 2002; Saridomichelakis, 2009).

Se han utilizado distintos métodos cuantitativos y cualitativos para detectar anticuerpos anti-*Leishmania* como la técnica de inmunofluorescencia indirecta (IFI), la técnica ensayo por inmunoabsorción ligado a enzimas (ELISA), la técnica Western Blot (WB), la prueba de aglutinación directa (DAT) o los sistemas de inmunocromatografía, con una sensibilidad y especificidad variable, dependiendo de la técnica utilizada (Paltrinieri et al., 2016; Maia y Campino, 2018; Alcover et al., 2021).

La técnica IFI se considera el método de referencia para serología anti-*Leishmania* en perros, basado en la alta sensibilidad y especificidad (cercana ambas al 100%) excepto en áreas endémicas de Latino América y Texas, donde el parásito *Trypanosoma cruzi* puede interferir dando resultados falsos positivos (Paltrinieri et al., 2016; Baneth et al., 2022). Los sueros problema se incuban a distintas diluciones, dependiendo de la especie de *Leishmania* que estemos analizando y la detección de anticuerpos anti-*Leishmania* se pone de manifiesto empleando un anticuerpo secundario marcado con un fluorocromo. Su evaluación se basa en la fluorescencia de los promastigotes de *L. infantum* detectada mediante microscopía ultravioleta y evaluada por un operador. Esto hace que esta técnica sea muy exigente a nivel técnico y de personal y además se requiere de la realización de diluciones seriadas de suero, lo que limita el número de muestras que se pueden analizar (Chatzis et al., 2014).

La técnica ELISA se considera un inmunoensayo muy sensible y específico que permite determinar la concentración de antígenos o de anticuerpos midiendo la formación del inmunocomplejo antígeno-anticuerpo con una reacción enzimática, siendo esta técnica más fácil de estandarizar puesto que los resultados son leídos por un espectrofotómetro automatizado. La sensibilidad y especificidad de la técnica dependen del antígeno que se emplee para el tapizado de las placas, y este puede variar utilizándose desde un extracto de promastigotes axénicos, unas proteínas de membrana purificadas o unas proteínas recombinantes entre otros (Porrozzi et al., 2007).

La técnica WB es un método serológico cualitativo que es capaz de distinguir el peso molecular de los antígenos de *L. infantum* que estimulan la producción de anticuerpos (Persichetti et al., 2017). Debido a su complejidad, esta técnica es

utilizada con menos frecuencia en la práctica veterinaria para el diagnóstico de la leishmaniosis.

Las técnicas basadas en inmunocromatografía son fáciles de utilizar y proporcionan resultados cualitativos rápidos, en el acto, aunque su rendimiento aún no es óptimo con un alta especificidad y variable sensibilidad (Mohebali et al., 2004; Mettler et al., 2005; Ferroglio et al., 2007; Villanueva-Saz et al., 2019) por lo que la interpretación de estas pruebas rápidas cualitativas o test rápidos debería ir acompañada siempre de una posterior técnica serológica cuantitativa que nos proporcione una titulación de anticuerpos (Solano-Gallego egt al., 2011; Paltrinieri et al., 2016). Este tipo de técnicas, en forma de test rápido, tienen la ventaja de encontrarse disponibles tanto para los veterinarios clínicos como para su empleo en estudios epidemiológicos de campo (Villanueva-Saz et al., 2022).

Los test rápidos como método cualitativo, y las técnicas IFI y ELISA, como métodos cuantitativos, son las pruebas diagnósticas utilizadas habitualmente para el manejo clínico de la leishmaniosis canina (Solano-Gallego et al., 2014). Los títulos altos de anticuerpos en presencia de signos clínicos y/o anomalías clinicopatológicas asociadas a la leishmaniosis son diagnósticos de infección activa, mientras que los títulos bajos de anticuerpos indican infección subclínica o exposición sin infección (Solano-Gallego et al., 2011; Travi et al., 2018). No obstante, perros con títulos de anticuerpos altos, pero con un nivel de antígeno de *L. infantum* circulante limitado podría no tener suficientes complejos inmunes solubles en circulación para desencadenar una respuesta inflamatoria y, por tanto, producirse la enfermedad clínica o daño a órganos (Day, 1999).

De acuerdo con las guías publicadas, los perros con niveles altos de anticuerpos de anti-*Leishmania* (es decir, 2 a 4 veces más altos que el valor umbral considerado positivo) con al menos un signo clínico relacionado con la leishmaniosis, y/o alteraciones laboratoriales asociadas a la misma, por lo general, se clasifican como 'enfermos' y requieren tratamiento anti-*Leishmania* apropiado (Calavera et al., 2021).

Por otro lado, se ha demostrado que se producen variaciones en los títulos de anticuerpos contra *L. infantum* en perros en áreas endémicas de leishmaniosis

canina caracterizadas por la estacionalidad del vector, con variaciones entre los periodos de transmisión y los periodos de no transmisión de los flebótomos (Cavalera et al, 2021).

Según los últimos estudios serológicos realizados, se estima que 2,5 millones de perros podrían estar infectados por *L. infantum* en Portugal, España, Francia e Italia (Baneth, 2022); siendo la incidencia media de casos de leishmaniosis canina por cada 1000 perros de 31/1000 perros/año en la clínica veterinaria en España durante los años 2016-2017 (Le Rutte et al., 2021). La seroprevalencia de *L. infantum* en perros en España oscila entre el 2% y el 57,1% según la región geográfica (Galvez et al, 2020). Esta variación de la seroprevalencia entre regiones podría explicarse por las diferencias geográficas en el clima y la estacionalidad asociada a los requisitos ecológicos y bioclimáticos de los vectores responsables de la transmisión de la enfermedad.

En gatos (*Felix silvestris catus*), la seroprevalencia en el continente europeo varía entre el 0 y el 68,5%, según las áreas endémicas estudiadas (Pennisi et al., 2015). En España, los estudios de prevalencia son escasos. En un estudio realizado en Ibiza se detectó un 13,2% de seroprevalencia en gatos que vivían en exterior (Sherry et al., 2011). Por otro lado, en un estudio realizado en Madrid, la seroprevalencia de *L. infantum* en gatos callejeros fue del 3,2% (Miró et al., 2014). En otro estudio reciente se obtuvo que la tasa general de gatos callejeros infectados en la ciudad de Zaragoza fue del 15,6% (calculada como el número de gatos seropositivos y/o PCR cuantitativa positivos) (Alcover et al., 2021).

Según la Organización Mundial de la Salud (OMS), anualmente se calculan entre 1,5 y 2 millones de nuevos casos de leishmaniasis humana y aproximadamente 70.000 muertes por esta enfermedad en todo el mundo (Jain et al., 2022). La leishmaniosis afecta a todos los continentes, a excepción de Oceanía, viéndose principalmente afectados América central y del sur, norte de África, Asia, Oriente Medio y sur de Europa.

En Europa afecta a la mayor parte de los países de la cuenca mediterránea de forma endémica, diagnosticándose anualmente entre 1200 y 2000 casos humanos de leishmaniasis visceral (Alvar et al., 2012).

En España la leishmaniasis es una enfermedad endémica de declaración obligatoria desde 2015 a través de la Red Nacional de Vigilancia Epidemiológica (RENAVE) (Fernández et al., 2019). Además, en las últimas dos décadas se ha descrito una propagación de la infección hacia el norte de Europa (Ferroglio et al., 2005; Maroli et al., 2008), detectándose flebótomos en Europa Central (Aspöck et al.,2005; Poeppl et al., 2013). Los cambios ambientales y el calentamiento global parecen estar repercutiendo en la distribución geográfica de la infección por *L. infantum* y sus vectores en todo el continente (Galvez et al, 2020).

El control de la leishmaniasis se basa en la detección precoz, el tratamiento de los casos y el control de los reservorios y vectores. Por ello, la identificación de los reservorios zoonóticos (es decir, poblaciones animales que pueden ser fuentes de *Leishmania*) es de gran importancia para los programas de control de leishmaniasis, en particular a la hora de diseñar programas de intervención adecuados (Cardoso et al., 2021). Además del perro, considerado el principal reservorio de la enfermedad (Quinnell et al., 2009; Morales-Yuste et al., 2022), recientemente también se han involucrado como posibles reservorios a otros animales domésticos y silvestres como otros cánidos (Alcover et al., 2020), gatos (Vioti et al., 2022), roedores (Alcover et al., 2021), y otras especies silvestres como mustélidos (Alcover et al., 2020), conejos y liebres (Carrillo et al., 2013; Díaz-Sáez et al, 2014).

El papel como reservorio activo de conejos (*Oryctolagus cuniculus*) y liebres (*Lepus granatensis*) ha sido descrito recientemente en la Comunidad de Madrid. Tras el brote de leishmaniasis que se produjo en el suroeste de esta comunidad, con más de 450 casos humanos diagnosticados entre 2009 y 2014. Se confirmó *L. infantum* como agente causal y el principal vector transmisor, *P. perniciosus* (Martín-Martín et al, 2014). El sistema de vigilancia para la leishmaniosis canina, incluida en la red epidemiológica de la Comunidad de Madrid, no detectó ningún aumento de prevalencia durante ese período en esta especie, lo que provocó una búsqueda de reservorios alternativos de la infección en la fauna silvestre de la zona afectada (Aguado et al., 2013). A raíz de esta búsqueda, se pudo determinar, por primera vez, un nuevo ciclo de transmisión selvático, en el que liebres y conejos eran los

reservorios activos y origen de la enfermedad en humanos en este brote de leishmaniasis (Aguado et al., 2013; Jiménez et al., 2013; González et al., 2017).

Este hallazgo se corroboró mediante diferentes estudios que incluyeron el xenodiagnóstico directo, documentando la presencia del parásito en liebres (Molina et al, 2012) y conejos (Jiménez et al., 2014); mediante la detección de anticuerpos anti-*Leishmania infantum* en el 74,1% de las liebres y el 45,7% de los conejos silvestres capturados en el área afectada por el brote (Moreno et al., 2014); y mediante la demostración de la exposición de las liebres y los conejos a la picadura de *P. perniciosus*, con la detección de niveles altos de anticuerpos anti-saliva de *P. perniciosus* en el suero de liebres capturadas en el área afectada por el brote (Martín-Martín et al., 2014).

A raíz de estos hallazgos, en la última década, se han realizado múltiples estudios con la finalidad de comprender el papel que juega la vida silvestre en la epidemiología de la leishmaniasis y poder así diseñar estrategias para reducir su prevalencia (Alcover et al., 2020; Azami-Conesa et al., 2021). El gran número de especies de mamíferos silvestres que existen en Europa dificulta la estandarización de pruebas serológicas y la comparación de datos serológicos entre estudios (Millan et al., 2014). Uno de los problemas surgidos para la identificación de reservorios alternativos de *L. infantum* mediante técnicas serológicas es la falta de anticuerpos específicos de especie (Giner et al., 2020). Por ello, la mayor parte de los estudios realizados se basan en la detección de *L. infantum* mediante la prueba de reacción en cadena de la polimerasa cuantitativa en tiempo real (Morales-Yuste et al., 2021).

Un estudio detectó una prevalencia de un 28% de *L. infantum* mediante PCR cuantitativa en muestras de hígado y bazo de especímenes de carnívoros silvestres testados (*Canis lupus*, *Felis silvestris*, *Genetta genetta*, *Martes foina*, *Martes martes*, *Meles meles*, *Vulpes vulpes*, *Mustela lutreola*, *Mustela nivalis* and *Mustela putorius*) en el País Vasco (del Río et al., 2014). Otro estudio realizado en Cataluña detectó un 29,49% de prevalencia en mamíferos salvajes (*Mus spretus*, *Erinaceus europaeus*, *Sciurus vulgaris*, *Vulpes vulpes*, *Felis catus*, *Meles meles*, *Martes foina* y *Mustela vison*) mediante PCR cuantitativa y medición de anticuerpos anti-*Leishmania* con la técnica ELISA (Alcover, 2020).

Entre estos carnívoros silvestres se encuentran los mustélidos. La familia *Mustelidae* corresponde a la más grande dentro del orden de los mamíferos del Orden Carnivora y se compone de 5 subfamilias, 22 géneros y 57 especies (Lariviere et al., 2009) que incluyen varios mamíferos de mediano y pequeño tamaño como lo son las comadrejas (*Mustela nivalis*), turones (*Mustela putorius*), garduñas (*Martes foina*), hurones (*Mustela putorius furo*), visones europeos (*Mustela lutreola*), visones americanos (*Neovison vison*), martas (*Martes martes*), nutrias (*Lutra lutra*), armiños (*Mustela erminea*) y tejones (*Meles meles*) entre otros.

Como representantes de los mustélidos en nuestro país, destacan principalmente tres de ellos: el hurón doméstico por su popularidad como mascota (d´Ovidio et al., 2014); el visón europeo) por ser una especie que se encuentra en peligro crítico de extinción (Palomares et al., 2017); y el visón americano por considerarse una especie invasora (Bonesi et al., 2007).

El visón europeo (Clase: mamíferos, Orden: carnívoros, Familia: Mustelidae; Género: Mustela, Especie: *Mustela lutreola*) es un pequeño mustélido que vive en medios acuáticos de muy variada tipología: ríos, arroyos, canales y lagunas, en los que las riberas cuentan con vegetación densa. Actualmente, el visón europeo se encuentra entre las especies de mamífero más amenazadas en Europa, catalogada en España "En Peligro de Extinción" desde 2011 e incluida en el Libro Rojo de los Mamíferos Terrestres (Palomo, 2011) y considerada como especie 'en peligro crítico' a nivel internacional por la Unión Internacional para la Conservación de la Naturaleza (UICN) desde octubre de 2018 (IUCN, 2022). En la actualidad, se estima que quedan menos de 500 individuos en España, distribuidos por el norte de la península ibérica (La Rioja, Navarra y País Vasco, principalmente), y su principal amenaza es la expansión del visón americano, especie exótica invasora que compite y desplaza al visón autóctono (Bonesi et al., 2007).

En 2005 se elaboró un plan denominado "Estrategia para la conservación del visón europeo en España" que promueve llevar a cabo un programa de estudio sanitario sobre la incidencia de patologías y procesos eco-toxicológicos que amenazan a la especie, promoviendo la investigación sobre sistemas de diagnosis, prevención y control de todas estas afecciones (Ministerio de Medio ambiente y Medio rural y marino del Gobierno de España, 2015). En este marco, se ha

especulado que la infección por el virus de la enfermedad Aleutiana del visón(VEAV) pudiera contribuir al declive del visón europeo. Para ello se evaluaron los efectos potenciales de la infección por el VEAV en la conservación del visón europeo mediante un estudio de prevalencia de anticuerpos frente a dicha enfermedad, tanto en visones europeos como visones americanos capturados en España (Mañas et al., 2016). La infección por VEAV parece ser endémica y distribuida entre la población de ambas especies. En este estudio no se observaron efectos en la dinámica de población de ninguna de las especies. Igualmente se ha realizado un estudio sobre la exposición de los visones europeos y americanos silvestres al virus SARS-Cov-2 en el norte de la Península Ibérica, área geográfica seriamente afectada por el COVID-19, no detectándose anticuerpos frente al virus en las muestras analizadas (Villanueva-Saz et al., 2022). En este sentido, la prevalencia de infección por *L. infantum* en visones europeos en España no ha sido estudiada, así como la implicación de la enfermedad en la disminución de la población de esta especie en el territorio de la Península Ibérica.

El hurón doméstico (Clase: mamíferos, Orden: carnívoros, Familia: Mustelidae; Género: Mustela, Especie: *Mustela putorius furo*) es un pequeño mustélido domesticado en Europa hace unos 2000 años y utilizado desde entonces para controlar poblaciones de roedores, en la caza de conejos y como modelo de investigación biomédica (Lauren et al., 2020). Actualmente, los hurones son mascotas en muchas partes del mundo (d´Ovidio et al., 2014) alcanzando una gran popularidad como animal de compañía en España.

Entre el año 2012 y el año 2015 se ha incrementado un 774% la presencia de hurones en los hogares españoles. Según los últimos datos del Gobierno, existe una población registrada oficialmente de 20.000 hurones domésticos en nuestro país (Ministerio de Agricultura, Alimentación y Medio ambiente, 2015). No existen estudios analizando el papel epidemiológico que podría tener el hurón doméstico en la infección, ni tampoco la seroprevalencia de *L.infantum* en esta especie.

Entre los mustélidos, la infección por *L. infantum* ha sido identificada por PCR cuantitativa en el pelo, hígado y / o muestras de tejido del bazo de un turón, un visón europeo, en nutrias y en otros mustélidos salvajes (tejón común (*Meles meles*), garduña (*Martes foina*) y marta (*Martes martes*))en el norte y oeste de la Península

Ibérica (Muñoz-Madrid et al., 2013; Del Río et al., 2014; Oleaga et al., 2018; Pereira et al., 2020) así como en garduñas y en un visón americano en el sur y este de la Península (Risueño et al., 2018; Alcover et al., 2019; Ortuño et al., 2019), aunque ninguno de los animales mostró lesiones típicas de leishmaniosis canina en el examen post mortem. En Grecia, tres de catorce visones americanos afectados por neumonía hemorrágica en una granja, el 21,4%, resultaron positivos para el ADN de *L. infantum* en el cerebro, el hígado o el bazo detectado mediante ITS1-nPCR (Filioussis et al., 2018). La gravedad de la enfermedad neumónica, que se debió a *Pseudomonas aerugionosa*, podría atribuirse a la inmunosupresión causada por *L. infantum*, aunque no ha podido confirmarse. En otro estudio realizado en 200 visones americanos en este país evidenció un 20% de seroprevalencia y la infección fue confirmada por técnicas moleculares en el 2,1% de los animales (Tsakmakidis, 2019).

Los primeros casos clínicos de leishmaniosis provocada por una infección natural de *L. infantum* en mustélidos han sido publicados recientemente. El primer caso descrito se presentó en la provincia de Valencia. Se trató de un hurón doméstico como animal de compañía con una lesión papular en el pabellón auricular derecho (Giner et al. 2020). El segundo caso clínico de infección natural por *L. infantum* se detectó en una nutria euroasiática criada en cautividad en un parque zoológico de Alicante con epistaxis, además de signos de anorexia, apatía y pérdida de peso (Cantos-Barreda et al. 2020).

En cuanto al diagnóstico de la leishmaniosis clínica en mustélidos, es importante diferenciar la infección de la enfermedad y aplicar las diferentes técnicas diagnósticas en cada caso. El diagnóstico es difícil y complejo, ya que el espectro de manifestaciones clínicas y alteraciones clínico-patológicas descritas en otras especies es amplio e inespecífico.

En el único caso descrito en un hurón doméstico, el paciente acudió con una lesión papular en un pabellón auricular y la infección por *L. infantum* se diagnosticó mediante examen histopatológico e inmunohistoquímica y cultivo de material aspirado en medio NNN. Además, se realizó un diagnóstico molecular mediante PCR cuantitativa y se detectó la presencia de anticuerpos anti-*Leishmania* mediante ELISA, IFI y WB (Giner et al., 2020).

En el caso clínico descrito en una nutria, el diagnóstico se realizó mediante visualización de amastigotes de *Leishmania* en una citología con muestra procedente de aspirado con aguja fina de bazo, PCR cuantitativa en tiempo real de sangre periférica y médula ósea y mediante serología ELISA e Inmunoensayo (Cantos-Barreda et al. 2020).

Igualmente, el tratamiento de la leishmaniosis en hurones domésticos, no ha sido descrito hasta el momento.

2.1 JUSTIFICACIÓN:

Según la Organización Mundial de la Salud la leishmaniasis es una de las enfermedades parasitarias más importantes a nivel mundial. Aunque el perro es el principal reservorio natural de la infección, la identificación de otros animales domésticos que puedan actuar como posibles reservorios del parásito podría tener un impacto significativo en la salud pública.

Pese a que no se dispone de datos oficiales sobre la población de hurones en Europa, el hurón doméstico es hoy en día una mascota común en muchos países del mundo. Aunque sólo existe un caso clínico descrito de la infección natural por *L. infantum* en un hurón doméstico; ante la falta de información científica al respecto, y conociendo que la capacidad por parte de un reservorio de *L. infantum* de infectar a flebótomos, principal vector transmisor de la enfermedad, no depende del estado clínico del reservorio (demostrado en el perro), se decide llevar a cabo este trabajo de investigación. En él, uno de los objetivos principales debería dirigirse a conocer el papel epidemiológico que podría tener el hurón doméstico en la enfermedad, dado su contacto estrecho con los seres humanos como animal de compañía. Por otro lado, también es interesante conocer la seroprevalencia de esta enfermedad en otros mustélidos silvestres como el visón europeo, por las consecuencias que podría suponer esta enfermedad para su conservación, dado el peligro crítico de extinción en el que se encuentra; así como en el visón americano, especie invasora que convive en las áreas geográficas con el visón europeo.

Del mismo modo, la detección de enfermedades no descritas previamente en una especie, es esencial para incluirlas en el diagnóstico diferencial de aquellos pacientes con clínica compatible, y poder realizar un diagnóstico, para aplicar un tratamiento adecuado.

2.2 OBJETIVOS:

En base a la evidencia de los primeros casos clínicos de infección por *L. infantum* en mustélidos, así como la detección de *L. infantum* en diversos mustélidos silvestres en la Península Ibérica y en visones americanos en Grecia, los objetivos del presente trabajo de tesis doctoral se centran en la caracterización de la infección de *L. infantum* en mustélidos, concretamente en el hurón doméstico y en el visón europeo, estudiando lo aspectos epidemiológicos, aspectos clínicos y la respuesta inmunitaria de los mismos hacia la infección. Con todo ello, los objetivos principales de la tesis doctoral son los siguientes:

1. Evaluación de la situación epidemiológica de *L. infantum* en el hurón doméstico mediante un estudio de seroprevalencia en la zona endémica donde fueron detectados los primeros casos clínicos de leishmaniosis.
2. Evaluación de la situación epidemiológica de *L. infantum* en visón europeo y visón americano en el Norte de la Península Ibérica; una de las tres poblaciones existentes en todo el mundo de visón europeo, uno de los mamíferos más amenazados que actualmente existen en el mundo, en la que estos conviven con poblaciones asilvestradas de visón americano.
3. Caracterización de la evolución de los anticuerpos anti-*L. infantum* en hurones seropositivos en una zona endémica de la enfermedad.
4. Caracterización clínica de los hurones seropositivos a *L. infantum*. Detectar los signos clínicos en hurones seropositivos compatibles con infección natural por *L. infantum* así como estudiar la evoluciónserológica y clínico-patológica tras el tratamiento de la infección clínica por *L. infantum*.

El objetivo final de esta tesis doctoral es profundizar en diversos aspectos de la infección por *L. infantum* en mustélidos.

3.Relación de publicaciones:

CAPÍTULO 1: First epidemiological survey of *Leishmania infantum* in the domestic ferret (*Mustela putorius furo*) in a canine leishmaniosis endemic area using serology and PCR.

Parasites & Vectors

RESEARCH Open Access

First epidemiological survey of *Leishmania infantum* in the domestic ferret (*Mustela putorius furo*) in a canine leishmaniosis endemic area using serology and PCR

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Abstract

Background: Leishmaniosis, a vector-borne disease caused by *Leishmania infantum*, is one of the most important parasitic zoonoses in Europe. The transmission cycle of leishmaniosis is maintained by both domestic and wild animals. However, few data are available on the role of wild mammals in transmitting the parasite in the European Mediterranean basin. As feline leishmaniosis, diagnosis of the infection in ferrets can be a challenge, the use of different serological and molecular methods combined is a recommended approach. Our aim was to investigate the prevalence of infection of *L. infantum* in apparently healthy domestic ferrets (*Mustela putorius furo*) in an endemic region of Spain (Community of Valencia), using serological and molecular methods and to evaluate the results comparing the different techniques.

Methods: The prevalence of *Leishmania* infection was studied in domestic ferrets. Blood was collected from each animal for serology and molecular analysis. Two serological methods, enzyme-linked immunosorbent assay (ELISA) and western blot (WB), were used for the detection of *L. infantum* antibodies, and real-time polymerase chain reaction (qPCR) was used for the detection of *L. infantum* DNA.

Results: Blood samples from 102 apparently healthy ferrets were analyzed. In the serological study, 25.5% of the animals tested positive by western blot, and 9.0% by enzyme-linked immunosorbent assays. The seroprevalence of *L. infantum* infection, based on a positive result in any serological test, was 28.4% (95% confidence interval [CI] 20.6-S37.9%). No kinetoplast DNA (kDNA) was detected by qPCR in peripheral blood samples from the ferrets tested.

Conclusions: The immunological response revealed by these tests indicates that the ferrets are exposed to repeated inoculations with the endemic parasite *L. infantum*. Although the low population of domestic ferrets means their reservoir potential is limited in the absence of a primary host, it would be of interest to carry out further studies using xenodiagnosis to determine whether they are accidental or reservoir host species capable of spreading infection.

Keywords: ELISA, Ferret, *Leishmania infantum*, Prevalence, PCR, Serology, Western blot

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Background

Leishmaniosis, a vector-borne disease caused by *Leishmania infantum*, is one of the most important parasitic zoonoses in Europe [1]. In the past decade, several wild animals, including lagomorphs and canids, have been described as playing a significant role in the transmission cycle of *L. infantum*. Moreover, an increasing number of reports suggest that leishmaniosis is not restricted to dogs (main reservoir) but may also affect other mammalian and avian species [2]. For instance, immunological positivity to *L. infantum* in cats from European endemic areas suggests they are frequently exposed to the parasite [3–6] and subjected to repeated inoculations. Additionally, several studies have detected *L. infantum* kinetoplast DNA (kDNA) in species of the Mustelidae family [7–10] and anti-*L. infantum* antibodies in American [11] and European mink in Spain [12].

The most common clinical signs of leishmaniosis described in ferrets *(Mustela putorius furo*) are periph- eral lymphadenomegaly and cutaneous lesions, includ- ing papular and ulcerative dermatitis [13]. Frequently observed clinicopathological abnormalities are hyperpro- teinemia with hyperglobulinemia and polyclonal gam- mopathy [13]. In ferrets, clinical data for leishmaniosisare still very scarce. Moreover, there is no epidemiologi- cal information about the prevalence and seroprevalenceof leishmaniosis in domestic ferrets in areas endemic for *L. infantum*, including the Community of Valencia (Spain), where seropositive clinically sick animals have been reported [14, 15].

Epidemiological surveys are an important step in establishing the presence/absence of a pathogen in a specific region, and the results are of great value to clinicians when infections are potentially underdiagnosed, as in the case of leishmaniosis in domestic ferrets. Epidemiological studies of canine and feline leishmaniosis have combined serological and molecular methods for maximum diagnostic efficiency, an approach that could also be applied to ferrets [3, 16–19]. However, serological methods need to be adapted and validated for each species to avoid negative results in seropositive animals [13].

Given the absence of studies on leishmaniosis in ferrets in the Community of Valencia, where cases have been recently reported, the present study aimed (1) to provide the first epidemiological data on the role of domestic ferrets in the epidemiology of *L. infantum* infection in an endemic region; (2) to investigate the prevalence of *L. infantum* infection in domestic ferrets using serology and quantitative molecular assays (quantitative polymerase chain reaction [qPCR]); and (3) to evaluate the screening results of apparently healthy domestic ferrets living in an endemic region by comparing serological and qPCR data.

Methods

Study areas, ferrets, and sampling

Blood samples were collected from September 2019to February 2021 from 102 ferrets in different towns of the Province of Valencia (39° 99 28′12.864″ N, 0° 22′36.48″ W), an area on the east coast of the Iberian Peninsula with a high incidence of canine leishmanio- sis (50–100/1000 dogs/year) [20, 21]. Based on epidemiological studies, 17.1% of dogs are seropositive in the Community of Valencia in Spain [21, 22]. However, the presence of human cutaneous leishmaniosis outbreak has also been detected in the Valencia region [22]

Before sampling, information was obtained about each animal regarding age, cohabitation with a dog, lifestyle (indoor, outdoor, or mixed) and gender, and a complete physical examination was carried out to establish health status (sick versus healthy). Whenever possible, one ml of blood was collected aseptically by cranial cava venipuncture from each ferret. The collected volume was divided equally between a sterile blood collection tube (to obtain the serum) and a second tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant (for molecular analysis). EDTA-blood and separated sera were stored at −20 °C until processing. Routine laboratory tests, such as a complete blood count and biochemistry profile, were not performed.

In total, 102 client-owned ferrets were sampled. A single sample was obtained from 94 ferrets. The other eight client-owned ferrets were tested an additional one ($n = 5$) to two ($n = 3$) different times during the study period. A total of 113 serum samples were included.

Diagnostic serological tests

Detection of specific antibodies was performed using two in-house serological techniques: enzyme-linked immunosorbent assay (ELISA) and western blot (WB).

Detection of L. infantum antibodies by a quantitative ELISA

The ELISA was performed on all sera as described previously, with some modifications [14]. Briefly, each plate was coated with 20 µg/ml of crude antigen obtained from *L. infantum* promastigote forms (MHOM/MON-1/LEM 75) in 0.1 M carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Next, 100 µl of cat sera, diluted 1:200 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M), was added to each well. The plates were incubated for 1 h at 37 °C in a moist chamber. Then they were washed, and 100 µl of protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:8000 in PBST-M was added. The plates

were incubated for 1 h at 37 °C in the moist chamber and were washed again with PBST and PBS as described above. The substrate solution (ortho-phenylenediamine) and stable peroxide substrate buffer (Thermo Fisher Scientific, Waltham, MA, USA) was added per well and developed for 20 ± 5 min at room temperature in the dark. The reaction was stopped by adding 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (Multiskan ELISA reader, Labsystems, Midland, Canada).

As a positive control (calibrator), each plate included serum from a ferret from Spain diagnosed with leishmaniosis, confirmed by a positive culture, and as a negative control, serum from a healthy, non-infected ferret. The same positive control serum was used for all assays and plates, with a constant inter-assay variation of < 10%. Plates with an inter-assay variation of > 10% were discarded. All samples and controls were run in duplicate. The cut-off was set to 0.180 optical density units (OD) (mean + 3 standard deviations of values from 30 indoor ferrets from northern Spain), and results above this value were considered positive.

Detection of L. infantum antibodies by WB

Anti-*Leishmania* antibodies were detected by WB using a whole antigen of *L. infantum* promastigotes (MHOM/FR/78/LEM75 zymodeme MON-1), as described by Alcover et al. [3], with some modifications. The protocol used for WB is based on the technique described by Aisa et al. [23], with sensitivity of 95.8% and specificity of 100% in dogs. Moreover, the specificity has been analyzed including a group of healthy cats (n = 20) from a non-endemic area (Switzerland) with a value of 100%. Antigen electrophoresis on 0.1% sodium dodecyl sulfate (SDS)-15% polyacrylamide gels together with molecu-lar mass protein standards (low-range standard; Bio- Rad, Hercules, CA, USA) was performed on a Mini-Gel AE-6400 Dual Mini Slab Kit (Atto, Bunkyo-ku, Japan).

Gels were run at 100 V for 1 h at room temperature. Polypeptides were transblotted onto nitrocellulose sheets (0.45-mm pore size, HAWP 304 FO; Millipore, Bed- ford, MA, USA), which were blocked with 20 mM Tris, 0.13 mM NaCl, pH 7.6 (TS), and 5% skimmed milk overnight at 4 °C.

The sheets were washed in TS and introduced into a multiscreen apparatus (Mini-PROTEAN II; Bio-Rad, Hercules, CA, USA). Sera were diluted 1:200 in TS-1% skimmed milk and 0.2% Tween 20. Then 500 µl of each sample was introduced into each channel of the multiscreen apparatus and incubated for 2 h at 37 °C. Bound immunoglobulins were developed by incubation with a 1:1000 dilution of protein A peroxidase conjugate (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. After the sheets were washed three times with TST and a final time with TS, color was developed with 4-chloro-1-naphthol substrate (Thermo Fisher Scientific, Waltham, MA, USA). Based on our experience and the literature, a serum sample was considered positive when immunoreactivity against the *L. infantum* antigen fraction 14 and / or 16 kDa was observed, and indeterminate when molecular weight bands of 18, 20, 24, 28, 30, 36, 38, and 46 kDa appeared, as reported previously [3].

Detection of L. infantum DNA by qPCR

DNA was extracted from 200 µl of mammalian bloodusing the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany), which allows genomic DNA to be isolated rapidly and easily from a wide variety of sample materials. All extractions were performed following the manufacturer's instruc- tions, and multiple PCR templates were obtained in min- utes using efficient High Pure spin columns.

The detection and quantification of *Leishmania* kDNA was carried out by amplification of kinetoplast minicircle DNA sequences by qPCR [13]. Each amplification was performed in triplicate in 10 µl of reaction mixture containing 1× iTaq Supermix with ROX (Bio-Rad, Hercules, CA, USA), 15 pmol of direct primer Leim1 (5′-CTT TTC TGG TCC TCC GGG TAG G-3′), 15 pmol of reverse primer Leim2 (5′- CCA CCC GGC CCT ATT TTA CAC CAA-3′), 50 pmol of the labeled TaqMan probe Leim3 (5′- FAM-TTT TCG CAG AAC GCC CCT ACC CGC TAMRA-3′), and 2.5 µl of sample DNA.

The ABI Prism 7900 HT thermocycler (Applied Biosystems, Waltham, MA, USA) was used at 94 °C and 55 °C cycling over 40 cycles with a FAM detector. A non-template control was used in each run as the qPCR negative control. A 10-fold dilution series of DNA from promastigotes (MHOM/ES/04/BCN-61, *L. infantum)* was used for calibration (serial dilution from 10^5 parasites/ml to 10^{-3} parasites/ml), allowing the plotting of a standard curve. The qPCR was considered positive when the threshold cycle (Ct) was lower than 40 and when the amplification was detected in all the replicates [3, 24, 25].

Statistical analysis

Data collected for the entire population were analyzedusing descriptive statistics. Associations between *L. infantum* and the recorded variables were analyzed. The significance of this difference was assessed using the Chi-square or Fisher's exact test. A value of $P \leq 0.05$ was con-sidered significant. The SPSS v.22 software grogram was used (IBM Corporation, Armonk, NY, USA).

Results

Animals studied

All the tested ferrets ($n = 102$; 49 females and 53 males) had a mixture of coat colors, and none had been surgically neutered. All ferrets were classified as apparently healthy, with no systemic signs of disease found in the general physical examination. The mean age of the animals was 4 years (ranging from 1 to 8), and they were classified as young (<2 years), adult (from ≥ 2 yearsto ≤ 6 years), or senior (> 6 years). None of the ferrets had been treated with a long-acting topical anti-parasitic repellent against sand flies (Table 1).

No significant association ($P > 0.05$) was detected between *Leishmania* positivity and gender (male/female), age (young, adult, senior), cohabitation with a dog, or lifestyle (indoor, outdoor, or mixed). All statistical analysis can be found in Additional file 1: Table S1.

For the serological study, a total of 113 serum samples were analyzed, 11 of which were obtained from the eight seropositive animals that were followed up. Moleculartests were applied to 62 peripheral blood samples, sevenof which belonged to five seropositive animals that were followed up. EDTA-blood could not be obtained fromsome of the domestic ferrets ($n = 47$) due to their small size and the absence of an anesthetic procedure during blood extraction.

Serology and qPCR for *L. infantum*

In the serological study, 100% of the animals were analyzed by both ELISA and WB. By ELISA, nine positive animals were detected, the seropositive rate being 8.8% (95% CI 4.5–16.1%). Using WB, 26 were positive, which constitutes a seropositivity of 25.5% (95% CI 18.0–34.8%), and nine ferrets (8.8%) gave indeterminate results (i.e., no bands were observed at 14 and/or 16 kDa). The WB data for sensitivity to the *L. infantum* antigen are pro- vided in Fig. 1. Among the 26 positive ferrets, bandswere observed at both 14 and 16 kDa for nine animals,at 16 kDa and other molecular weights for 12, and only at 16 kDa for five ferrets.

Of the nine animals diagnosed as indeterminate by WB, eight were negative by other techniques, and one with a band at 46 kDa tested positive by ELISA. Twenty-nine ferrets (28.4%) tested positive by at least one of the three techniques (95% CI 20.6–37.9%). Twenty of these were diagnosed as positive only by WB, and three only by ELISA (including one of the three WB indeterminate sera), whereas six ferrets tested positive by both serological techniques. Eight of the 29 positive animals were followed up, four of them testing negative in the second sample; two of the four were analyzed only by serological techniques and two by ELISA, WB, and PCR. The WB result for one of these four animals was indeterminate, yielding a band only at 20 kDa.

Table 1 Evidence of contact with *L. infantum* using ELISA and western blot

Variable	Animals studied (%)	ELISA		Western blot			Positive animals for both techniques (%)
		Positive (%)	Negative (%)	Positive (%) [a]	Indeterminate (%) [b]	Negative (%) [c]	
Sex							
Female	49 (48.0)	4 (8.2)	45 (91.8)	15 (30.6)	2 (4.1)	32 (65.3)	16 (32.7)
Male	53 (52.0)	5 (9.4)	48 (90.6)	11 (20.8)	7 (13.2)	35 (66.0)	13 (24.5)
Total	102	9 (8.8)	93 (91.2)	26 (25.5)	9 (8.8)	67 (65.7)	29 (28.4)
Age							
Young	15 (14.7)	0 (0)	15 (100)	4 (26.7)	2 (13.3)	9 (60)	4 (26.7)
Adult	48 (47.1)	5 (10.4)	43 (89.6)	16 (33.3)	4 (8.3)	28 (58.3)	17 (35.4)
Senior	39 (38.2)	4 (10.23)	35 (89.7)	6 (15.4)	3 (7.7)	30 (76.9)	8 (20.5)
Housing shel er							
Inside	55 (53.9)	4 (7.2)	51 (92.72)	10 (18.2)	3 (5.5)	42 (76.3)	13 (23.6)
Outside	6 (5.9)	1 (16.7)	5 (83.33)	2 (33.3)	2 (33.3)	2 (33.3)	2 (33.3)
Mixed	41 (40.2)	4 (9.8)	37 (90.24)	14 (34.2)	4 (9.8)	23 (56.1)	14 (34.2)
Cohabitation with a dog							
Yes	28 (27.5)	3 (10.7)	25 (89.3)	8 (28.6)	2 (7.1)	18 (64.3)	8 (28.6)
No	74 (72.6)	6 (8.1)	68 (91.9)	18 (24.3)	7 (9.5)	49 (66.2)	21 (28.4)

[a] 14 and/or 16 kDa bands were present

[b] Band patterns observed but not for 14 and/or 16 kDa

[c] No band observed

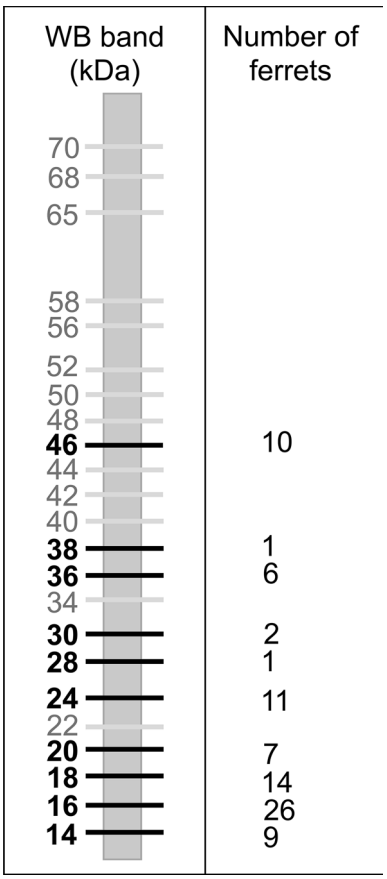

Fig. 1 Western blot results in 102 domestic ferrets from Valencia (Spain). A positive result was considered when bands of 14 and/or 16 kDa were observed. When bands of 18, 20, 24, 28, 30, 36, 38, and 46 kDa appeared, the results were regarded as undetermined

During the follow-up of eight animals originally found positive by WB, three remained positive by WB in the second analysis, and only one (M5706) tested positive in a third analysis (Table 2). One of the three ferrets (M5931) was also positive by ELISA, and again tested positive by both serological techniques in the second analysis (ELISA titer of 0.241 OD and WB band at 16 kDa); however, in the subsequent follow-up, both tests were negative (Table 2). The third of the three ferrets (M6188) tested negative in the second analysis. This animal was classified as apparently healthy ferret without evidence clinical signs detected during physical examination. In this sense, it is possible to detect seasonal variation in anti-*Leishmania* antibodies in dogs [26] and ferrets [27] in endemic areas of canine leishmaniosis. Finally, all the asymptomatic ferrets with full blood samples, previously screened for *Leishmania* DNA, tested negative.

Discussion

In this study, 102 domestic ferrets in the Community of Valencia (Spain) were tested for *L. infantum* infection using serological and molecular techniques. The rate of seropositivity was high by both ELISA and WB, although the number of positive cases detected by WB was statistically significantly higher ($P < 0.008$). WB is known to be a more sensitive method than ELISA, which is more specific [28]. Generally used as a confirmatory test of ELISA results, WB is more difficult to perform and requires higher laboratory skills. However, the results of the pre- sent study do not reflect these differences in sensitivity and specificity.

The detection of *L. infantum* kDNA by qPCR has been previously evaluated in a range of matrices [3, 24, 25]. In veterinary medicine, it is known, especially in dogs, that blood is not the best matrix for *Leishmania* qPCR [29]. Noninvasive samples including oral and conjunctival swabs, hair, or saliva have been evaluated, but there is a lack of studies that support the usefulness of such sam- ples applied in clinical practice as follow-up and clinical prognostic parameters [30–32]. In Spain, blood samples from stray cats have been tested by qPCR to detect the parasite, and variations in prevalence between studies and endemic regions have been reported [3]. Parasite

Table 2 Follow-up of ferrets that had contact with *L. infantum*

	First determination			First follow-up			Second follow-up		
Identification	PCR	ELISA (OD)	WB (kDa)	PCR	ELISA (OD)	WB (kDa)	PCR	ELISA (OD)	WB (kDa)
M5798	–	–	+ (14/16/18/24/36/46)	NP	–	–	NP	NP	NP
M6314	–	–	+ (14/16/18)	–	–	–	NP	NP	NP
M5706	–	–	+ (16/20/46)	–	–	+ (16/118/24)	NP	NP	NP
M5741	–	–	+ (16)	–	–	Undetermined (20)	NP	NP	NP
M6626	–	–	+ (16/18)	NP	–	–	NP	NP	NP
M6188	NP	–	+ (16/18)	NP	+ (0.226)	+ (14/16/18/20/24)	–	–	–
M5412	–	–	+ (16)	NP	+ (0.186)	–	NP	NP	NP
M5931	NP	+ (0.201)	+ (14/16)	–	+ (16)	–	–	–	–

NP not performed, *OD* optical density; +: positive; –: negative

detection using qPCR assays based on highly repeti- tive gene loci or extrachromosomal kDNA sequences is restricted to the genus level. In the present study, we used extremely sensitive genus-specific primers to be able to detect asymptomatic animals. Even so, the qPCR results of peripheral blood samples were all negative, possibly because the parasite load in the circulatory system was insufficient for detection by this technique or was non-existent. *Leishmania* protozoa mainly multiply in macrophages of the skin and spleen, and therefore in mild infections their levels in blood are low [33, 34]. Thus, although peripheral blood samples are easy to obtain and are minimally invasive, they proved unsuitable for detecting kDNA of *Leishmania* spp. in asymptomatic ferrets, which may have low or unstable parasitemia over time. A study analyzing *Leishmania* infection in mustelids using tissue macerates reported high percentages of kDNA detection by qPCR [7], suggesting that this type of sample is more suitable for genetic analysis, although it is also more invasive. In ferrets, we suspect that *L. infantum* would be more prevalent in organ tissues than in blood. This may imply that ferrets are not efficient *Leishmania* reservoirs and cannot sustain the parasite transmission cycle alone. For further clarification of the nature of *Leishmania* infection in ferrets, xenodiagnostic studies would be useful [3, 35–37].

In general, seroprevalence studies on specific patho-gens in animals produce variable results depending onfactors such as the geographical location, lifestyle, age, or analytical methodology. The data obtained in the present study provide an estimation of *L. infantum* seroprevalence in domestic ferrets in Spain. Although we obtained data demonstrating ferret exposure to *L. infantum* inoculations, in accord with a previous report [27], the mere presence of antibodies or parasite DNA is not suf- ficient evidence of reservoir host status [38]. To confirm whether an animal is an accidental or reservoir (primary or secondary) host of *L. infantum* requires xenodiagno- sis. Also, in areas with a high density of sand fly vectors and biting rates, if a suitable vertebrate reservoir hostspecies for *Leishmania* is absent or rare, the transmission cycle of the parasite is not sustained in the long term.Such a case has been reported in a peri-urban zoologi-cal park in southern Spain, where most sand flies feed on large herbivores that do not act as *Leishmania* reservoirs [37].

Although ferrets may not play a crucial role as reser- voir hosts, their involvement in the parasite cycle maystill have a significant impact. More studies are needed to elucidate the epidemiological role of domestic ferrets in the spread of leishmaniosis in *L. infantum* endemic areas such as the Community of Valencia in Spain.

Conclusions

Our serological results revealed that domestic ferretsin the Mediterranean basin are exposed to the endemic parasite *L. infantum*. To our knowledge, this study demonstrates for the first time the seroprevalence and prevalence rates of *L. infantum* in domestic ferrets in an endemic region of Spain. Further prevalence surveys in other endemic regions, using other diagnostic methodsand more suitable matrices for molecular analysis, would be useful to clarify the role of this popular pet in the epidemiology of *L. infantum* transmission.

Abbreviations

Ct: Threshold cycle; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; qPCR: Quantitative real-time PCR; PCR: Polymerase chain reaction; WB: Western blot.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05517-y.

Additional file 1: Table S1. Statistical analysis of the positivity to IgG antibodies against *L. infantum* in ferrets in Spain, in relation to sex, age, housing shelter, and cohabitation with a dog.

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Author contributions

SVS, CR, MA and RF designed the survey. CR, MA, RF and SVS supervised technical work. MA, JG, JR, XR, MV, AF, CR, RF and SVS contributed with data analysis. MA, JG, JR, CR, RF and SVS and wrote the manuscript. JG and SVS performed physical examination and collected samples from ferrets. MA, JR, MV and SVS performed serological testing and JR and XR performed the molecular work of this study. All authors read and approved the manuscript.

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Availability of data and materials

The datasets supporting the findings of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate

This survey was included under Project Licence PI25/20 approved by the Ethics Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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CAPÍTULO 2: Detection of Anti-*Leishmania infantum* Antibodies in Wild European and American Mink (*Mustela lutreola* and *Neovison vison*) from Northern Spain, 2014-20.

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Detection of Anti-*Leishmania infantum* Antibodies in Wild European and American Mink (*Mustela lutreola* and *Neovison vison*) from Northern Spain, 2014-20

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ABSTRACT: The European mink (*Mustela lutreo-la*) is listed as a critically endangered species because of ongoing population reduction from habitat degradation and the effects of introducedspecies, such as American mink (*Neovison vison*). This small, fragmented population becomes vulnerable to many other threats, including diseases. Leishmaniosis is a zoonotic disease caused by the protozoan parasite *Leishmania infantum* found in the Mediterranean area, which affects many mammals, including wild small mammals. Furthermore, clinical disease caused by *L. infantum* has recently been described in other mustelids. To assess the exposure to *Leishmania* sp. infectionin mink species in northern Spain, blood samples from 139 feral American mink and 42 native European mink from north Spain were evaluated for *Leishmania* sp. infection using enzyme-linked immunosorbent assays against *Leishmania* spp. antibodies, with 52.4% of American mink and 45.3% of European mink being found seropositive. This finding raises questions regarding how the disease may affect these species and the potential repercussions for conservation efforts. Despite a high seroprevalence being observed in wild mink of both species in this study, association with clinical or pathologic signs of disease has yet to be elucidated.

Key words: Leishmania infantum, serologic survey, wild minks.

Leishmaniosis, caused by *Leishmania infantum*, is a vector-borne, zoonotic disease endemic in southern Europe, which is spreading to northern regions (Pennisi et al. 2015). This parasite is transmitted under natural conditions by female phlebotomine sand flies during blood feeding. In Spain, dogs (*Canis familiaris*) are considered to be the main reservoir for *L. infantum*. However, the role of other potential reservoirs for thisparasite, such as wild small mammals, is being investigated (Alcover et al. 2020). Detection of the parasite infection in wild carnivores in Spain has been shown, suggesting the exis- tence of a sylvatic cycle of the *L. infantum* independent of dogs (Sobrino et al. 2008). A recent study detected a seroprevalence of 20% among 200 farmed American mink (*Neovison vison*) without any skin or visceral lesions. Nevertheless, seropositivity was asso- ciated with poor body condition (Tsakmakidis et al. 2019).

Recently, the first clinical cases of leishmaniosis in mustelids were published in a domestic ferret (*Mustela putorius furo*), and a captive Eurasian otter (*Lutra lutra*). The ferret had a papular lesion in the right pinna (Giner et al. 2020), and the otter had bilateral epistaxis, plus signs of anorexia, apathy, and weight loss (Cantos-Barreda et al. 2020).

The European mink (*Mustela lutreola*) belongs to the Mustelidae family (Carnivora) and is classified as a critically endangered species, according to the International Union for Conservation of Nature Red List (Maran et al. 2016). During the 20th century, numbers of European mink declined, andthe range of distribution has been reduced to a few fragmented populations; today, this

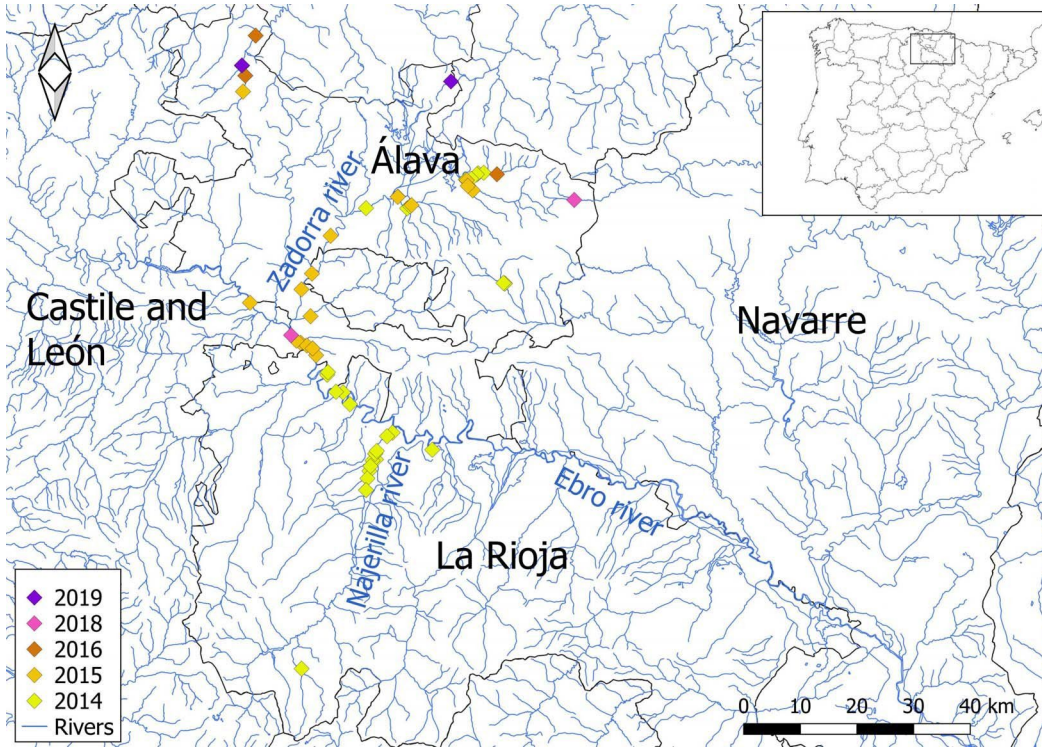

FIGURE 1. Location of *Leishmania*-seropositive American minks detected by enzyme-linked immunosorbent assay, from 137 individuals from the Cantabrian and Ebro basins, northern Spain, 2014–20. The main map shows the region of Castile and León; see inset map of Spain for the area depicted.

species faces extinction (Amstislavsky et al. 2008). Several causes have been put forwardto explain the disappearance of the species in different time periods. Overhunting was the most critical cause during the first half of the 20th century; at present, climate change, destruction of habitat, and the presence of the introduced American mink in the same region in which European mink reside aggravate the situation and often make it irreversible (Frankham 2003).

Our study aimed to determine the prevalence of natural infection with *L. infantum* in wild European and American mink in north-ern Spain using an in-house enzyme-linked immunosorbent assay. The results would help ascertain the degree of exposure to the parasite in both mink species (native and introduced) in their two distribution areas in northern Spain: the Ebro basin, with a semiarid climate with dry, hot summers and cold winters; and the Cantabrian basin, characterized by mild winters and warm summers.

From 2014 to 2020, a total of 181 animals (139 American mink and 42 European mink) were examined. For each animal, information that included geographic coordinates, river basin, sex, and body scoring was obtained. Blood samples from native European mink were obtained from various sources: population surveys of the European mink in the Spanish distribution areas; periodic mink population controls in river drainages; campaigns to capture founders for the European mink breeding program in Spain; and accidental trapping during culling campaigns of feral American mink. Samples from feral American mink were collected during population control operations conducted by several governmental authorities and performed by rangers and biologists acting as trappers. This survey was included under the LIFE project, approved by the European Commission for

TABLE 1. Summary of *Leishmania* seropositivity based on enzyme-linked immunosorbent assay of American mink (*Neovison vison*) and European mink (*Mustela lutreola*) from the Cantabrian and Ebro basins, northernSpain, 2014-2020.

River	River basin	No. mink	Year (*n*)[a]	No. seropositive mink	Serology classification (no.)	Sex seropositive (no.)	Year seropositive (*n*)
American mink							
Alegria	Ebro	1	2014	0			
Aramayona	Cantabrian	2	2019 (2)	1	Low (1)	Female	2019 (1)
Ayuda	Ebro	5	2014 (1) 2015 (4)	1	Low (1)	Male	2015 (1)
Barrundia	Ebro	13	2014 (10) 2015 (2) 2016 (1)	7	Low (7)	Male (3) Female (4)	2014 (6) 2016 (1)
Bayas	Ebro	1	2014	0			
Berron	Ebro	6	2014 (6)	3	Low (3)	Female (3)	2014 (3)
Ebro	Ebro	28	2014 (11) 2015 (15) 2016 (2) 2018 (1)	14	Low (13) High (1)	Female (7) Male (7)	2014 (6) 2015 (7) 2018 (1)
Ega	Ebro	2	2014 (1) 2015 (1)	1	Low (1)	Female (1)	2014 (1)
Errekabarri	Ebro	1	2015 (1)	1	Low (1)	Female (1)	2015 (1)
Izoria	Cantabrian	7	2015 (7)	0			
Najerilla	Ebro	16	2014 (16)	11	Low (11)	Female (5) Male (6)	2014 (11)
Nervion	Cantabrian	16	2014 (3) 2015 (6) 2016 (4) 2017 (1) 2018 (1) 2019 (1)	7	Low (7) Moderate (1)	Female (2) Male (5)	2015 (4) 2016 (2) 2019 (1)
Salburua	Ebro	3	2014 (3)	2	Low (2)	Female (1) Male (1)	2014 (2)
Urbion	Ebro	1	2014 (1)	1	Low (1)	Female (1)	2014 (1)
Yalde	Ebro	1	2014 (1)	1	Low (1)	Male (1)	2014 (1)
Zadorra	Ebro	34	2014 (12) 2015 (19) 2016 (3)	12	Low (12)	Female (6) Male (12)	2014 (5) 2015 (7)
Zirautza	Ebro	1	2018 (1)	1	Low (1)	Male (1)	2019 (1)
European mink							
Alegr´ıa	Ebro	2	2014 (1) 2019 (1)	1	Low (1)	Female (1)	2019 (1)
Alhama	Ebro	1	2017 (1)				
Arroy	Ebro	2	2020 (2)	2	Low (2)	Female (2)	2020 (2)
Bayas	Ebro	2	2016 (2)	2	Low (2)	Male (2)	2016 (2)
Cidacos	Ebro	3	2017 (1) 2019 (2)	0			
Ea	Ebro	1	2019 (1)	0			
Ebro	Ebro	7	2015 (1) 2016 (1) 2017 (2) 2020 (3)	6	Low (6)	Female (1) Male (5)	2015 (1) 2017 (2) 2020 (3)

TABLE 1. Continued.

River	River basin	No. mink	Year (*n*)[a]	No. seropositive mink	Serology classification (no.)	Sex seropositive (no.)	Year seropositive (*n*)
Ega	Ebro	5	2015 (1) 2016 (1) 2017 (2) 2020 (1)	3	Low (3)	Female (2) Male (1)	2015 (1) 2017 (1) 2020 (1)
Iregua	Ebro	1	2017 (1)	1	Low (1)	Female (1)	2017 (1)
Laguna de los dos Reinos	Ebro	1	2019 (1)	1	Low (1)	Male (1)	2019 (1)
Leza	Ebro	2	2017 (2)	2	Low (2)	Female (2)	2017 (2)
Najerilla	Ebro	3	2018 (1) 2019 (1) 2020 (1)	2	Low (2)	Female (1) Male (1)	2018 (1) 2020 (1)
Oja	Ebro	2	2014 (2)	0			
Salburu´a	Ebro	1	2014 (1)	0			
Tiron	Ebro	2	2014 (2)	1	Low (1)	Female (1)	2014 (1)
Zadorra	Ebro	3	2014 (1) 2017 (1) 2018 (1)	0			
Zirauntza	Ebro	4	2016 (2) 2018 (1) 2020 (1)	1	Low (1)	Female (1)	2016 (1)

[a] *n* ¼ number of mink tested in a given year.

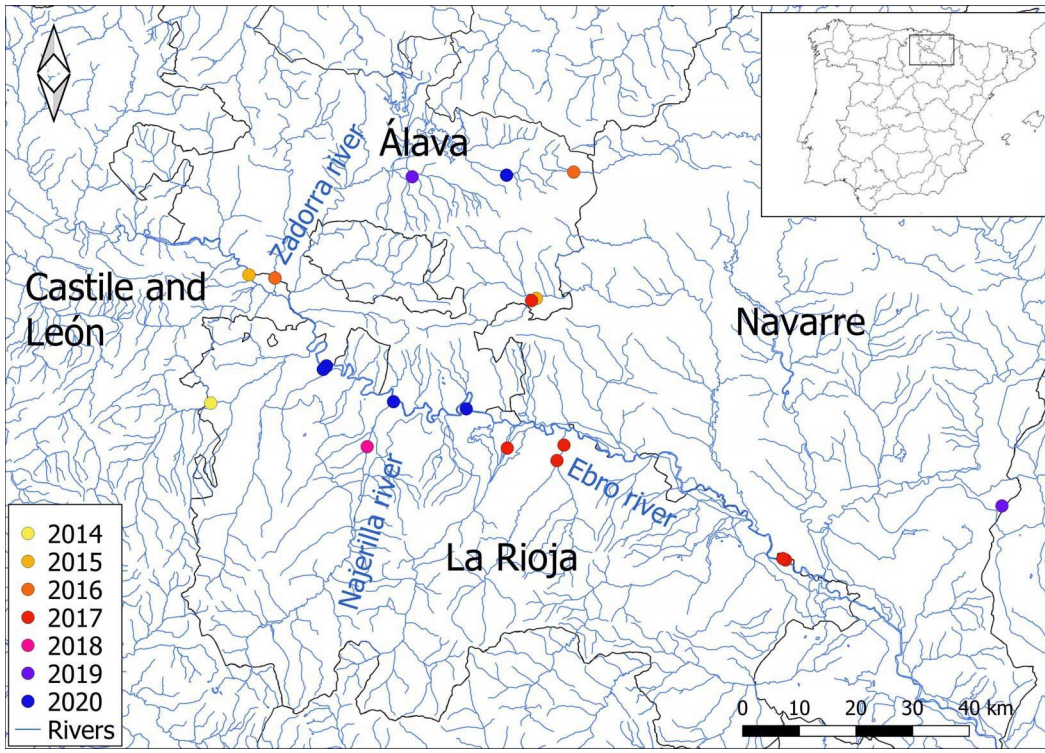

FIGURE 2. Location of *Leishmania*-seropositive European minks detected by enzyme-linked immunosorbent assay, from 42 individuals from the Cantabrian and Ebro basins, northern Spain, 2014–20. The main map shows the region of Castile and León; see inset map of Spain for the area depicted.

TABLE 2. Seroprevalence of *Leishmania infantum* in American mink (*Neovison vison*) and European mink (*Mustela lutreola*) from the Cantabrian and Ebro basins, northern Spain, 2014–20 by gender, species, and habitat.

	European mink[a]			American mink		
	22/42			63/137		
Animals (Seropositive animals/total)	*n*	%	95% CI	*n*	%	95% CI
Gender						
Male	10	23.8	(13.5–38.5)	31	22.6	(16.4–30.3)
Female	12	28.6	(17.2–43.6)	32	23.4	(17.1–31.1)
River basin						
Ebro	22	52.4	(37.7–66.6)	55	40.1	(32.3–48.5)
Cantabrian	0	0	NA	8	5.8	(3.0–11.1)

[a] *n* ¼ number seropositive; 95% CI ¼ 95% confidence internal; NA ¼ not available.

the conservation of the European mink (00NAT/E/7299, 00NAT/E/7335, and 00NAT/E/7331). The care and use of animals were carried out according to the Spanish Policy for Animal Protection (RD 53/2013), which meets European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

A total of 139 American mink were included (72 females and 67 males), whereas 42 European mink were evaluated (24 females and 18 males). These animals came from various riverbanks in northern Spain. The total number of samples processed in the sampling period (2014–20) ranged from 1 to67 in each year. All animals in this study were apparently healthy and presented an ideal condition (3/5) using a body scoring system based on a five-point scale (Rouvinen-Watt and Armstrong 2002). For that scale, animals in an ideal condition have the following characteristics: the mink has a slender neckand a straight body shape, there is a slightamount of subcutaneous body fat, and the shoulder, hip bones, and the ribs can be easily felt.

Both species were captured in single-entry 15315360-cm wire-cage traps. Captured European mink were anesthetized intramuscularly with a combination of 5 mg/kg ketamine hydrochloride (Imalgene 1000, Merial, Lyon, France) and 0.10 mg/kg medetomidine hydrochloride (Domtor, Orion Corporation, Espoo, Finland). Atipamezole (Antisedans, Orion) was used for reversal of the medetomidine at five times the medetomidine dose. All European mink were clinically examined and bled by jugular puncture; sex, weight, and body condition score were recorded, and they were marked with subcutaneous, passive transponder tags for identification. After recovery from anesthesia, they were released at their capture locations.

American minks were also anesthetized, and blood samples were collected from the jugular vein or by cardiac puncture. Routine laboratory tests, such as a complete blood cell count and a biochemistry profile were not

TABLE 3. Factors evaluated regarding the presence of anti-*Leishmania* antibodies in American mink (*Neovison vison*) and European mink (*Mustela lutreola*) from the Cantabrian and Ebro basins, northern Spain, 2014–20.[a]

	All minks			American mink		European mink	
	Sex	River basin	Species	Sex	River basin	Sex	River basin
Leishmania seropositivity	0.767	0.132	0.482	0.867	0.183	0.763	Not available

[a] Fisher exact test. Associations with a P , 0.05 were considered to be statistically significant.

performed. After data collection, and when still under anesthesia, these animals were sacrificed following the welfare legal standards.

An enzyme-linked immunosorbent assay was performed on all sera as described previously, with some modifications using 100 lL of mink sera diluted 1:50 (Giner et al. 2020). Each plate included as a positive control serum from a ferret (*Mustela putorius furo*) from Spain diagnosed with leishmaniosis (Giner et al. 2020) and a negative control serum from a healthy, noninfected ferret. The cutoff was set to 0.200 optical density (OD) units (mean63 SD values from 40 healthy, indoor ferrets). Sera with an OD≥1.00 were classified as high-positive, those with an OD≥0.60 and ,1.00 as moderate-positive, and those with an OD . 0.20 and ,0.60 as low-positive.

Data were analyzed using SPSS version 22 software (SPSS Inc., Chicago, Illinois, USA). Descriptive analysis of the variables (sex, Ebro basin or Cantabrian basin, and species) was carried out considering the proportion of the qualitative variables. the Fisher exact test and 95% confidence interval (% CI) were used to compare proportions. In all analyses, the significance level was established at P , 0.05.

Among the American mink, 63/139 were seropositive for *L. infantum* at variable antibody levels, including low-positive (n¼61), moderate-positive (0.610 OD value, n¼1), and high-positive levels (1.59 OD value, n¼1; Fig. 1). We found 44.4% of females (32/72) and 46.3% of males (31/67) were seropositive. In contrast, 22/42 European mink were seropositive for *L. infantum*, all with low antibody levels (Fig. 2); 50.0% of females (12/24) and 55.6% of males (10/18) were seropositive. Real seroprevalence values of 45.3% (95% CI, 34–52.4) and 52.4% (95% CI, 36.4–66.6) of *L. infantum* infection in American and European mink, respectively, were obtained (Tables 1, 2). No significant association (P . 0.05) was found between seropositivity for anti-*Leishmania* antibodies and the variables studied: river basin, sex, and body score (Table 3).

In Spain, the seroprevalence of canine leishmaniosis differs from one area to another and varies from 3.7% to 34.6%, with the highest prevalence cited for southern and eastern Spain and substantially lower preva- lence (3.7–4.4%) in the northern provinces of the Iberian Peninsula (Miro´ et al. 2012; Montoya et al. 2020).

During the past two decades, many wild mammals have been diagnosed with *Leishmania* infection by serologic and/or molecular methods (Oleaga et al. 2018). In the same way, studies have provided evidence of the wide presence of *L. infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain, with the presence of *Leishmania* in 28% (44/156) of animals in the Basque Country: in 26% of Eurasian badgers (*Meles meles*; n¼53), 29% of foxes (*Vulpes vulpes*; n¼48), 29% of beech martens (*Martes foina*; n¼21), and in 25–50% of less-abundant species, including genets (*Genetta genetta*), wild cats (*Felis silvestris*), pole cats (*Mustela putorius*), weasels (*Mustela nivalis*), and European mink (del Rio et al. 2014). Oleaga et al. (2018) reported a prevalence of 33% for wolves (*Canis lupus*) and an overall prevalence of 40% for all the wild carnivores studied in northwestern Spain, including a prevalence of 70% for the Eurasian otter (*Lutra lutra*), 62% of European pine marten (*Martes martes*), and 67% of beech marten (Oleaga et al. 2018). In Catalonia, a 29.5% prevalence has been detected in wild mammals by *Leishmania* DNA, and specific anti-*Leishmania* antibodies were detected (Alcover et al. 2020).

The high occurrence of *L. infantum* in American mink in this study suggests that further studies are needed to develop a deeper knowledge to avoid an added potential risk for European mink. Such studies should include animal monitoring using PCR, xenodiagnostic experiments to confirm that sandflies take blood meals from minks, and traps for the capture of adult *Phlebotomus* spp.

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CAPÍTULO 3: Antibodies to *Leishmania* in naturally exposed domestic ferrets (*Mustela putorius furo*) in Spain.

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Antibodies to *Leishmania* in naturally exposed domestic ferrets (*Mustela putorius furo*) in Spain

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ABSTRACT

Zoonotic leishmaniosis due to *Leishmania infantum* is a vector-borne disease endemic in southern Europe and dogs are the main reservoir for this infection. Seasonal variations in antibody titers in this species in areas where phlebotomine vectors have seasonal patterns of activity are important for epidemiological, preventive and clinical studies related with canine leishmaniosis. It has been suggested that cats, rabbits and ferrets may act as peridomestic reservoirs and not only as accidental hosts. The aim of this study was to determine if seropositive ferrets (*Mustela putorius furo*) to *Leishmania* could be affected by seasonal variations of anti-*Leishmania* antibodies. A group of seropositive clinically healthy ferrets (n = 21) were included in this study. A significant reduction in anti-*Leishmania infantum* antibodies was detected during non-transmission period (December 2020-February 2021) in comparison to transmission period (April-October 2020). This study describes for the first time a sea- sonal variation in the anti-*Leishmania* antibodies detected in domestic ferrets following natural exposure during sand fly transmission period and the following non-sand fly transmission period in a Mediterranean area considered as an area where *L. infantum* is endemic.

1. Introduction

Leishmaniosis is a vector-borne disease caused by *Leishmania infantum*, with the parasite being transmitted by phlebotomine sand flies under natural conditions. It is considered one of the most important vector-borne zoonoses in Europe (Baneth et al., 2016). Although various phlebotomine species are implicated in the transmission of *L. infantum* in Europe, only two of them are found in Spain: *Phlebotomus ariasi* and *Phlebotomus perniciosus* (Lucientes et al., 2005), with *P. perniciosus* being the prevalent vector in eastern Spain with a seasonal activity pattern from the end of March to November, with two peaks from June to July and from September to October (Lucientes et al., 2005).

Among pets, dogs are the primary domestic reservoir for human infection. However, other animals could be infected by the parasite, including domestic animals such as cats (Fernandez-Gallego et al., 2020). Recently, leishmaniosis has been diagnosed in a domestic ferret (*Mustela putorius furo*) using a wide range of confirmatory techniques (Giner et al., 2020). However, there is no information about the detection of anti-*Leishmania* antibodies in ferrets. In dogs, seroconversion after *Leishmania*-infected female *Phlebotomus* spp. bites is variable from 1 to 22 months, being shorter in experimental infection in comparison to natural infection (Moreno and Alvar, 2002). By contrast, timing of seroconversion is unknown for cats when the causative agent is *L. infantum*, whilst experimental infection with *Leishmania braziliensis* has shown that skin lesions usually occur before antibody response, and seroconversion can be detected when the lesions are improving and the size of the lesions decreased (Simões-Mattos et al., 2005).

In areas where *L. infantum* is endemic, the majority of dogs are exposed to infection and this exposure can be detected by serological methods based on detecting a specific antibody response against

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L. infantum (Baneth and Aroch, 2008). For dogs, enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT), and the immunochromatographic rapid tests represent the most common methods used for the detection of the anti-*Leishmania* antibodies (Maia and Campino, 2018). In the same way, the presence of anti-*Leishmania* antibodies in cats can be detected by IFAT, ELISA, direct agglutination test (DAT) and Western Blot (WB) techniques (Pennisi and Persichetti, 2018).

In this context, the detection of high antibody levels is often associated with a high parasitic load and disease in dogs (Reis et al., 2006). Conversely, low antibody levels in clinically normal dogs with a negative result on molecular and/or parasitological tests may indicate exposure without direct detection of the parasite or early stages of *Leishmania* infection (Paltrinieri et al., 2010). Changes in anti--*Leishmania* antibodies titers with a seasonal variation during transmission or non-transmission period have been observed in dogs in Spain (Acedo-Sánchez et al., 1998) and Italy (Cavalera et al., 2021), associated with the seasonal pattern of sand fly activity.

This study describes for the first time a seasonal variation in anti-*Leishmania infantum* antibodies detected by a quantitative serological test in seropositive healthy ferrets living in Valencia, an area where leishmaniosis is endemic.

2. Material and methods

2.1. Study area

All ferrets included in this study were from the Province of Valencia (39° 28′ 12.864″ N, 0° 22′ 36.48″ W), on the east coast of Iberian Peninsula, which is an area with a high prevalence of canine leishmaniosis.

2.2. Characterization of the ferrets under study

From a total of 330 ferrets analysed to detect the presence of anti-Leishmania antibodies during one year, only some animals fulfilled the following criteria: seropositive to *Leishmania* in the transmission period and a second serum sample of the same ferret obtained in the non-transmission period. Twenty-one ferrets seropositive to *Leishmania* were included in this study. A complete physical examination was carried out before sampling. For each ferret, serum samples were collected aseptically by cranial cava venipuncture in two different temporal points, one sample was obtained during sand fly transmission period (April- October 2020) and the second sample during sand fly non-transmission period (December 2020- February 2021). Data on age, gender, cohabitation with a dog and lifestyle were recorded. Separated serum were stored at -20 °C until processing. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Zaragoza (protocol code PI25/20).

2.3. Serology

Anti-*Leishmania* antibodies were detected by ELISA using a crude *L. infantum* antigen (MHOM/FR/78/LEM75 zymodeme MON-1), as described by Giner et al. (2020). Sera were diluted at 1/50 and a protein A peroxidase conjugate (dilution, 1/8,000; Pierce) was used. The cutoff was set to 0.200 optical density units (OD) (mean 3 standard deviations of values from 20 indoor ferrets from northern Spain). Each test included serum from a *L. infantum* confirmed sick ferret from Spain with a *L. infantum* isolation as positive control and serum from a healthy, non-infected ferret as negative control. Sera with an OD ≥ 0.700 were classified as high positive, with an OD ≥ 0.400 and < 0.700 as medium positive, and with an OD > 0.200 and ≤ 0.400 as low positive. OD between results obtained during sand fly transmission period and sand fly non-transmission period were considered different when OD variation was greater than 10 %. All samples and controls were analyzed in duplicate in the same ELISA plate to avoid inter- and intratest variability.

2.4. Statistical analysis

SPSS software 22.0 for Windows (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. A Kolmogorov-Smirnov test was performed and this test determine that the data were not normally distributed. Comparison of anti-*Leishmania* antibodies levels between periods (transmission and non-transmission) was tested with Wilcoxon signed rank test. Association between variation in anti-*Leishmania* antibodies (stable/increased/decreased) and the recorded variables (age, gender, cohabitation with a dog and lifestyle) were analyzed. The significance of these differences was assessed using chi-square test. A P-value <0.05 was considered significant.

3. Results

3.1. Characterization of the ferrets under study

All the tested ferrets (5 females and 16 males) had a mixture of coat colors and no ferrets had been neutered surgically. The age of the ferrets ranged from 3 to 9 years old. These ferrets had neither clinical signs nor laboratory abnormalities compatible with clinical leishmaniosis. None of the ferrets had been treated with long-acting topical anti-parasitic repellent against sand flies during the entire season of risk of expo- sure. Among the 21 seropositive ferrets, 10 animals lived with a dog at the same place. Considering the lifestyle, nine animals had an indoor lifestyle, two animals had an outdoor lifestyle and the remaining ferrets had a mix lifestyle.

3.2. Variation of anti-Leishmania antibodies between the transmission period and the non-transmission period

Overall, 12 ferrets of 21 examined showed a decrease of ELISA OD during the non-transmission period compared with the values observed during the transmission one. Table 1 shows the *L. infantum* serological results obtained by ELISA. The OD during transmission period was 0.331 ± 0.118 (mean ± standard deviation), being the maximum value 0.731 and the minimum value was 0.202. By contrast, OD during non-transmission period was 0.267 ± 0.098 with a maximum value of

Table 1
OD variation detected by ELISA during the sand fly transmission period, and follow-up during the sand fly non-transmission period.

Animal	OD during transmission period (classification)	OD during non-transmission period (classification)	Follow-up variation
1	0.260 (low)	0.273 (low)	Stable OD
2	0.247 (low)	0.196 (negative)	Reduced OD
3	0.276 (low)	0.239 (low)	Reduced OD
4	0.336 (low)	0.471(medium)	Increased OD
5	0.220 (low)	0.227 (low)	Stable OD
6	0.343 (low)	0.345 (low)	Stable OD
7	0.329 (low)	0.169 (negative)	Reduced OD
8	0.346 (low)	0.430 (medium)	Increased OD
9	0.397 (low)	0.145 (negative)	Reduced OD
10	0.256 (low)	0.190 (negative)	Reduced OD
11	0.202 (low)	0.319 (low)	Increased OD
12	0.731 (high)	0.375 (low)	Reduced OD
13	0.274 (low)	0.239 (low)	Reduced OD
14	0.318 (low)	0.153 (negative)	Reduced OD
15	0.463 (medium)	0.288 (low)	Reduced OD
16	0.382 (low)	0.242 (low)	Reduced OD
17	0.231 (low)	0.215 (low)	Stable OD
18	0.278 (low)	0.326 (low)	Increased OD
19	0.421 (medium)	0.426 (medium)	Stable OD
20	0.417 (medium)	0.174 (negative)	Reduced OD
21	0.227 (low)	0.173 (negative)	Reduced OD

0.471 and the minimum value of 0.145. No significant association was found between positivity for *L. infantum* infection and the factors evaluated ($P > 0.05$). In contrast, a statistically significant association was found between OD positivity during transmission period and OD positivity during non-transmission period ($P = 0.04$)

Considering antibody variation between sand fly transmission period and sand fly non-transmission period, five ferrets (23.8 %) had a similar anti-*Leishmania* antibodies during both periods. In general, during the transmission period 17 ferrets presented low positive values, three medium positive values and one ferret a high positive value. When comparing with the non-transmission period, a decrease in antibody titers was observed: seven ferrets became seronegative, 11 ferrets had a low positive and finally three ferrets had a medium positive titer.

During the non-transmission period, anti-*Leishmania* antibodies increased in four ferrets (19 %). Two of them increased from low levels to medium levels, whilst in the remaining ferrets OD was slightly increased, but both animals were classified as low positive. Anti-*Leishmania* antibody levels decreased in 12 of 21 (57,14 %), with seven becoming seronegative (initially low positive during transmission period), and in five ferrets with a low positive result in the transmission period, OD decreased in the non-transmission period being classified as low positive. During the transmission period, one ferret with a high positive result and another ferret with a medium positive result shifted into low positive results during the non-transmission period.

4. Discussion

To the authors' knowledge, this study describes for the first time a seasonal variation in the anti-*Leishmania* antibodies detected in domestic ferrets (*Mustela putorius furo*) following natural exposure, including sand fly transmission period and the following non-sand fly transmission period.

Female *Phlebotomus* spp. feed on a variety of vertebrate reservoirs (Killick-Kendrick, 1999), including humans, livestock, dogs, wild rabbits, hares, rodents and cats, with variable impacts on the epidemiology of leishmaniosis. The opportunistic feeding behavior of *P. perniciosus*, taking blood meals from a range of reservoirs, has been demonstrated in Menorca (De Colmenares et al., 1995) and other Mediterranean foci (Branco et al., 2013; Risueño et al., 2017).

There are some reports describing seasonal variations in anti-*Leishmania* antibodies from dogs. Reduction of anti-*Leishmania* antibodies has been detected within the same transmission season (Acedo-Sánchez et al., 1998) or between transmission seasons (Cavalera et al., 2021). Our results reinforce the importance of considering an antibody reduc-tion level in other animals such as ferrets between transmission and non-transmission periods with a significant difference (P 0.04).

A potential limitation of this study was the reduced number of animals included to extrapolate the results obtained to the general ferret population located in endemic areas of *L. infantum*. Studies with higher number of subjects are necessary to better establish the most adequate tools to be used in the context of this study. The seropositive ferrets were classified as clinically healthy animals according to the absence of laboratory abnormalities detected by routine red blood cell count and clinical chemistry. In this sense, for a better characterization of *Leishmania* infection, an additional confirmatory including a qPCR technique should be performed in bone narrow or lymph node samples. Nevertheless, the procedure to obtain these two different samples is difficult to be accepted by the owner when the animal is apparently healthy without clinical signs and laboratory alterations. The problem of the phenomenon of cross-reaction could be a common situation with other trypanosomatids, but according to our knowledge, all ferrets lived in Valencia, a region in Spain where *Trypanosoma cruzi* is not present and *L. infantum* is the parasite responsible for canine leishmaniosis in Europe.

During blood feeding, saliva inoculated stimulates a species-specific antibody response because this saliva comprises proteins with high immunogenicity property (Rohousova et al., 2005; Vlkova et al., 2011). The detection of this immune response could be useful in an epidemiological setting as marker of sand fly exposure. Differences in the detection of exposure between endemic regions could be due to the sand fly density pattens influenced by temperature, latitude, elevation, season and annual pattern among others.

Detection of anti-sand fly saliva antibodies in canine samples are mainly based on ELISA techniques that use different types of antigens, including the salivary gland homogenate (SGH), which is considered as the gold standard (Burnham et al., 2020), or other recombinant protein such as yellow-related protein rSP03B (Kostalova et al., 2015). Both type of antibodies recognizing SGH and rSP03B are associated to sand fly abundance and it is characterized by a seasonal dynamics of the *P. perniciosus*: increasing anti-salivary antibodies during summer timeand decreasing during winter months when sand flies are not active (Kostalova et al., 2015). Further studies are needed to investigate the immunological properties of the antibodies detected in ferrets as these would have important implications for seroprevalence studies.

In conclusion, the results observed in ferrets suggest a variation of anti-*Leishmania* antibodies between the transmission period and no transmission period as previously observed in dogs. Further studies are needed to expand the knowledge about sand fly exposure in other animals such domestic ferrets, detecting the presence of anti-saliva antibodies against *P. perniciosus* and their correlation with other parameters including clinicopathological and immunological information.

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CRediT authorship contribution statement

Sergio Villanueva-Saz: Conceptualization, Writing - original draft, Writing - review & editing, Project administration. **Jacobo Giner:** Conceptualization, Writing - original draft, Writing - review & editing. **Maite Verde:** Resources. **Andrés Yzuel:** Writing - original draft. **Héctor Ruiz:** Resources. **Delia Lacasta:** Resources. **Cristina Riera:** Resources. **Roser Fisa:** Resources. **María Magdalena Alcover:** Supervision. **Antonio Fernández:** Writing - original draft, Visualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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CAPÍTULO 4: Leishmaniosis caused by *Leishmania infantum* in ferrets: Update review.

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Leishmaniosis caused by *Leishmania infantum* in ferrets: Update review

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ABSTRACT

Leishmaniosis in domestic ferrets (*Mustela putorius furo*) is a disease caused by *Leishmania infantum*, a parasite transmitted through the bite of an infected female phlebotomine sand fly. Among vertebrates, the dog is the primary domestic reservoir of the parasite; however, other domestic animals can be implicated such as cats. The first description of a clinical case of leishmaniosis in domestic ferrets was reported recently. As a result, new knowledge has been published including empirically based treatment protocols, confirmatory techniques to detect the presence of the parasite infection and seasonal variation in the antibodies against *Leishmania* in apparently healthy domestic ferrets. The most common clinical signs observed are enlargement of peripheral lymph nodes and skin lesions such as papular and/or ulcerative dermatitis. Additionally, the most frequent laboratory alterations seen are hyperproteinaemia with hyperglobulinaemia and biochemical analytes alterations depending on the affected tissue. Two different therapeutic protocols have been described to treat domestic ferrets with leishmaniosis: meglumine antimoniate plus allopurinol protocol or miltefosine plus allopurinol protocol. These treatment protocols seemed to be able to control the *Leishmania* infection, although the presence of xanthinuria could be detected. The susceptibility of domestic ferrets to *Leishmania infantum*, the clinical picture, treatment of infected animals and prevention are poorly understood, due to the scarcity of recent description in the literature. Different proposed diagnostic algorithms have been included for domestic ferrets with suspected leishmaniosis, clinically healthy domestic ferrets and animals as blood donors. In this sense, the present review provides updated data on scientific knowledge of leishmaniosis in ferrets.

1. Introduction

Leishmaniosis is a zoonotic infection caused by *Leishmania infantum*, which mainly occurs with chronic clinical forms, and transmitted through the bite of infected females phlebotomine sand flies in Southern Europe. Dogs are considered the primary domestic reservoir of the parasite causing human visceral leishmaniasis. However, other animals including cats can be implicated as additional reservoir.

In Tunisia, the first report of *Leishmania* infection in a dog was described in 1908 (Nicolle & Comte, 1908), whilst the first report of feline leishmaniosis (FeL) was described in 1912 in Algeria (Sergent, Lonbard, & Quilichini, 1912). Since then, the scientific information about *L. infantum* infection focused on dogs, and more recently in cats, has increased. One-hundred-eight years after the first record of FeL, the first clinical case of leishmaniosis in a domestic ferret (Giner et al., 2020a) was published in Spain. However, the susceptibility of domestic ferrets (*Mustela putorius furo*) to *L. infantum*, the clinical pictures, management and treatment of infected animals are poorly understood, associated to the recent description in the literature.

2. Material and methods

2.1. Search and eligibility criteria

A bibliographic search was carried out in the database of Pubmed. The following combination of keywords was used/cross referenced:

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Leish* AND (control OR disease OR diagnosis OR epidem* OR infection OR one health OR reservoir OR transmission OR treatment) AND (ferret). Other inclusion criteria were the language (English) and date of publication (between January 1, 1990 and September 31, 2021). This review was carried out essentially based on guidelines outilined in the study published in Research Synthesis Methods (Polanin, Piggot, Espelage, & Grotpeter, 2019).

3. Results and discussion

A total of 4 articles were included in this review. The number of domestic ferrets in all reported studies was 23. The most relevant information obtained is presented based on the following topics including epidemiology, clinical manifestations and laboratory abnormalities, diagnosis, treatment and prevention.

3.1. Epidemiology

There is a lack of clinical description of the disease and epidemiological information of the infection in terms of seroprevalence and prevalence rates. As in cats, the prevalence of infection in endemic areas should be considered lower compared to dogs, due to the lack of infection surveys and clinical descriptions of the disease. Cases of FeL have been reported and described in several countries in Europe, South America, the US and Asia (Pereira & Maia, 2021). These cases have been described from traditionally endemic areas of canine leishmaniosis (CanL) where sand fly transmission can occur during most parts of the year, and the main mode of transmission route is through the bite of the infected female sand fly to human or animals.

Other non-vectorial transmission routes have not been described; however, it is possible to detect the presence of *Leishmania* spp. DNA in peripheral blood samples obtained from a clinically affected domestic ferret (Giner et al., 2020a), transfusion-transmitted leishmaniosis should be taken account in this species as well as it can occur in humans and dogs.

Leishmaniosis in domestic ferrets could be underdiagnosed because of lack of specific commercially available confirmatory techniques for domestic ferrets to detect the infection and the scarce clinical description of the disease. Immune response could be playing an important role in terms of susceptibility/resistance to *L. infantum* infection. In domestic ferrets with leishmaniosis, the presence of concurrent diseases was reported in a clinical case associated with impaired immune response (Giner et al., 2020b), whilst a second clinical case was not associated with concurrent immunosuppressive conditions (Giner et al., 2021a). In this regard, it is possible that the immune response elicited against the parasite in domestic ferrets was similar to that of dogs (Ordeix, Montserrat-Sangrà, Martinez-Orellana, Baxarias, & Solano-Gallego, 2019) and cats with *L. infantum* specific IFN-γ production (Priolo et al., 2019)

Although dogs are the most important hosts of the parasite, domestic ferrets could be among the potential domestic reservoirs for *L. infantum*. However, conducting research is necessary for the evaluation of the infectiveness of domestic ferret with leishmaniosis to sand flies based on xenodiagnosis. Further studies are necessary to elucidate their epidemiological roles (Giner et al., 2020a, 2021a).

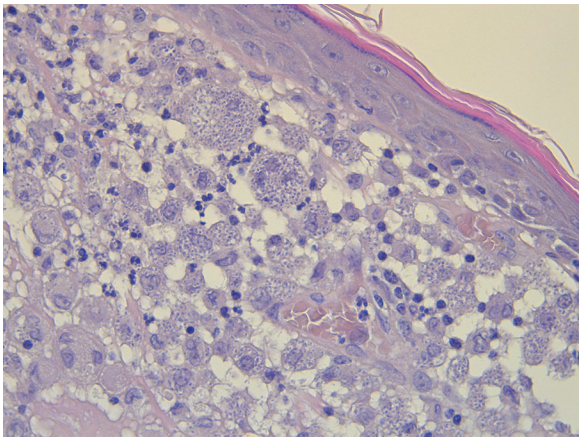

Fig. 1. Histological section of skin from a domestic ferret with suspected of leishmaniosis. Inflammatory lesion reveals the presence of macrophages and multinucleate giant cells. The cytoplasm of these cells is laden with *Leishmania* spp. amastigotes. Hematoxylin and eosin stain (x40 objective).

3.2. Clinical manifestations and laboratory abnormalities

Domestic ferret leishmaniosis is a multiorgan disease that affects different organs and tissues with the presence of non-specific clinicopathological abnormalities detected and clinical signs observed in two clinical cases at the moment. The more relevant clinical manifestation found in these domestic ferrets is skin lesions which could be detected along with other laboratory abnormalities (Giner et al., 2020a, 2021a).

One domestic ferret presented a nonpruritic erythematous and an edematous papular painless skin lesion on the right ear pinna compatible with papular dermatitis or nodular lesion associated to the inoculation site. The other domestic ferret presented a nonpruritic ulceration of the lower lip. In both cases, a severe chronic diffuse pyogranulomatous dermatitis was detected by histological examination. In this sense, leishmaniosis could induce in domestic ferrets a pyogranulomatous and granulomatous inflammation, being necessary to rule out other causes.

In the first clinical case reported, no other apparent clinical signs were detected. However, an immunosuppressive status and elevation of the parasitic load was related to immunosuppressive therapy. Furthermore, *Leishmania* infection probably could exacerbate the immunosuppression status of the patient because cryptosporidiosis with intestinal and pulmonary signs was detected several months after *Leishmania* infection had been confirmed (Giner et al., 2020b). In the second clinical case, other clinical signs detected were peripheral lymphadenomegaly and splenomegaly.

There is a published case report about clinical leishmaniosis in another animal belonging to the same carnivorous mammals' family, Mustelidae, a captive Eurasian otter (*Lutra lutra*) with epistaxis and non-specific clinical signs such as anorexia, apathy, and weight loss (Cantos-Barreda et al., 2020).

Hyperglobulinemia was the most common laboratory abnormality detected in both domestic ferrets as well as in the captive Eurasian otter leishmaniosis clinical case. Clinical leishmaniosis should be considered in the differential diagnosis of hyperglobulinemia in domestic ferrets, a clinicopathological abnormality usually associated with a variety of infections in this species including systemic mycoses, viruses and finally certain neoplasia (Melillo, 2013). Serum protein electrophoresis is a crucial biochemical technique used for the investigation of a normal distribution of serum protein fractions (albumin, α 1, α 2, β 1, β 2 and γ). In small animal veterinary medicine, different serum protein electrophoresis patterns could be detected, from normal pattern to acute-phase protein responses, polyclonal gammopathies, oligoclonal gammopathies or also called restricted polyclonal gammopathies and finally monoclonal or paraproteinemias. In canine leishmaniosis, three different gammopathies patterns are associated to the disease including polyclonal, oligoclonal, biclonal or monoclonal gammpathy, being the polyclonal pattern, the most common gammopathy detected (Paltrinieri, Gradoni, Roura, Zatelli, & Zini, 2016).

Furthermore, another laboratory finding detected in one of the patients was a high serum enzymes activity including alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transferase. The cause of the elevation of these liver parameters and whether these were related to leishmaniosis as occurs occasionally in dogs (Villanueva- Saz, Peréz, Yzuel, Fernández, & Verde, 2020) cannot be determined because liver biopsies were not obtained in the domestic ferret. Bile culture and

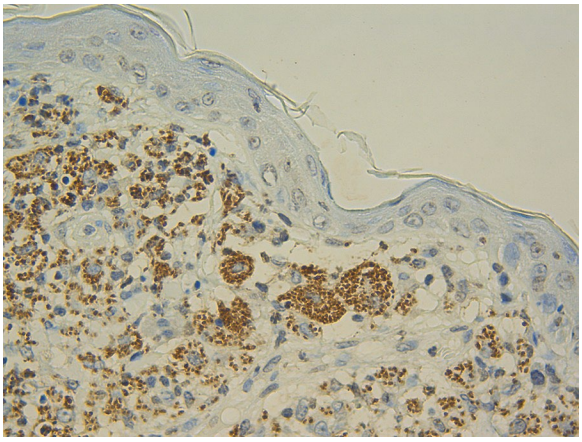

Fig. 2. Immunohistochemical staining labeling of *Leishmania* spp. amastigotes (skin of a domestic ferret with suspected of leishmaniosis). The amastigotes parasites labelled in brown (x40 objective).

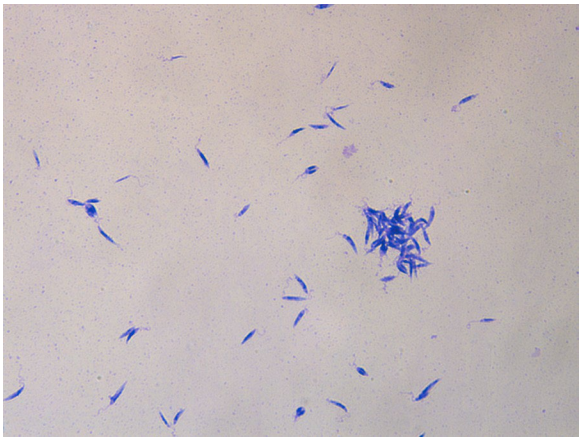

Fig. 3. *Leishmania* spp. promastigotes isolated by NNN culture obtained from a domestic ferret with suspected of leishmaniosis. Giemsa stain (x40 objective).

abdominal ultrasound were performed in the domestic ferret of this report with negative results; however, the favourable response to anti-*Leishmania* treatment could be considered as indirect indicator that *Leishmania* parasites might have some role in the pathogenesis of the hepatic disease.

3.3. Diagnosis

The same confirmatory techniques to detect the presence of *L. infantum* infection in dogs and cats can be available for domestic ferrets. Parasitological methods include all confirmatory techniques with direct observation of the parasite such as cytology, where amastigotes can be found in infected macrophages. Cytological study from lymph nodes samples in case of lymphadenomegaly can confirm the presence of the *Leishmania* parasite. Other tissues such as bone marrow are not always accessible in domestic ferrets due to the small size of the patient. Histology (Fig. 1) and histopathological evaluation are recommended in cases where cytology of the lesions is classified as a negative result for the presence of *Leishmania* amastigotes. The presence of granulomatous inflammation pattern in absence of intracellular *Leishmania* amastigotes should be evaluated considering specific immunohistochemistry using a hyperimmune serum obtained by experimental animal immunized with *L. infantum* antigen due to the lack of commercial specific primary antibodies to be used in the immunohistochemical diagnosis of leishmaniosis (Fig. 2).

Culture and *Leishmania* isolation are another parasitological method that can be used and different types of media can be employed, being the most common the biphasic Novy-McNeal-Nicolle medium (NNN) and the Schneider´s medium. The NNN is a culture medium prepared preferably with rabbit blood and agar, although other blood from different animals can be used, whilst the liquid phase contains fetal calf serum, antibiotics and other supplementary components necessary to produce a great number of promastigotes. By contrast, Schneider´s medium is a monophasic liquid medium prepared with different components, the most important medium being Schneider´s insect medium. (Castelli et al., 2014). This type of diagnostic procedure requires special laboratories including trained personnel and class II biosafety cabinets, and two main disadvantages: the possibility of bacterial contamination of the medium and the time consuming for *Leishmania* species to be able to grow. Nevertheless, the isolation and subsequent positive *Leishmania* culture allows to establish the cause-effect relationship in the animal with leishmaniosis (Fig. 3).

Serology is a methodology based on the detection of specific anti-IgG antibodies against *L. infantum* using different techniques including ELISA, immunofluorescence antibody test (IFAT) and western blot (WB). When the disease is described for the first time in other species, it is necessary to establish the cut off setting. This cut off could be similar or different to other species such as dogs and cats. In domestic ferrets (Giner, 2020a) and cats (Iatta et al., 2020), cut off was set at 1:80 dilution for IFAT, whilst, in dogs was set from 1/20 to 1/160 (Santarém et al., 2020). In general, serological techniques must be adapted to the animal species, first, for example, using different FITC-conjugate for IFAT and then validated to evaluate diagnostic measures including sensitivity and specificity.

Among serological tests to investigate *Leishmania* infection in cats, WB technique has the best diagnostic performance in terms of sensitivity (Alcover et al., 2021a; Persichetti et al., 2017). In cats infected with *L. infantum*, WB can detect different antigen bands including 14 kDa, 16 kDa, 18 kDa, 20 kDa, 24 kDa, 36 kDa and 46 kDa, the most frequent positive bands detected were 46 kDa and 16 kDa (Alcover, Riera, & Fisa, 2021). In particular, WB in domestic ferrets with clinical leishmaniosis presented reactivity against the 14 and/or 16 kDa bands as described previously in cats (Alcover et al., 2021a; Giner et al., 2020a).

Recent evidence suggests that a variation in the anti-*Leishmania* antibodies can be detected during the sand fly transmission period and the following non sand fly transmission period in apparently healthy domestic ferrets in natural conditions (Villanueva-Saz et al., 2021). This fact is important from a point of sampling time due to the presence of apparently healthy seropositive domestic ferrets during the transmission period, making it necessary to reassess the serological status during the non-transmission period to avoid unnecessary treatment based on antibody titers obtained during transmission period (Villanueva-Saz et al., 2021). Other factors that should be taken into account is the fact that serological methods should be validated to be used in domestic ferrets. For this purpose, two different steps should be carried out including a first step based on design, adaptation an preparation of the techniques and the second step with a field validation on a domestic ferret population, possibly with two subpopulations from endemic and non-endemic areas. Finally, serological methods for use in cats or dogs would give a negative result in seropositive domestic ferrets.

The presence of *Leishmania* spp. DNA in different types of matrix (EDTA-blood, paraffin-embedded tissue specimens, Whatman filter paper, bone marrow and lymph node fine-needle aspiration, among others) could be evaluated by real time PCR to determine the parasitic load (Giner et al., 2020a). For a better diagnostic management, a combination of confirmatory techniques with different nature, including a qPCR technique and a quantitative serology, should be performed.

In conclusion, the diagnosis of the disease in domestic ferrets is similar to dogs and cats. It requires the integration of the clinical picture and the laboratory abnormalities detected in the laboratory tests performed (complete blood count, complete biochemical profile, urinalysis

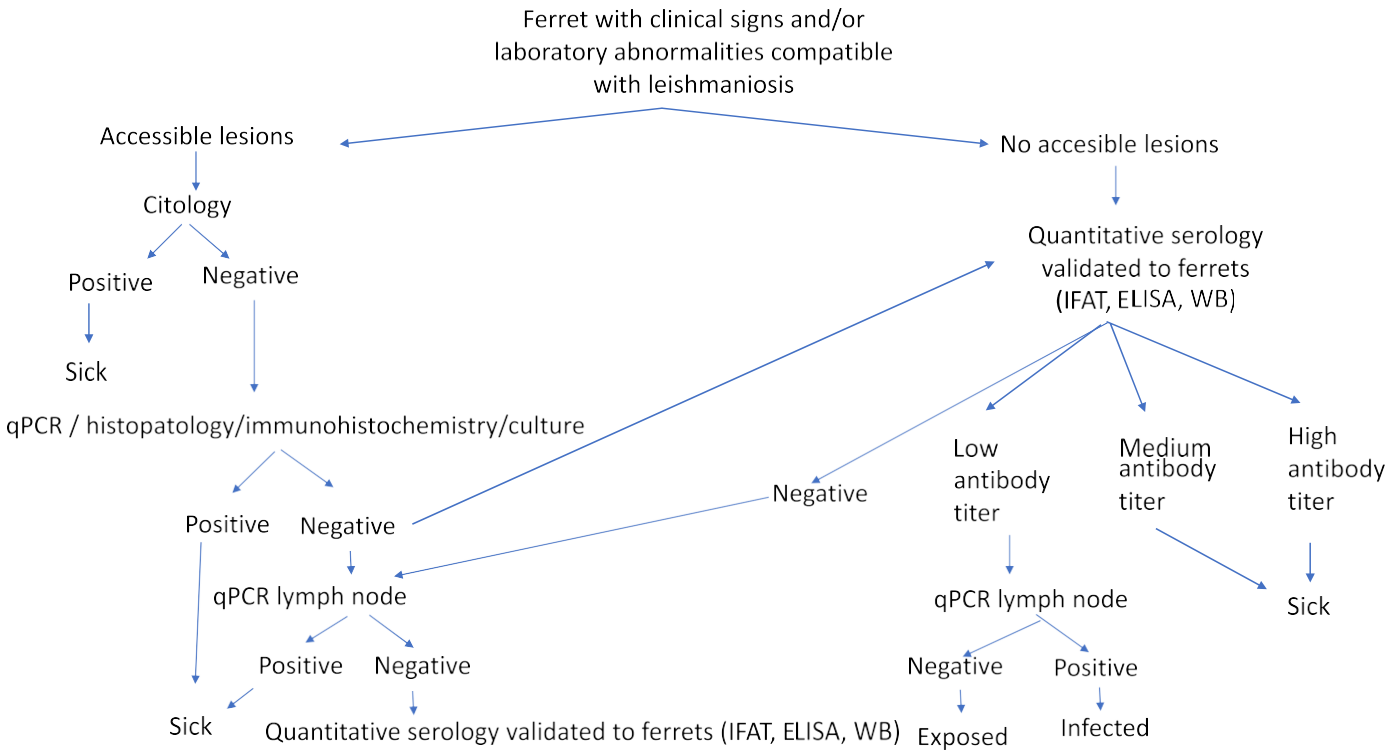

Fig. 4. Diagnostic algorithm for domestic ferrets with suspected of leishmaniosis.

and serum protein electrophoresis) together with a positive result of any of the confirmatory techniques described previously. In this sense, the diagnostic algorithm for domestic ferrets with suspected leishmaniosis is included (Fig. 4).

3.5. Treatment

The information on the treatment of domestic ferrets with leishmaniosis is extremely scarce because only two reports have been published. Two different therapeutic protocols have been described to treat domestic ferret with leishmaniosis: meglumine antimoniate plus allopurinol (Giner et al., 2020b) or miltefosine plus allopurinol (Giner et al., 2021a). Although combined therapeutic protocols based on these drugs were well tolerated in those patients, information is lacking on pharmacological characteristics of these drugs in domestic ferrets. In addition, in the captive Eurasian otter clinical case, allopurinol alone was administrated as treatment protocol (Cantos-Barreda et al., 2020).

Allopurinol is a compound that blocks RNA synthesis in *Leishmania*. Consequently, a negative effect in parasite multiplication is produced. The length of allopurinol treatment in dogs and cats depends on the response to treatment and the individual tolerance to this drug (Pennisi et al., 2015; Solano-Gallego et al., 2011). Although it is well tolerated by dogs, different side effects are associated with its administration including xanthinuria, and the occurrence of itching specifically in some dogs with continuous long-term allopurinol therapy (Manna et al., 2014; Torres et al., 2016). In dogs, xanthinuria can develop during the first few weeks after starting allopurinol treatment (Torres et al., 2016). Increased levels of xanthine could produce more severe situations such as xanthine urolithiasis and renal mineralization. The presence of xanthine crystals is a result of the inhibition of the xanthine oxidase enzyme, which is part of the pathway to allopurinol degradation. Usually, xanthine crystalluria is identified with the morphology of crystals without the need of evaluation by optical crystallography, although sometimes these crystals are impossible to distinguish from others (ammonium biurate and amorphous urate crystals) with similar appearance by light microscopy. In domestic ferrets, the presence of

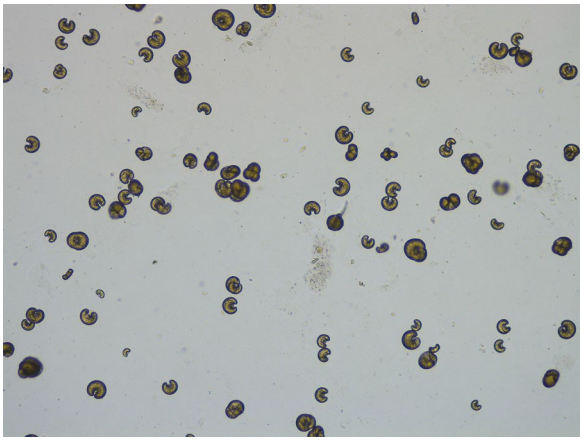

Fig. 5. Xanthine crystals from a ferret treated with allopurinol. Unstained sediment (x40 objective).

xanthinuria could be detected associated to the allopurinol treatment in some animals, whilst, other domestic ferrets did not develop this type of urine crystal. For thus, domestic ferrets receiving therapy should be monitored for the development of urinary adverse effects from the beginning of treatment and urinalysis should be systematically used in follow-up.

Meglumine antimoniate is one of the first-choice drug for the treatment of CanL (Oliva et al., 2010). This compound causes a marked decrease in the parasitic load in dogs during the treatment (Manna et al., 2014). The main side effects in dogs are potential nephrotoxicity and cutaneous abscesses/cellulitis (Solano-Gallego et al., 2011).

In the first report on leishmaniosis in naturally infected domestic ferret an empirically based protocol with allopurinol plus meglumine antimoniate was established (Giner et al., 2020b). Meglumine antimoniate was prolonged during 8 weeks due to the fact that the parasite culture was still positive and allopurinol was administered *sine die*. After

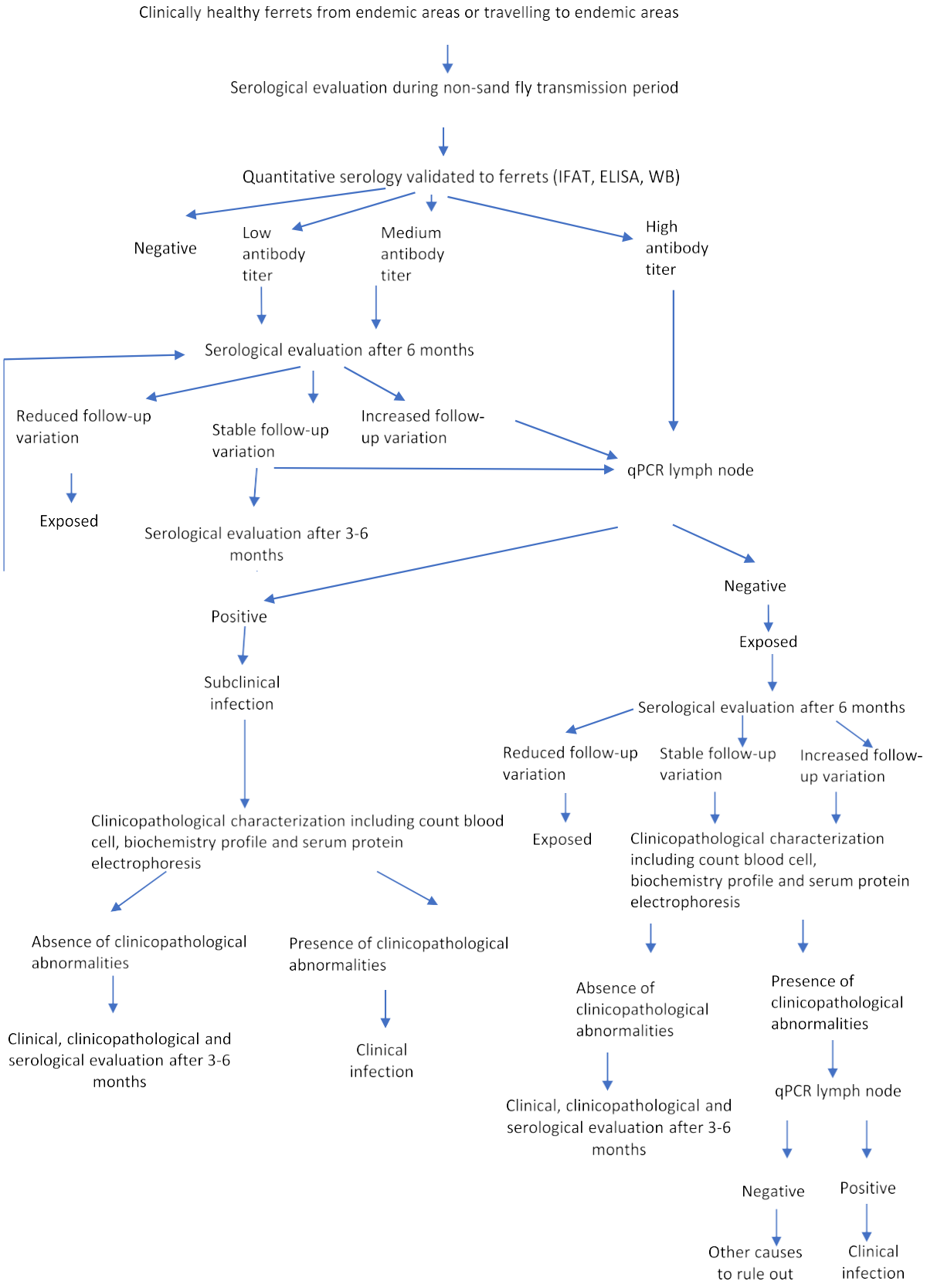

Fig. 6. Diagnostic algorithm for clinically healthy domestic ferrets.

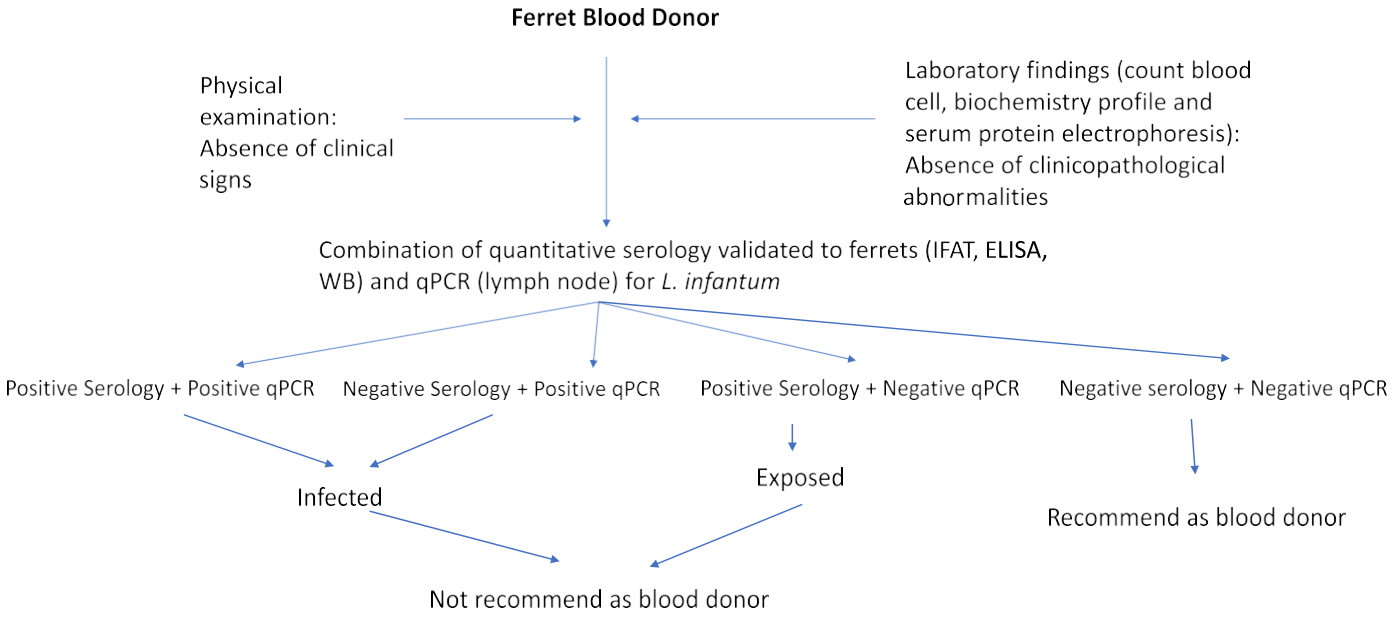

Fig. 7. Diagnostic algorithm for clinically healthy domestic ferrets used as blood donors.

finishing meglumine antimoniate administration, the domestic ferret was classified as apparently healthy. Six months since treatment was initiated, xanthinuria was detected which was related to the allopurinol treatment (Fig. 5).

In the second domestic ferret leishmaniosis case report published, an anti-*Leishmania* therapeutic protocol was established with miltefosine during 28 days and allopurinol *sine die* (Giner et al., 2021a). In contrast, in this second case report, the presence of xanthinuria associated with allopurinol treatment was not observed in urine sediment during the follow-up. This urinary adverse effect of allopurinol should be considered as dependent of each treated animal (Giner et al., 2020b). In the other mustelid with clinical leishmaniosis, allopurinol alone was administered during 3 months and the clinical signs disappeared.

Miltefosine interferes in parasite metabolic pathways and the induction of apoptosis (Soto & Soto, 2006). It is considered the second-choice drug used for leishmaniosis in dogs (Dias et al., 2020; Solano-Gallego et al., 2011). The main side effects in dogs are usually vomiting and diarrhea (Solano-Gallego et al., 2011).

The most commonly used treatments for CanL are two different protocols including meglumine antimoniate plus allopurinol protocol, or miltefosine plus allopurinol protocol. Dogs treated with meglumine antimoniate plus allopurinol protocol have a lower risk of relapse compared to dogs treated with miltefosine plus allopurinol protocol (Manna et al., 2014). In FeL, treatment information is very limited based on empirically based treatment protocols. Allopurinol alone and meglumine antimoniate alone are the most common drugs used (Pennisi et al., 2015; Pereira & Maia, 2021).

3.6. Prevention

Although future research is needed to analyze the role of domestic ferrets as hosts for *L. infantum*, it is necessary to develop preventive measures against the parasite in this species to contribute to the decline in the infection prevalence in endemic areas and to reduce the *Leishmania´*s impact of developing a clinical disease in these animals.

Current preventative measures in dogs are based on vaccines against *Leishmania,* the administration of domperidone as immune-modulator agent (Travi & Miró, 2018) and the use of registered products with a repellent activity against sand flies (Solano-Gallego et al., 2011). In domestic ferrets, the use of these products is off-label and the application of concentrated pyrethrin and pyrethroids spot-on products labelled for dogs may cause tremors or seizures in that species (Dunayer, 2008). However, an imidacloprid 10%/permethrin 50% solution was tested in a farmed mink (*Neovison vison*) flea control study (Larsen, Siggurdsson, & Mencke, 2005) which was found to be effective without toxicity effects. Minks are a close relative species of domestic ferrets; therefore these results could be extrapolated and this solution could be used in this species. In cats, flumethrin, a synthetic piretroid with a repellent activity against sand flies can be safely used (Brianti et al., 2017). Nevertheless, further studies are required to investigate these agents as repellent effect against sand flies in domestic ferrets.

Serological screening for early detection of *Leishmania* antibodies as serologic marker of infection during non-transmission sand fly period to monitor the serological status is another preventative measure that can be implemented every year (Fig. 6). As in dogs and cats, transfusion transmitted infection associated to *Leishmania* should be considered, being necessary to test blood donors by serology and blood qPCR (Fig. 7). Another preventative measure to avoid sand fly bites could be tokeep domestic ferrets indoors because sand flies are more active in the evening.

7. Conclusions

Leishmaniosis caused by *L. infantum* is a chronic infection affecting mainly dogs, cats, lagomorphs (Tsakmakidis et al., 2019) rodents (Alcover et al., 2021b) and other mustelids including minks (Giner et al., 2021b) and domestic ferrets could be infected. This short review highlights the need for xenodiagnosis to evaluate the infectiousness of domestic ferrets to sand flies and the possibility that infected domestic ferrets may represent an additional domestic reservoir for the parasite impelling the study and detection of clinically affected and subclinically seropositive domestic ferrets. Some further studies to adaptation and validation of serological techniques for ferrets are necessary, as serology is still the main tool of the monitoring and control the *L. infantum* infection in all animal species. In this sense, research should be carried out to expand the knowledge about *L. infantum* in mustelids.

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CAPÍTULO 5: Treatment and follow-up of a domestic ferret (*Mustela putorius furo*) with clinical leishmaniosis caused by *Leishmania infantum*.

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Short Communication

Treatment and follow-up of a domestic ferret (*Mustela putorius furo*) with clinical leishmaniosis caused by *Leishmania infantum*

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ABSTRACT

Leishmania infantum infection including treatment and follow up in domestic animals other than dogs and cats has not been described at this moment. This article describes the anti-*Leishmania* treatment and follow-up of a ferret (*Mustela putorius furo*) with leishmaniosis. A combined therapeutic protocol established for the patient, notyet approved for ferrets, was a combination of meglumine antimoniate plus allopurinol. A follow-up was es- tablished monthly during the first year in order to monitor the health condition of the patient. Six months after commencing allopurinol therapy, xanthine crystalluria was observed in urine sediment with no other urine alterations detected by urine analysis. The ferret worsened progressively with diarrhoea and weight loss after cohabiting with another ferret diagnosed with cryptosporidiosis. *Cryptosporidium parvum* was isolated in faecal samples from the patient detected by three different methods including Ziehl-Neelsen staining, a qualitative test to detection of *C. parvum* antigens and finally a specific molecular analysis to characterize the species. To the best of the authors´ knowledge, this is the first report providing information about anti-*Leishmania* protocol therapy used and follow-up in a domestic ferret with clinical leishmaniosis. Veterinarians practicing in endemic areas should be aware of this infection in ferrets at risk and their susceptibility especially when im- munosuppressive conditions are present.

1. Introduction

Zoonotic leishmaniosis due to *Leishmania infantum* is a vector-borne disease endemic in Southern Europe, Asia, North Africa and South America. Dogs are the main reservoir for this infection and the disease can be fatal some circumstances if not treated in people and dogs (Solano-Gallego et al., 2011). It has been estimated, based on ser- oprevalence studies from Italy, Spain, France and Portugal, that 2.5 million dogs in these countries are infected by *L. infantum* (Moreno and Alvar, 2002). In European Mediterranean countries, canine and human leishmaniosis are mainly caused by the same zymodeme MON-1 (Baneth et al., 2008). In areas where *L. infantum* is transmitted to dogs, other animals such as cats, and other non-conventional household pets are likely to be in contact with the parasite and can also be potentially infected (Pennisi, 2015), although with lesser impact in comparison to the role of the dog in the epidemiology of this infection. It has beensuggested that cats may act as a peridomestic reservoir and not only as accidental hosts (Solano-Gallego et al., 2007). In the same way, wild mammals have been considered as potential silent reservoirs of *L. in- fantum* in the Mediterranean area, suggesting the presence of chronic subclinical infection without evidence of clinical signs and they may contribute to maintaining the zoonotic cycle in an area where the presence of the dog is limited (Alcover et al., 2020). However, one of the common problems in the identification of alternative reservoirs is

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the lack of reagents to detect the infection, furthermore specific techniques such as xenodiagnosis is not always possible to be applied.

In endemic areas with high canine leishmaniosis prevalence, the detection of the *Leishmania* infection in other household pets like cats has been described, although information concerning the epidemiology and clinical picture is scarce. Moreover, the proportion of infected catsis inferior in comparison to dogs in the same endemic area. Immunosuppressive conditions in this species have been involved with promoting factors to increase the susceptibility to develop clinical disease (Solano-Gallego et al., 2011; Pennisi and Persichetti, 2018).

Allopurinol and meglumine antimoniate are the two main drugs used for the treatment of *L. infantum* in dogs (Solano-Gallego et al., 2011) and cats (Pennisi et al., 2015). Nevertheless, the use of these two drugs is off-label and there is no information in cats being empirically evidence-based (Pennisi and Persichetti, 2018).

The domestic ferret (*Mustela putorius furo*) is a small mustelid considered to be the same species as the European polecat and was domesticated between 2000 and 3000 years ago (Talbot et al., 2013). The present report, which describes therapeutically findings observed in a domestic ferret with leishmaniosis along with follow-up data thus provides information on the disease in ferrets, becomes highly relevant owing to the increasingly popularity of ferret as household pets since about the 1970´s across Europe and the United States of America (Shepherd, 2008; Talbot et al., 2013), with an official registered population of 20.000 pet-ferrets in Spain (Ministerio de Agricultura, Alimentación y Medio Ambiente, Spanish Government, 2015), which could be considered potential natural host for *Leishmania*.

2. Methods

A 4-year-old intact female ferret from Valencia (39° 28′ 12.864"N, 0° 22′ 36.48"W), on the east coast of Iberian Peninsula, was clinically evaluated in February 2019 because of a presence of a nonpruritic dermal lesion in right pinna. This ferret was adopted at the age of two years with unknown previous history. The patient lives in an apartment with other ferrets and has outdoor terrace access. The owner provides the pet-ferret an environmental enrichment with mental stimulation and outlets for its activity needs and an indoor housing appropriate for agile active, ferrets with climbing levels and multiple sleeping areas. Furthermore, ferret care is supply with a highly digestible diet consisting of a high-quality animal protein and fat, with minimal carbohydrate and fiber commercial ferret diet. It was under chronic medical management with prednisolone 0,5 mg/kg *Per os* (PO), twice a day (BID), and cyclosporine 7 mg/kg, PO once a day (SID) because of inflammatory bowel disease diagnosed one year earlier and also it was diagnosed a suppurative cholangitis six months prior to presentation.

On physical examination, the ferret presented an erythematous and edematous papular painless lesion with a diameter of 5 mm in the right ear pinna (Fig. 1a) with no other apparent clinical signs.

A skin lesion sample was taken by fine needle aspiration and stained with Diff-Quick stain for cytological examination. Cytology results revealed pyogranulomatous inflammation in which infectious agents were not visualized. Topical therapy with was iniciated with marbofloxacin 3 mg/clotrimazole 10 mg/dexamethasone plus 0.9 mg per ml suspension (Marbodex®, Ecuphar, Belgium) BID. Equally, the animal was treated with marbofloxacin (Marbocyl® 5 mg, Vetoquinol, France)2 mg/kg PO BID during 3 weeks (Fig. 1b, c). A follow-up exam three weeks later revealed little improvement of the dermatitis.

A full thickness biopsy of the lesion was taken under short general anesthesia. The lesion was completely excised, fixed in 10% neutral-buffered formalin, and submitted for histological examination. A severe chronic diffuse pyogranulomatous dermatitis with proteinaceous edema, serocellular crusts, and intramacrophagic oval organisms with eccentrical nuclei and pale cytoplasm, approximately 3 to 4 μm in size, interpreted as protozoas (*Leishmania* spp.) or fungal organisms (*Histoplasma* spp.), was observed microscopically (Fig. 2).

Anti-*Leishmania* treatment was not initiated because lesion was completely excised and any anti-*Leishmania* therapeutic protocol for ferrets was available or documented in that moment. Immune-modulating drugs dosage were reduced to avoid possible drug immunosuppression (prednisolone 0.5 mg/kg PO SID and cyclosporine7 mg/kg PO SID) and oral nutritional supplements based on S-adenosyl methionine 31.25 mg, silybin 3.75 mg and vitamin E 1.5 mg (Hepatosil Plus®, Opko health, Spain) SID were added to the treatment (Fig. 1d, 3a, b). During follow-up, four weeks later, a new papular dermatitis was detected on cicatricial edge from previous surgical incision on the right ear pinna (Fig. 3c). A sample from the cutaneous lesion was taken by needle aspiration for parasite culture in Novy-MCNeal-Nicolle medium (NNN), which tested positive for *Leishmania* parasites (Fig. 3d). Additional diagnostic procedures included detection of parasite DNA by PCR from peripheral blood sample and Whatman filter paper number 3 with aspirated material from the perilesional excised area, immunohistochemistry specific against *L. infantum* and anti-*Leishmania* antibodies detected by western blot, immunofluorescence indirect test and enzyme-linked immunosorbent assay (Giner et al., 2020). Delayed type hypersensitivity (DTH) reaction to leishmanin was evaluated using an inactivated suspension of 3×10^8 *L. infantum* promastigotes (MHOM/FR/78/LEM75) per ml in 0.2% phenol-saline, with a protein content of 30 μg/ml. The solution (100 μl) was intradermally injected in the skin of the groin. Skin reactions were recorded after 72 h and an induration or eritematous area > 5 mm in diameter was considered positive.

An anti-*Leishmania* therapeutic protocol was established with allopurinol at 10 mg/kg BID PO *sine die* (Zyloric® 100 mg, Faes Farma, Spain) and meglumine antimoniate (Antishmania®, Fatro, Italy) during three weeks at increasing doses every week to control possible drugadverse effects from 25 mg/kg BID the first week to 50 mg/kg BID the third week subcutaneously (Fig. 4a and b). Anticoagulated blood sample was analysed by an automated haematology analyser (LaserCyte IdexX, Westbrook, USA) to perform a complete cells blood count. Clinical biochemistry was analysed with an automatic analyser (Cata-lyst One IdexX, Westbrook, USA) including the following parameters: alanine amino-transferase (ALT), alkaline phosphatase (ALKP), serum gamma glutamyl transferase (GGT), total bilirubin (TBil), total choles- terol (CHO), glucose (GLU), total protein concentrations (TP), creati- nine (CRE), blood urea nitrogen (BUN), calcium (Ca), inorganic phos-phorus (P). Urine analysis was performed including urine specific gravity (USG) and urine protein to creatinine ratio (UPC). Serum pro- tein electrophoresis was run manually with agarose gels (Sebia, Evry, France) and densitometer (Shimadzu CS-9000, Kyoto, Japan) was used for scanning the electrophoretograms.

Cryptosporidium infection was detected by faecal smear by modified Ziehl-Neelsen staining method according to World Organization for Animal Health (OIE) (OIE, 2018). Later, a rapid immunochromato- graphic test for the qualitative detection of *Cryptosporidium* spp. anti- gens in faeces (*FAST*est® CRYPTO Strip, MEGACOR Diagnostik GmbH, Hörbranz, Austria) was used to confirm the diagnosis. Finally, mole- cular analysis was performed to characterize and determine the species of *Cryptosporidium* parasite. For this purpose, total genomic DNA was extracted from 180 to 200 mg of stool samples from the ferret using a QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified DNA was stored at 4 °C until used in polymerase chain reaction (PCR). Real-time PCR amplification specific for *Cryptosporidium parvum* DNA was performed according to Jothikumar et al. (2008). Real-time PCR amplifications were performed using a LightCycler 96 instrument (Roche, Basel, Switzerland).

3. Results

A follow-up visit one month since the treatment was initiated demonstrated clinical response and it was observed that the ear pinna lesions had almost disappeared (Fig. 4c). At any rate, a new sample was

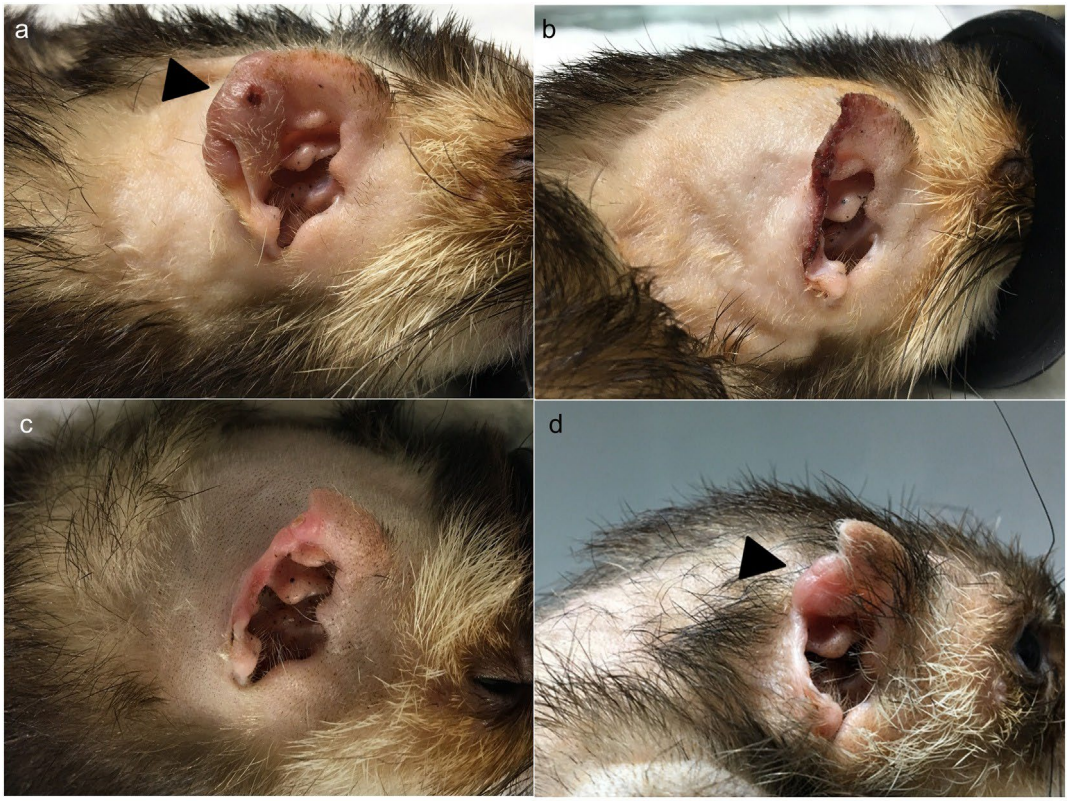

Fig. 1. Right ear pinna from the affected ferret with dermatological lesion associate to *L. infantum*. a Edematous papular lesion 5 mm in diameter before the lesion was excised. b Right ear pinna after full thickness biopsy for histopathological evaluation. c Cicatricial edge 10 days after lesion was excised. d Papular dermatitis detected on cicatricial edge from previous surgery during a follow-up one-month after surgical incision (lateral view).

taken by needle aspiration for parasite culture in NNN which was again positive for *Leishmania* promastigotes. The same biochemical abnormalities were observed as initially with persistent alteration of protein electrophoresis with hypergammaglobulinemia and elevated serum enzyme activities (ALT, ALKP and GGT).

Because the anti-*Leishmania* protocol therapy was tolerated well with no renal compromise or clinically apparent side effects noted, treatment with allopurinol at 10 mg/kg BID PO *sine die* and meglumine antimoniate at 50 mg/kg BID was continued. Three weeks following completion of the treatment, blood PCR was negative for L. *infantum* and new sample for parasite culture in NNN was taken by needle aspiration from affected area which was negative for *Leishmania* parasites. Equally, on this follow-up the ferret was appeared healthy and haematological and biochemical tests were repeated observing a partial resolution of the alteration in protein's electrophoresis (Tables 1 and 2). For these reasons, meglumine antimoniate treatment was discontinued after 56 days. The ferret continued to be treated with allopurinol at the same dose for five months. Follow-up visits (Fig. 4d) to the attending veterinarian were made every month monitoring clinicopathological parameters including complete blood count (CBC), biochemistry, urine analysis, anti-*Leishmania* antibody levels by serology and PCR (Table 3). On a follow-up visit six months since treatment was initiated, xanthinuria was observed in urine sediment and no other urine alterations were detected by urine analysis (Fig. 5). The presence of xanthine crystals was associated to the allopurinol treatment. Abdominal ultrasound before starting allopurinol was performed and no urinary tract alterations were observed.

Because serology and protein electrophoresis (Fig. 6) revealed an improvement of the animal status, allopurinol therapy was dis- continued temporarily to prevent xanthine urolithiasis. Xanthine crys- tals were not persisted after withdrawal of allopurinol two weeks later observed by urine analysis. Response to treatment after withdrawal of allopurinol was considered as effective way to evaluate if the xanthi-nuria was or not persistent.

Seven months after leishmaniosis diagnosis was made, the ferret was examined because of severe weight loss, apathy, diarrhoea and dyspnoea with tachypnea. The ferret's owner reported that the patient lived with two new ferrets diagnosed with cryptosporidiosis. A blood sample was collected and submitted for follow-up CBC and serum biochemical profile. The CBC showed neutrophilia and marked monocytosis. Serum biochemical profile revealed increases in ALT, GGT, ALKP activities, and TBil (10.3 mg/dl) concentration Table 1, November 2019). Equally, hyperproteinemia and alteration of electrophoresis with hypergammaglobulinemia and a reduced albumin/globulin ratio was detected. Thoracic radiographs revealed a diffuse broncointerstitial pattern with alveolar infiltrates compatible with a pulmonary parenchyma disorder such an infectious pneumonia (bacterial, fungal, viral protozoal or parasitic in origin) or neoplasia. *Cryptosporidium* infection was detected by faecal smear stained by modified Ziehl-Neelsen, a rapid immunochromatographic test for the qualitative detection of *Cryptosporidium* spp. antigens in faeces (Fig. 7) and a positive result obtained in molecular analysis. Immunocompromised hosts are more likely to develop clinical signs of cryptosporidiosis (Scorza and Tangtrongsup, 2010; Kumar et al., 2016). Therefore, immune-modulating drugs were discontinued because they were considered a possible cause of immunosuppression in this patient. DTH reaction to leishmanin was negative and no induration or eritematous area was observed. Azithromycin (EFG, Cinfa, Spain) was administered at 10 mg/kg PO once a day for one month. Because immunosuppressive status is one of the strongest risk factors for overt clinical disease and serology in the patient still remained positive, allopurinol 5 mg/kg PO BID *sine die* was added to the therapy. Respiratory constants and stools were normal within a week of initiating therapy. Currently, the animal's general clinical status is stable and the

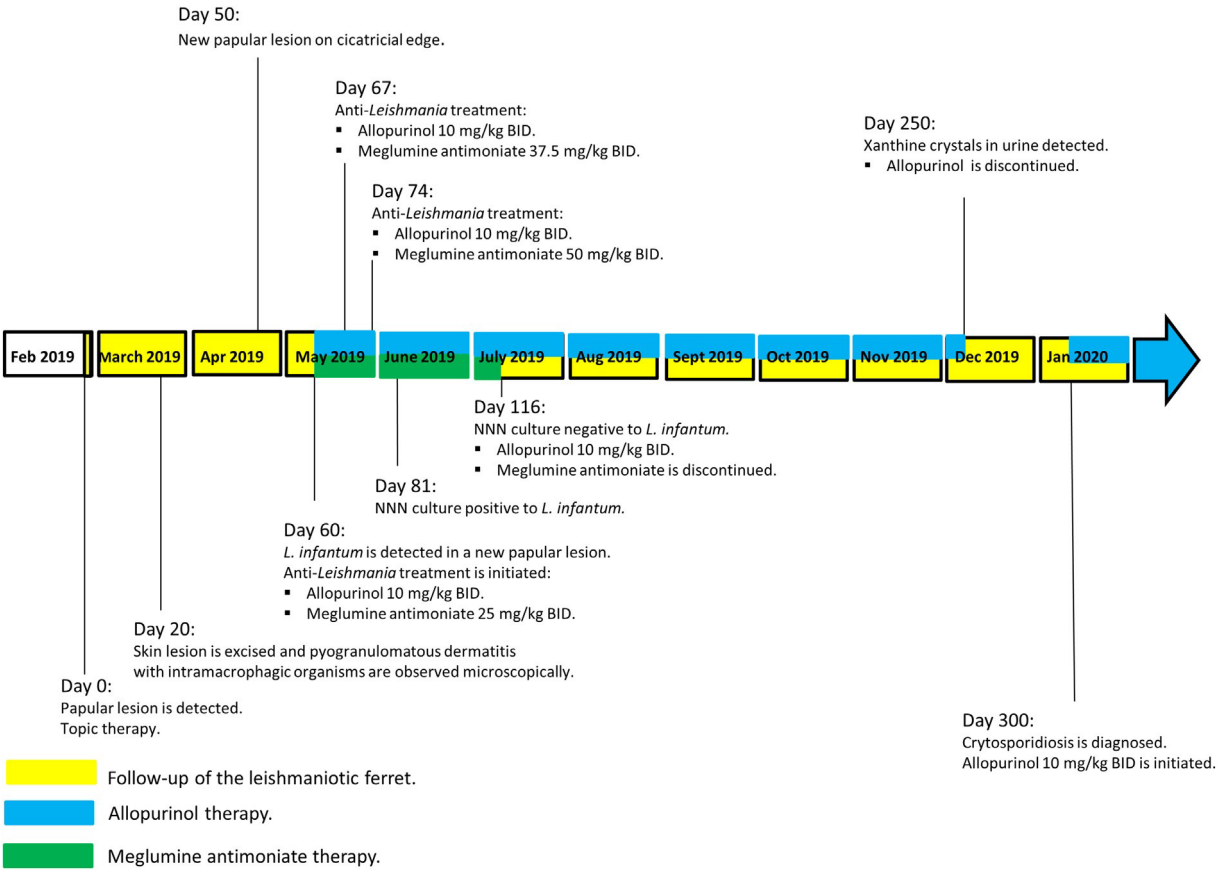

Fig. 2. Timeline of the treatment periods and following up visits.

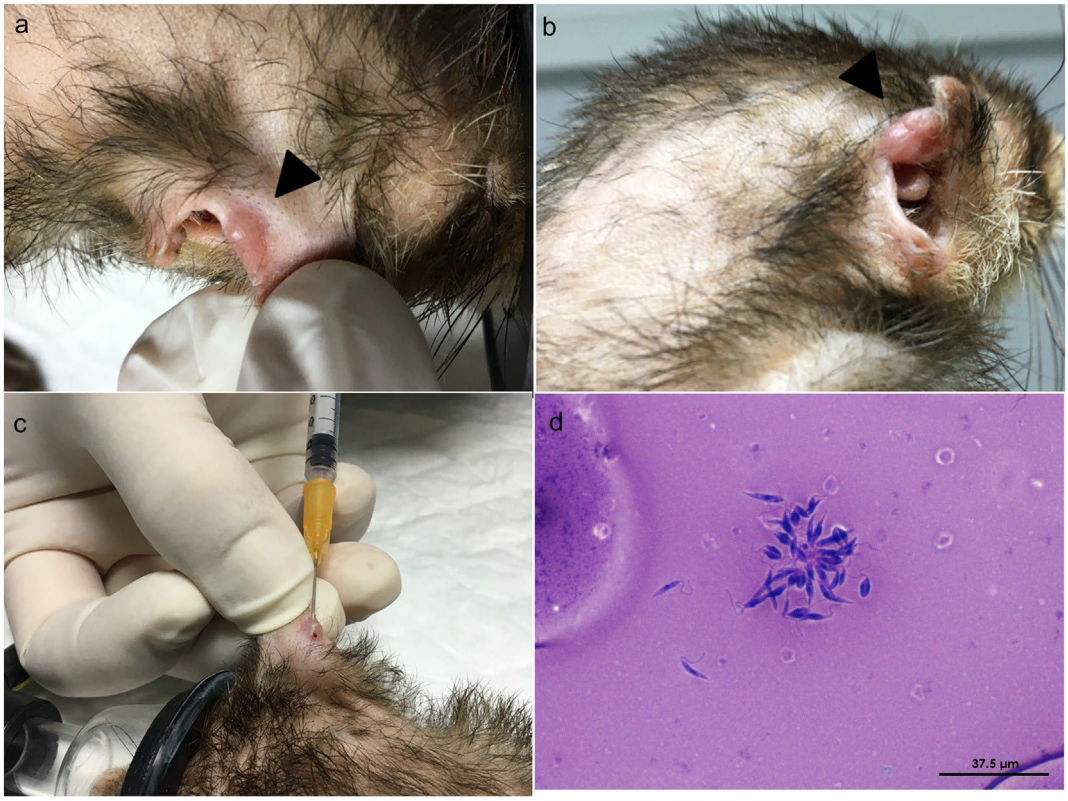

Fig. 3. Clinical lesion detected in the ferret before anti-*Leishmania* treatment was initiated. a-b Clinical sign observed at the relapse; medial view (a) and dorsal view (b). c Fine needle aspiration from papular lesion for parasite culture. d *Leishmania* promastigotes forming in rossetes from parasite culture in NNN medium. Giemsastain (40×).

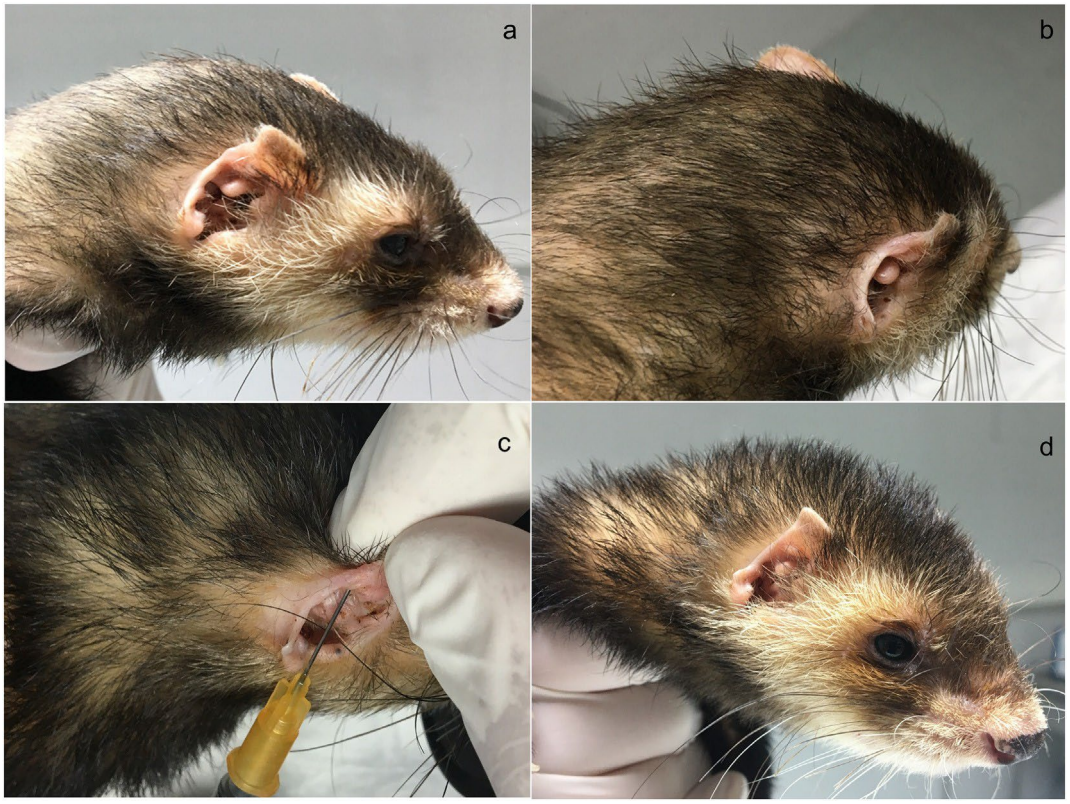

Fig. 4. Dermatological clinical sign improvement during anti-*Leishmania* treatment. a-b Different right ear pinna views on a follow-up visit one month after the initiation of treatment showing clinical response. c Fine needle aspiration from affected area for a new parasite culture in NNN medium. d Ear pinna four months since anti-*Leishmania* therapy was initiated.

Table 1
Body weight, haematological and biochemical parameters determined in the leishmaniotic ferret at the first veterinary examination before treatment (March 2019) and during the follow-up.

Parameter	Dates											Reference range
	March 2019	April 2019	May 2019	June 2019	July 2019	August 2019	September 2019	October 2019	November 2019	December 2020	January 2020	
Body weight (g)	625	635	665	620	640	530	505	520	500	420	405	500–900
Haematology												
WBC (K/μl)	4.46	5.56	5.34	4.97	4.91	5.88	3.57	5.99	6.25	nd	15.29	2–10
Neutrophils (K/μl)	3.35	3.41	3.80	3.54	2.61	4.91	2.37	4.48	4.55	nd	11.05	0.62–3.30
Lymphocytes (K/μl)	0.37	1.16	0.32	0.34	1.37	0.17	0.41	0.36	0.53	nd	0.61	1–8
Monocites (K/μl)	0.61	0.83	0.95	0.89	0.79	0.51	0.66	0.90	0.92	nd	3.12	0.18–0.90
Eosinophils (K/μl)	0.11	0.12	0.22	0.12	0.11	0.21	0.08	0.16	0.25	nd	0.41	0.10–0.60
Basophils (K/μl)	0.02	0.05	0.05	0.07	0.03	0.08	0.05	0.09	0.01	nd	0.10	0.00–0.10
RBC (M/μl)	9.89	8.17	9.28	11.43	8.82	8.57	8.42	7.59	9.27	nd	6.65	6.35–11.20
Haematocrit (%)	47.6	44,1	43.2	59.9	47.2	57.0	45.3	43.5	49.9	nd	35.2	37.0–55.0
Haemoglobin (g/dl)	13.9	14.3	14.7	15.2	15.2	12.4	15.6	16.0	18.9	nd	11.8	11.0–17.0
MCV (fl)	48.1	54.0	46.5	52.4	53.5	66.5	53.8	57.3	53.8	nd	52.9	45.0–55.0
MCH (pg)	14.1	17.5	15.9	13.3	17.2	14.5	18.5	21.1	20.4	nd	17.7	14.0–18.0
MCHC (g/dl)	29.3	32.5	34.1	25.4	32.1	31.8	34.4	36.7	38.0	nd	33.4	32.0–35.0
RDW (%)	17.0	16.9	18.1	17.7	16.6	17.5	17.1	16.4	16.8	nd	17.3	19.0–25.0
Platelets (K/μl)	357	327	278	230	207	102	271	171	309	nd	306	270–880
Blood Chemistry												
ALT (U/L)	> 1000	> 1000	634	> 1000	657	370	> 1000	> 1000	> 1000	nd	700	82–289
ALKP (U/L)	239	255	193	154	169	194	389	538	649	nd	748	9–84
GGT (U/L)	250	205	216	137	119	436	735	876	752	nd	952	0.2–14
TBil (mg/dl)	1.2	0.6	0.8	0.6	0.6	0.9	0.8	0.9	10.3	nd	1.4	0.1–1.0
Cholesterol (mg/dl)	369	379	377	321	228	296	365	360	362	nd	257	64–296
Glu (mg/dl)	131	152	107	113	119	126	102	100	155	nd	112	94–207
Crea (mg/dl)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	nd	0.4	0.4–0.9
BUN (mg/dl)	21	20	34	19	32	31	28	19	34	nd	31	10–45
P (mg/dl)	5.8	7.7	7.0	5.9	5.6	7.5	6.7	7.0	7.8	nd	6.0	4.8–8.9
Ca (mg/dl)	9.4	9.7	9.3	9.2	8.2	8.4	8.9	8.7	8.8	nd	8.1	8.0–11.8

Abbreviations: WBC White Blood Count, *RBC* Red Blood Count, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *MCHC* mean corpuscular haemoglobin concentration, *RDW* red *blood* cell distribution width *nd* not determined. Abnormalities are highlighted in bold.

Table 2
Electrophoretograms of serum proteins and urine analysis determined in the leishmaniotic ferret at the first veterinary examination before treatment (March 2019) and during the follow-up.

Parameter	Dates											Reference range
	March 2019	April 2019	May 2019	June 2019	July 2019	August 2019	September 2019	October 2019	November 2019	December 2020	January 2020	
Electrophoretograms of serum proteins												
Total protein (g/dl)	8.7	10.5	9.7	7.9	7.8	7.0	8.4	8.9	7.4	nd	7.3	4.9–7.3
Albumin (g/dl)	3.54	3.78	3.43	2.91	2.53	2.26	3.21	3.10	2.79	nd	2.58	2.30–3.60
Total globulins (g/dl)	5.16	6.72	6.27	4.99	5.27	4.74	5.19	5.80	4.60	nd	4.72	1.8–3.1
Alpha 1 globulins (g/dl)	0.22	0.24	0.74	0.21	0.18	0.14	0.19	0.40	0.93	nd	0.99	0.10–0.60
Alpha 2 globulins (g/dl)	1.11	0.75	0.55	0.75	0.97	0.56	1.19	1.00	0.44	nd	0.40	0.40–0.90
Beta globulins (g/dl)	1.63	1.26	0.75	0.91	2.71	1.23	1.70	2.30	1.27	nd	1.18	1.00–1.90
Gamma globulins (g/dl)	2.20	4.47	4.23	3.12	3.24	2.81	2.11	2.10	1.96	nd	2.15	0.30–0.90
A/G	0.69	0.56	0.55	0.58	0.48	0.48	0.62	0.53	0.61	nd	0.54	
Urine analysis												
UPC	nd	nd	nd	nd	nd	nd	0.21	nd	nd	0.35	0.41	< 0.5
USG	1.029	1.028	1.029	1.035	1.035	1.030	1.028	1.035	1.032	1.030	1.035	1.026–1.060
Sediment	–	–	–	–	–	–	–	–	–	Xanthine crystals	–	

Abbreviations: A/G albumin:globulin ratio, *UPC* urine protein to creatinine ratio, *USG* urine specific gravity, *nd* not determined, – negative.
Abnormalities are highlighted in bold.

ferret continues with the same oral daily dose of allopurinol.

4. Discussion

The present study represents the first report describing leishmaniosis treatment and one-year follow-up with routine physical examination, laboratory tests, serology and PCR in a domestic ferret (*Mustela putorius furo*) with a well-maintained quality of life.

Parasitic virulence, nutritional status, age, the host genetic and response factors in canine leishmaniosis are known to contribute to the disease although the progression of the disease after primary infection is only partly understood. Infection in dogs may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness (Solano-Gallego et al., 2009). Subclinical infection is not necessarily permanent and factors such as immunosuppression or concomitant diseases lead to the progression of clinical disease in dogs (Solano-Gallego et al., 2011) as in cats (Pennisi and Persichetti, 2018). Leishmaniosis has been associated in cats with an impaired immunocompetence due to several factors, including retroviral infections, immunosuppressive treatments and concomitant debilitating diseases, thus suggesting that these conditions may act as promoting factors (Pennisi and Persichetti, 2018; Fernández-Gallego et al., 2020). In humans, whereas most immunocompetent individuals will not develop disease after *Leishmania* infection, immunosuppression is a well-established risk factor for the development of the disease, as in patients with human immunodeficiency virus (HIV) mainly and a wide range of non-HIV-related immunosuppressive states falling under the realm of transplantation medicine, rheumatology, haematology, and oncology medicine (Van Griensven et al., 2014).

Immunosuppression induced by immunomodulating drugs such cyclosporine in dogs with atopic dermatitis have been described (Navarro et al., 2008). Although ferrets often are relatively resistant to the immunosuppressive effects of prednisolone (Chen, 2008; Chen, 2010), this clinical case reports a ferret with a suggested

Table 3
Results of *Leishmania* infection confirmation tests recorded during the follow-up.

Leishmania infantum confirmation tests			March 2019	April 2019	May 2019	June 2019	July 2019	August 2019	September 2019	October 2019	November 2019	January 2020
Parasitological methods	Cytology		—	nd	nd	nd	nd	nd	nd	nd	nd	nd
	H&E		+	nd	nd	nd	nd	nd	nd	nd	nd	nd
	IHQ		nd	+	nd	nd	nd	nd	nd	nd	nd	nd
	Culture		nd	+	nd	+	—	—	nd	nd	nd	nd
Molecular (qPCR)	Peripheral blood		nd	+	—	—	—	—	—	—	—	—
	Paraffin block		nd	+	nd	nd	nd	nd	nd	nd	nd	nd
	Whatman filter paper from the skin lesion		nd	+	nd	nd	nd	—	nd	nd	nd	nd
Serology	ELISA (OD)	Cut-off: 0.137	nd	nd	+ (0.599)	+ (0.443)	+ (0.485)	+ (0.684)	+ (0.619)	+ (0.522)	+ (0.455)	+ (0.237)
	IFAT(titer)	Cut-off: 1:20	nd	nd	+ (1:160)	+ (1:80)	+ (1:80)	+ (1:160)	+ (1:160)	+ (1:80)	+ (1:40)	+ (1:20)
	WB (bands)	Cut-off: 14 and/or 16 kDa	nd	nd	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)	+ (16 kDa)
Method for detecting cellular response against *L. infantum*	DTH test	An induration or eritematous area > 5 mm in diameter was considered positive	nd	nd	nd	nd	nd	nd	nd	nd	nd	—

Abbreviations: DTH delayed type hypersensitivity,*H&E* hematoxylin and eosin staining, *IFAT* immunofluorescence antibody test, *IHQ* specific immunohistochemistry

for *Leishmania* detection, *kDa* kilodaltons, *ELISA* enzyme-linked immunosorbent assay, *WB* western blot, + positive, − negative, *nd* not determined.

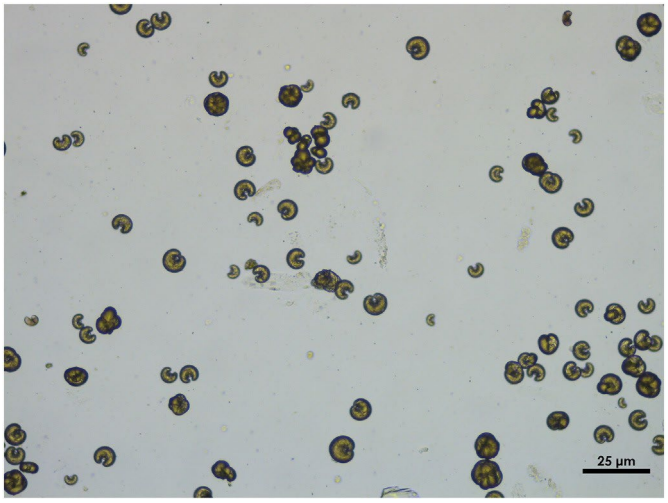

Fig. 5. A photomicrograph of urine sediment xanthine crystals. Unstained sediment, x40 objective.

immunosuppression condition initially associated with immune-modulating drugs therapy (prednisolone and cyclosporine). Furthermore, *Leishmania* infection probably could increase the immunosuppression status of the patient because an intestinal cryptosporidiosis and sup-posed pulmonary cryptosporidiosis was detected seven months since *Leishmania* infection was diagnosed. *Cryptosporidium* spp. are protozoa that inhabit the respiratory and intestinal tract epithelium though the disease is usually self-limiting and subclinical (Patterson and Fox, 2007; Powers, 2009). *Cryptosporidium* infections of the ileum are the most common, although gastric, respiratory, and conjunctival infections have been reported in immunosuppressed people (Scorza and Tangtrongsup, 2010).

According to a recent feline retrospective leishmaniosis study, median survival time in a group of cats treated specifically for leish-maniosis without concomitant diseases was longer than in another group with concomitant diseases or known immunosuppression status; however, no statistical differences were seen between groups (Fernández-Gallego et al., 2020).

A negative result in DTH test is observed in acute cases of visceral leishmaniasis in humans (Sundar and Rai, 2002) and dog with clinical leishmaniosis (Solano-Gallego et al., 2005), whilst, a positive result can be detected in human cases where kala-azar has been cured (Sundar and Rai, 2002) and in dogs that improve after anti-*Leishmania* treatment (Solano-Gallego et al., 2001). In this ferret, a negative result detected by DTH could be interpreted as potentially no resistant to *Leishmania* infection.

Laboratory findings revealed high serum enzymes activities (ALT, ALKP, GGT) on each follow up serum chemistry evaluation performed. Various hepatic diseases have been reported in ferrets; the most common liver disorders encountered in this species are inflammatory, infectious and toxic hepatic diseases; and less commonly hepatic lipidosis, and hepatic neoplasia. Owing to most previously mentioned liver diseases remained often subclinical may lead to difficulties in diagnosing those conditions accurately (Huynh and Laloi, 2013). Hepatobiliary failure due to *L. infantum* has been documented in dogs experimentally as well as in natural infection, recording laboratory alterations associated with liver injury and histopathological changes in the liver which are characterized by the presence of a mononuclear inflammatory cell infiltration (Valladares et al., 1997; Rallis et al., 2005; Moreira et al., 2016). Bile culture was performed in the ferret ofthis report with negative results and abdominal ultrasound did not revealed biliary tree or liver parenchyma abnormalities. The cause of ALT, ALKP and GGT elevations in the ferret, and if these were related to

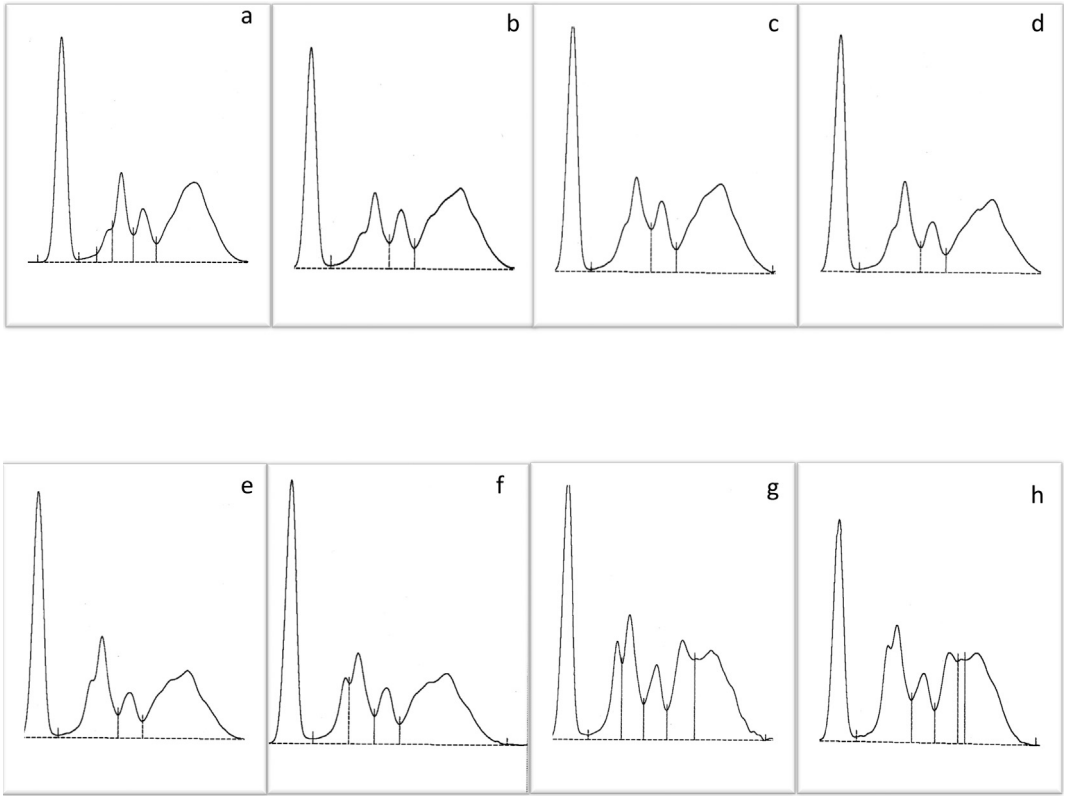

Fig. 6. Agarose gel electrophoretograms of serum proteins of the ferret before starting anti-*Leishmania* treatment (a) and during the follow up (b, c, d, e, f, g, h). b One-month follow-up (June 2019). c Two-month follow-up (July 2019). d Three-month follow-up (August 2019). e Four-month follow-up (September 2019). f Five- month follow-up (October 2019). g Six-month follow-up (November 2019). h Eight-month follow-up (January 2020).

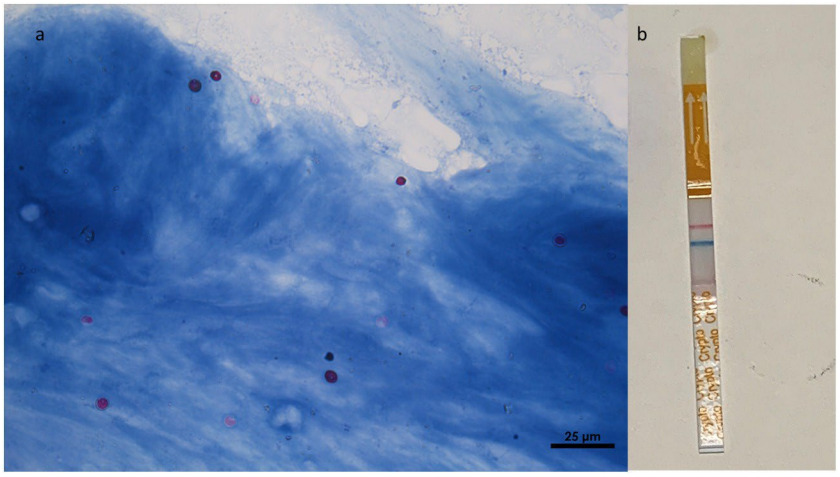

Fig. 7. a Oocysts of *Cryptosporidium* spp. Ziehl Neelsen, 40×. b Qualitative detection of *Cryptosporidium* antigens by a rapid test-kit. A red test line indicates a positive result whilst a blue test line indicates that the test has been performed properly. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

leishmaniosis, cannot be determined because liver biopsies could not be obtained.

Anti-*Leishmania* treatment selection between meglumine antimoniate *versus* miltefosine was based on the better clinical efficacy of meglumine antimoniate in long term clinical setting in dogs (Manna et al., 2014). Different treatment protocols with variable survival time have been described in cats with clinical leishmaniosis: allopurinol alone, a combination meglumine antimoniate plus allopurinol, a com-bination miltefosine plus allopurinol, and finally meglumine anti- moniate alone with very variable survival time between anti-*Leishmania* treatments (Fernández-Gallego et al., 2020).

Although combined therapeutic protocol based on allopurinol and meglumine antimoniate was well tolerated in this patient, information is lacking on pharmacokinetic and pharmacodynamic characteristics of these drugs in ferrets and also about their safety. Clinical improvement was observed in this ferret and papular dermatitis was resolved within a few weeks after treatment was initiated. In the same way, protein electrophoresis alterations were partially resolved until *Cryptosporidium* infection was detected in roughly seven months since anti-*Leishmania* treatment was initiated. The prolongation of the anti-*Leishmania* combined treatment by 2–3 weeks has been described in dogs and may be considered if patient improvement is inadequate (Solano-Gallego et al., 2011). In our patient, the clinical decision to increase the anti-*Leishmania* combined treatment for four more weeks was based on positive results in parasite culture of a new material aspirated from the previous lesion site.

However, xanthinuria was observed in urine sediment during a follow-up six months after long-term administration of allopurinol. Presence of xanthine crystals is a result of the inhibition of a specific enzyme, xanthine oxidase, which is part pathway of purine degradation and usually occurs secondary to therapy with allopurinol influenced by several variables including the dosage of allopurinol, quantify of dietary purine precursors, the rate of degradation and hepatic function. Our findings suggest that the presence of xanthinuria can be found during allopurinol therapy in ferrets. Treatment of leishmaniosis in this ferret was not based on scientific evidence reports. At any rate, the drugs protocol used in this animal seems to be effective and safe with pro- longed survival time.

This report demonstrates that meglumine antimoniate plus allopurinol seems to be effective as anti-*Leishmania* treatment in a ferret with clinical leishmaniosis. In general, close clinical and analytical monitoring should be performed to detect drugs side effects such as xanthinuria associate with allopurinol administration. Veterinarians practicing in endemic areas should be aware of this infection in ferretsat risk and their susceptibility especially when immunosuppressive conditions are present.

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Ethical statement

This study did not require official or institutional ethical approval. The animal was handled according to highly ethical standards and the national legislation. Nevertheless owner was required to sign an in- formed consent to allow use of samples in this study.

Declaration of Competing Interest

The authors have nothing to disclose.

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CAPÍTULO 6: Clinical leishmaniosis in a domestic ferret (*Mustela putorius furo*) treated with miltefosine plus allopurinol: Serological and clinical follow-up.

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Case Report

Clinical leishmaniosis in a domestic ferret (*Mustela putorius furo*) treated with miltefosine plus allopurinol: Serological and clinical follow-up

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ABSTRACT

The published information on the treatment of mustelid leishmaniosis is extremely scarce because there are only two case reports available. In one case, a domestic ferret (*Mustela putorius furo*) was treated with a combination of meglumine antimoniate plus allopurinol and, in the other case, a therapeutic regimen with allopurinol was administrated to a Eurasian otter (*Lutra lutra*). This article describes for the first time a combined therapeutic protocol with miltefosine (2 mg/kg once a day during 28 days *per os*), and allopurinol (10 mg/kg twice a day PO *sine die*) in a domestic ferret with splenomegaly, lymphadenomegaly and a facial pyogranulomatous dermatitis, with a moderate level of antibodies to *Leishmania infantum*.

1. Introduction

Leishmaniosis caused by *Leishmania infantum* is a parasitic zoonotic disease in Southern Europe transmitted by phlebotomine sand flies. The domestic dog is the main reservoir host for L. *infantum* and canine leishmaniosis is an important and complex disease extensively studied (Solano-Gallego et al., 2011). However, other domestic mammals are likely to be in contact with the parasite and can also be potentially infected such as cats (Alcover et al., 2021) and other conventional household pets as ferrets (Giner et al., 2020a). Moreover, the reports that leishmaniosis affects many other animals besides dogs and cats are increasing, with a recent review published including other mammals (Cardoso et al., 2021).

The domestic ferret (*Mustela putorius furo*) belongs to the family Mustelidae, the largest family within the mammalian order Carnivora. Recently, the first notification of natural L. *infantum* infection detected by parasite culture in mustelids was described (Giner et al., 2020a). In the same way, it has been published the first treatments and follow-up clinical cases of leishmaniosis in mustelids: a domestic ferret treated with a combined therapeutic protocol based on allopurinol and meglumine antimoniate (Giner et al., 2020b), and a captive Eurasian otter (*Lutra lutra*) with a therapeutic regimen based in allopurinol (Cantos-Barreda et al., 2020).

Different drugs are available as anti-*Leishmania* therapeutic protocols in dogs, including meglumine antimoniate, miltefosine y/or allopurinol (Solano-Gallego et al., 2011). Miltefosine is is considered as the only oral drug for the treatment of leishmaniasis in humans (Soto and Soto, 2006) and one of the drugs usually used for leishmaniosis treatment in dogs (Mateo et al., 2009; Manna et al., 2009). The combination of miltefosine plus allopurinol promoted better effects in comparison to miltefosine monotherapy (Dias et al., 2020).

2. Case presentation

A 3-year-old intact female ferret from the Province of Valencia (39° 28′ 12.864"N,0° 22′ 36.48"W), on the eastern coast of Spain, was clini-cally evaluated because of the presence of an inflammatory and non-pruritic lesion on facial skin near the chin on April 2020. The ferret lived with other ferrets and a cat in a house with an outside lifestyle.

On physical examination, it was in good body condition, active, alert,

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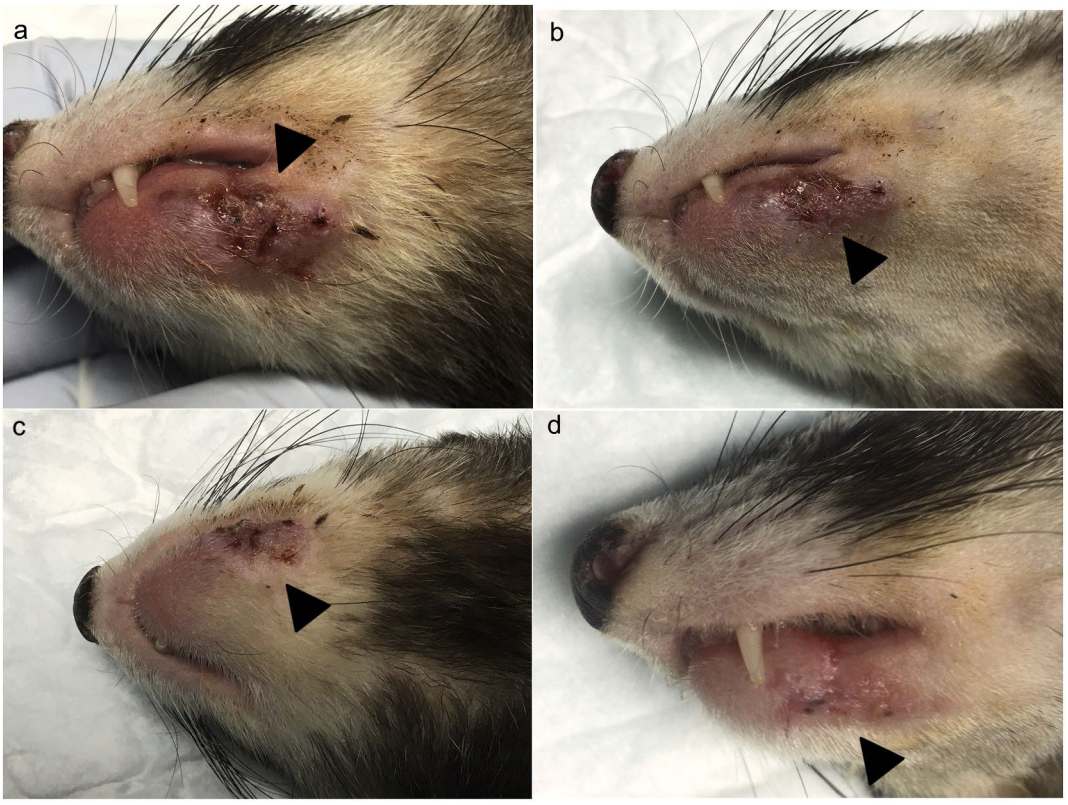

Fig. 1. Different views of dermatologic lesions detected in this ferret (a, b, c) and improvement during anti-*Leishmania* treatment (d). a-c. Erythematous, ulcerative and edematous lesion on the margin of the right lower lip. d. Dermatological lesion in a follow-up visit 3 weeks after the initiation of treatment showing clin-ical response.

Table 1
Body weight, haematological and biochemical parameters determined in the leishmaniotic ferret at the first veterinary examination before treatment (April 2020) and during the follow-up.

Parameter	April 2020	July 2020	September 2020	December 2020	February 2021	Reference ranges
Body weight (g)	660	705	775	755	660	500–900
Haematology						
WBC (K/μL)	5.62	4.43	3.92	3.41	4.68	2–10
Neutrophils (K/μL)	3.30	1.41	1.68	1.47	1.99	0.62–3.30
Lymphocytes (K/μL)	1.26	2.08	1.33	1.25	1.62	1–8
Monocites (K/μL)	0.85	0.73	0.63	0.49	0.85	0.18–0.90
Eosinophils (K/μL)	0.15	0.18	0.24	0.17	0.18	0.10–0.60
Basophils (K/μL)	0.05	0.04	0.03	0.03	0.05	0.00–0.10
RBC (M/μL)	10.13	10.74	10.70	9.66	8.36	6.35–11.20
Haematocrit (%)	51.20	51.7	50.1	45.3	40.6	37.0–55.0
Haemoglobin (g/dL)	**18.6**	**18.9**	18.9	15.9	13.5	11.0–17.0
MCV (fL)	50.6	48.1	46.8	46.9	48.6	45.0–55.0
MCH (pg)	**18.4**	**17.6**	**17.7**	16.5	16.2	14.0–18.0
MCHC (g/dL)	**36.3**	**36.6**	**37.8**	35.1	33.2	32.0–35.0
RDW (%)	**16.0**	**16.0**	**16.8**	**16.1**	**15.8**	19.0–25.0
Platelets (K/μL)	565	478	623	566	348	270–880
Blood Chemistry						
ALT (U/L)	88	280	207	248	260	82–289
ALKP (U/L)	<10	43	48	38	33	9–84
Glu (mg/dL)	114	96	94	95	99	94–207
Crea (mg/dL)	0.4	0.8	0.8	0.6	0.5	0.4–0.9
BUN (mg/dL)	17	28	28	26	25	10–45
PT (g/dL)	**8.1**	**7.7**	7.1	6.5	6.3	5.2–7.3
Alb (g/dL)	3.1	3.3	3.1	2.8	2.7	2.6–3.8
Glob (g/dL)	**5**	**4.4**	**4.1**	**3.6**	**3.5**	1.8–3.1
Alb/Glob ratio	0.6	0.8	0.8	0.8	0.8	
ELISA (OD)	**0.45**	**0.39**	**0.35**	**0.27**	**0.25**	Cut-off: 0.20

Abbreviations: *WBC* White Blood Count, *RBC* Red Blood Count, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *MCHC* mean corpuscular haemoglobin concentration, *RDW* red *blood* cell distribution, ALT alanine amino-transferase, ALKP alkaline phosphatase, GLU glucose, TP total protein concentrations, Alb albumin, Glob globulins, CREA creatinine, BUN blood urea nitrogen, ELISA enzyme-linked immunosorbent assay, OD optical density units. Abnormalities are highlighted in bold.

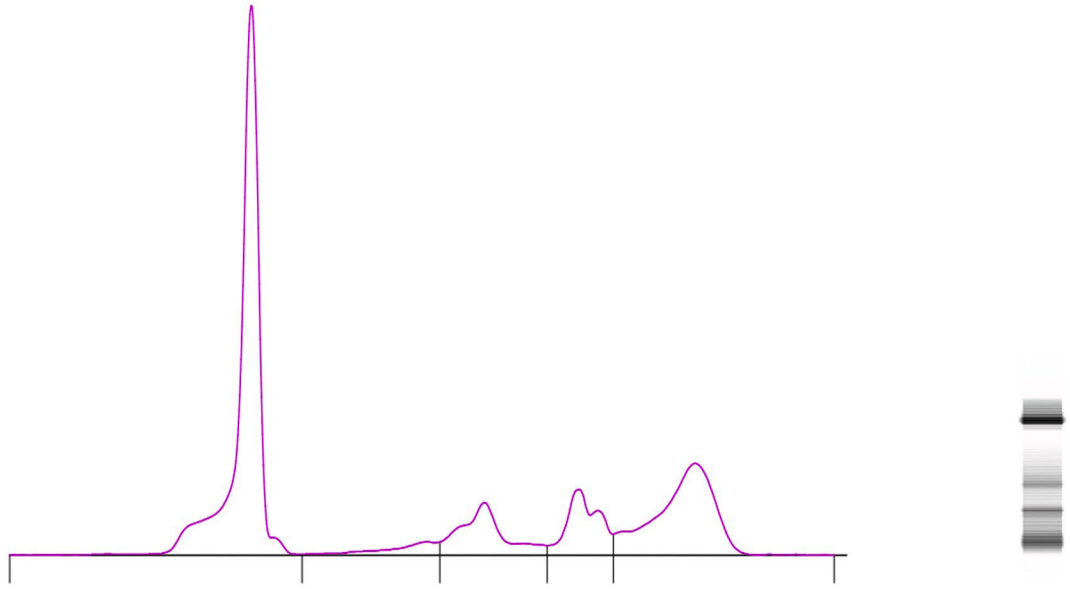

Fig. 2. Electrophoretogram using capillary zone electrophoresis. The electrophoresis revealed polyclonal gammopathy.

normothermic and not dehydrated. The patient presented an erythematous, ulcerative, edematous and painful lesion on the margin of the right lower lip (Fig. 1a, b, c), enlargement of loco-regional lymph nodes and splenomegaly with no other apparent clinical signs. A skin lesion sample and a lymph node sample were taken by fine needle aspiration and stained with Diff-Quick stain for cytological examination. Cytology results revealed a pyogranulomatous inflammation in which infectious agents were not visualized in the lesion sample and a reactive lymphoid hyperplasia in the lymph node sample. A complete blood cell count (LaserCyte Idexx, Westbrook, USA) and a biochemical profile (Catalyst One Idexx, Westbrook, USA) was performed with unremarkable results except a marked alteration of globulin levels (Table 1). An alteration of the electrophoretic profile of serum proteins, showing a polyclonal gammopathy, was detected (Fig. 2).

Anti-*Leishmania* antibodies were determined by an in-house enzyme-linked immunosorbent assay (ELISA) using sonicated L. *infantum* antigens as described previously (Giner et al., 2020a). As a positive control, a serum from a seropositive ferret was included (Giner et al., 2020a) and as a negative control, serum from a healthy, non-infected ferret. The cutoff was set to 0.200 Optical Density units (OD units) (mean 3 standard deviations of values from 40 healthy indoor ferrets). Medium levels of antibodies against L. *infantum* were detected in serum samples from this patient with an OD result of 0.45.

Equally, a full thickness incisional biopsy of the lesion was taken. Histopathological examination revealed a severe chronic pyogranulomatous dermatitis with fibrosis. No acid-fast organisms were identified by Zielh-Neelsen stain. A diagnosis of leishmaniosis was made based on clinical manifestations and clinicopathological findings including the detection of specific serum antibodies using a quantitative serological technique.

An anti-*Leishmania* therapeutic protocol was established with miltefosine (Milteforan®, Virbac Laboratories, Spain) at 2 mg/kg once a day during 28 days *per os* (PO) and allopurinol at 10 mg/kg twice a day PO *sine die* (Zyloric® 100 mg, Faes Farma, Spain). Marbofloxacin (Marbocyl® 5 mg, Vetoquinol, France) at 2 mg/kg twice a day PO was added to the treatment during the first 10 days of therapy to control possible secondary infections in the skin lesions detected. The pyogranulomatous lesions disappeared throughout the first month of treatment (Fig. 1d) and there was no relapse of the clinical signs after 10 months. Equally, there were a significant decrease of spleen and lymph nodes size during the first three months of therapy. Follow-up visits to the attending veterinarian were made monitoring clinicopathological parameters including complete blood count, biochemistry, urine analysis and anti-*Leishmania* antibody levels by serology. A decrease in serum globulin levels over time was detected: July 2020 (4.4 g/dL), September 2020 (4.1 g/dL), December 2020 (3.6 g/dL) and February 2021 (3.5 g/dL) (Table 1). On the other hand, a serological follow-up of the response to treatment was carried out in which a reduction in anti-*Leishmania* antibody levels was observed over time: July 2020 (0.39), September 2020 (0.35), December 2020 (0.27), February 2021 (0.25) and a decrease in serum globulin levels over time: July 2020 (4.4 g/dL), September 2020 (4.1 g/dL), December 2020 (3.6 g/dL) and February 2021 (3.5 g/dL) (Table 1).

3. Discussion and conclusion

To the authors´knowledge, this report describes the first clinical case of leishmaniosis in a domestic ferret (*Mustela putorius furo*) treated with a combination of miltefosine and allopurinol. Different therapeutic protocols are established for canine and feline leishmaniosis. Two different treatments protocols are described recently in mustelids (Giner et al., 2020b; Cantos-Barreda et al., 2020). In the case of the domestic ferret, the use of meglumine antimoniate during 8 weeks plus allopurinol during 4.5 months has been described with a clinical improvement 3 weeks after starting treatment, however, at 6 months after starting treatment, the presence of xanthinuria was observed. In the case report about the treatment of the Eurasian otter, it was based on the single use of allopurinol during 3 months, also observing a clinical improvement.

A combined therapeutic protocol based on miltefosine and allopurinol was well tolerated in our patient. Clinical improvement was observed in this ferret and pyogranulomatous dermatitis, splenomegaly and lymph nodes enlargement were resolved within a few weeks after treatment was initiated. In this case, after one year with allopurinol treatment, xanthinuria was not observed in urine sediment during the long-term administration of allopurinol. This finding suggests that urinary adverse effects of allopurinol treatment is variable depending on the individual response (Giner et al., 2020b).

Canine leishmaniosis is a systemic disease that may potentially involve any organ, tissue or body fluid and is manifested by nonspecific clinical signs (Villanueva-Saz et al., 2020). The diagnosis of clinical leishmaniosis in dogs and cats was based on the clinical manifestation and/or the laboratory abnormalities that were compatible with the disease as well as by the confirmation of L. *infantum* infection. In this sense, this patient presented a pyogranulomatous dermatitis,

lymphadenomegaly and splenomegaly. In ferrets, systemic coronavirus, atypical mycobacterias, *Pseudomona luteola* or *Criptococcus* spp. are pathogens that induce pyogranulomatous and granulomatous inflammation (Lucas et al., 2000; Garner et al., 2008; Morera et al., 2014; Baum et al., 2015). Splenic enlargement is a very common and nonspecific finding in adult ferrets and the causes are multiple, including extramedullary hematopoiesis, lymphosarcoma and other neoplasms such as hemangiosarcoma, cardiomyopathy or chronic infections. Equally, lymph nodes enlargement in ferrets is associated with chronic inflammation or chronic infections. Moreover, hyperglobulinemia is found in this species in many types of inflammation, determinate infections or certain neoplasms. Leishmaniosis could be a pathogen that cause those clinicopathological alterations commonly detected in ferrets as splenomegaly, lymphadenomegaly and hyperglobulinemia.

This report demonstrates that miltefosine plus allopurinol seems to be effective as anti-*Leishmania* treatment in a ferret with clinical leishmaniosis, as well as the possibility to detect the presence of anti-*Leishmania* antibodies over a long period of time.

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Declaration of Competing Interest

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4. Discusión:

La medicina de animales exóticos y silvestres se encuentre en vías de desarrollo en muchas áreas, en comparación con la medicina canina y felina; principalmente debido a la falta de técnicas específicas y valores de referencia para este tipo de animales, lo que hace que el estudio de enfermedades en estas especies suponga un reto para la medicina veterinaria. El objetivo general recogido en esta tesis doctoral fue profundizar en diversos aspectos de la Leishmaniosis en mustélidos. Para ello, se llevaron a cabo dos estudios sobre la evaluación de la situación epidemiológica de *L. infantum* en el hurón como animal de compañía en la Provincia de Valencia y en el visón europeo y americano como mustélidos silvestres en el Norte de España; la caracterización de la evolución de los anticuerpos anti-*L. infantum* en hurones seropositivos en una zona endémica de la enfermedad; y la caracterización clínica de los hurones seropositivos a *L. infantum.* Dada la amplitud y variabilidad de dichos estudios, se realiza una discusión general dividiendo esta en los diferentes aspectos estudiados:

I. Evaluación de la situación epidemiológica de *L. infantum* del hurón.

En este trabajo de investigación se realiza un estudio sobre la prevalencia de la infección por *L. infantum* en hurones domésticos en un área endémica de leishmaniosis canina, en la Provincia de Valencia (España), situada en la costa este de la Península Ibérica. Para ello se utilizaron muestras de sangre residuales extraídas durante chequeos rutinarios de salud de 102 hurones aparentemente sanos (49 hembras y 53 machos) que convivían con sus tutores como animales de compañía y que fueron llevados a consulta en el Centro Veterinario Menescalia de Valencia. Las muestras fueron recogidas asépticamente por venipunción de la vena cava craneal con el consentimiento de sus tutores. Se recogió sangre de cada animal para estudios serológicos y análisis molecular. Para la detección de anticuerpos contra *L. infantum* se utilizaron dos métodos serológicos, la técnica WB y la técnica ELISA utilizando antígenos sonificados de *L. infantum* (MHOM/FR/78/LEM75

zymodema MON-1), y para la detección de ADN de *L. infantum* se realizó la técnica de la reacción en cadena de la polimerasa en tiempo real cuantitativa.

La respuesta humoral específica en la leishmaniosis canina es, en general, muy intensa, con altos niveles de inmunoglobulinas específicas que permiten el diagnóstico serológico; además, la seroconversión se produce a los pocos meses de la infección. Sin embargo, la presencia de anticuerpos anti-*Leishmania* por sí solos no es un signo concluyente de enfermedad debido a la incapacidad de discriminar entre la inmunidad y la infecciosidad real (Morales-Yuste et al., 2022). Son múltiples las técnicas serológicas que se utilizan para detectar la presencia de anticuerpos anti-*Leishmania* en los estudios epidemiológicos en perros y gatos, como IFI, ELISA, WB y el test DAT. Importante destacar que la validación y adaptación de cada técnica serológica a las especies animales, así como los reactivos empleados para realizar la técnica requiere del uso de antisueros específicos para las especies animales analizadas.

El WB es una técnica analítica usada para detectar una proteína especifica en un extracto crudo mediante el uso de anticuerpos, que son proteínas producidas por el sistema inmune del animal (inmunoglobulinas) capaces de detectar proteínas ajenas (antígenos) y unirse específicamente a ellas. Se trata de una técnica utilizada para la inmunodetección y cuantificación de proteínas específicas que permite transferir las proteínas de un gel de poliacrilamida sodio dodecil sulfato (SDS) a una membrana absorbente, siendo las proteínas transferidas a la membrana una copia exacta del gel, donde han sido separadas por electroforesis, con el objetivo de identificar proteínas específicas en una mezcla compleja de proteínas (Kurien et al., 2006). El análisis WB es un método serológico cualitativo capaz de distinguir el peso molecular de los antígenos de *L. infantum* que estimulan la producción de anticuerpos (Persichetti et al., 2017). Se ha demostrado que la técnica WB es óptima para el diagnóstico y es considerada como un método serológico más sensible que la técnica ELISA, que es más específica (Riera et al., 2004). Por ello, generalmente se utiliza como prueba de confirmación de los resultados detectados mediante la técnica ELISA. No obstante, dado que la técnica WB es más difícil de realizar y requiere habilidades de laboratorio superiores, no se utiliza habitualmente en el diagnóstico rutinario. En este estudio la técnica de WB se realizó según lo descrito

por Alcover et al. (2021), con algunas modificaciones. Se consideró un suero positivo cuando se observó inmunoreactividad y por tanto presencia de bandas frente a la fracción del antígeno Leishmania de 14 y/o 16 kilodaltons (kDa) e inmunoreactividad intermedia cuando se observó presencia en las bandas 18, 20, 24, 28, 30, 36, 38, y 46 kDa.

La técnica ELISA es un ensayo que permite la cuantificación de un antígeno en una mezcla mediante el uso de un anticuerpo. Si bien el fundamento es la especificidad de la unión anticuerpo-antígeno al igual que el WB, el procedimiento es mucho más sencillo y rápido, aunque de menor sensibilidad con respecto a este último. En este estudio se realizó la técnica ELISA tal como lo describe Giner et al. (2020) en la publicación sobre el diagnóstico del primer caso de leishmaniosis clínica en un hurón doméstico, con algunas modificaciones. Esta técnica fue adaptada utilizando la proteína A como reactivo conjugado, ya que es capaz de interactuar con la región cristalizable del fragmento de inmunoglobulina G de varios animales incluyendo hurón, perro y gato. La proteína A siendo este reactivo muy útil para determinar la inmunoglobulina G sérica total en animales con leishmaniosis clínica en el momento del diagnóstico. Como control positivo se utilizó suero del primer hurón diagnosticado con leishmaniosis en España, confirmado por un cultivo positivo y, por el contrario, como control negativo, suero de un hurón sano no infectado. El corte se estableció en 0,180 unidades de densidad óptica (DO) (media + 3 desviaciones estándar de los valores de 30 hurones con un resultado negativo para la PCR de *Leishmania* en sangre) considerándose positivo aquellos resultados por encima de este valor.

La detección y cuantificación de ADN de *Leishmania* se llevó a cabo mediante una prueba de PCR con amplificación del ADN del kinetoplasto (kADN) a partir de sangre periférica. Existen diferentes sensibilidades para detectar ADN de *L. infantum* por las diferentes técnicas de PCR según la procedencia de la muestra, siendo más sensibles las muestras procedentes de linfonodos, bazo, médula ósea, piel o conjuntiva; mientras que se consideran poco sensibles las muestras procedentes de orina o sangre periférica (Solano-Gallego et al., 2011). Esto se debe principalmente a que los protozoos de *Leishmania* se multiplican principalmente en macrófagos de la piel y del bazo por lo que, en infecciones leves, sus niveles en

sangre son bajos (Di Pietro et al., 2020). En este estudio se decidió realizar con sangre puesto que la recolección de la muestra en este caso resulta mínimamente invasiva, a diferencia de la toma de muestras procedentes de otras partes del organismo reconocidas como más sensibles para la detección del ADN y como se ha llevado a cabo en otros estudios de prevalencia en gatos (Alcover et al., 2021)

Muestras provenientes de 29 hurones (28,4%) resultaron positivas en al menos una de las tres técnicas realizadas, siendo el Intervalo de Confianza (IC) del 95%; 20,6-37,9%. Veinte de estos fueron diagnosticados como positivos solo por WB, y solo tres por ELISA; mientras que seis hurones dieron positivo por ambas técnicas serológicas. La tasa de la seropositividad fue alta tanto por ELISA como por WB, aunque el número de casos positivos detectados por WB fue mayor estadísticamente significativo ($P < 0,008$).

En este estudió se pretendía una recogida de muestras mínimamente invasiva, por lo que se decidió utilizar una muestra de sangre periférica con EDTA para la detección de kADN de *L. infantum*. No se detectó kADN de *L. infantum* mediante qPCR en las muestras de sangre de los hurones analizados. La causa por la que todas las muestras analizadas resultaron negativas en este estudio se debió, posiblemente, a una parasitemia en sangre periférica insuficiente en hurones asintomáticos para la detección mediante la técnica utilizada; bien porque la parasitemia fuese inexistente en estas muestras, o bien porque el volumen de sangre fuese insuficiente. Existen estudios en otras especies, como en gatos, en los que tampoco se encontró ADN en las muestras sanguíneas pese a detectarse anticuerpos frente a *L. infantum* en esas mismas muestras (Miró et al., 2014). No obstante, en el primer caso clínico descrito de leishmaniosis en hurones sí que se detectó el ADN de *Leishmania*, tanto en muestras de piel como en muestras sanguíneas periféricas (Giner et al., 2020). Sería recomendable realizar más estudios moleculares para la detección de ADN de *Leishmania* en esta especie con el objetivo de determinar si diferentes tipos de muestras como médula ósea, linfonodos, piel o hisopos con muestras orales y/o conjuntivales pueden ser adecuados para el diagnóstico de la enfermedad en esta especie.

Otros resultados interesantes del estudio estadístico aportan que no se detectó asociación significativa ($P >0,05$) entre la positividad de *Leishmania* y el

género (masculino/femenino), edad (joven, adulto, senior), convivencia con un perro, o estilo de vida (interior, exterior o mixto).

Los resultados descritos en este estudio son altamente sugestivos de un posible papel de los hurones como reservorio de infección por *Leishmania*. No obstante, aunque los datos obtenidos en este estudio demuestran la exposición de los hurones al parásito *L. infantum*, la mera presencia de anticuerpos frente al mismo no es suficiente para considerar al hurón como reservorio de la enfermedad.Estos resultados deberían tenerse en cuenta ya que los hurones del estudio realizado proceden de una región donde la tasa de Leishmaniasis en humanos ha ido incrementándose en los últimos años, siendo la Comunitat Valenciana la zona geográfica española donde se ha detectado un mayor número de casos con una tasa anual de 2,63 casos/100000 habitantes, con un total de 471 casos entre 2014 y 2017, lo que supone un 34,7% del total de casos notificados a la Red Nacional de Vigilancia Epidemiológica (Fernández et al., 2019). Del mismo modo la incidencia de leishmaniosis canina en el sudeste de la Península Ibérica con una tasa anual entre 50 y 100 casos/1000 perros entre 2015 y 2017(Le Rutte et al., 2021) es de 17,1% (Montoya-Alonso et al., 2020) considerándose la Comunitat Valenciana como una zona hiperendemica con un riesgo alto de infección por *L. infantum* en perros (Galvez et al., 2020). Por todo ello, sería recomendable realizar un xenodiagnóstico para confirmar si esta especie es un hospedador accidental o un hospedador reservorio (primario o secundario) de *L. infantum* y así poder llevar a cabo medidas preventivas necesarias para la detección temprana de los hurones infectados, seguida de diversas medidas que permitan que la carga infecciosa de estos huéspedes sea lo más reducida posible. Además, serían necesarios estudios con una mayor información epidemiológica para comprender el papel de los hurones como reservorio potencial para la infección humana; así como estudios de cómo la infección por *L. infantum* podría actuar como enfermedad en esta especie.

II. Evaluación de la situación epidemiológica de *L. infantum* en visón europeo (*Mustela lutreola*) y visón americano (*Neovison vison*) en el Norte de la Península Ibérica.

En este trabajo se realiza un estudio sobre la prevalencia de la infección por *L. infantum* en visones europeos y americanos en dos áreas del Norte de la Península Ibérica, concretamente en el área de la cuenca del Ebro y de la cuenca cantábrica, una zona geográfica donde reside gran parte de la población europea del visón europeo. Esta es una de las especies animales en mayor riesgo de desaparición de todo el planeta. Además, se está provocando un agravamiento de la situación debido tanto al cambio climático, la degradación del ecosistema, y también a la presencia del visón americano introducido en la misma región en la que reside el visón europeo. Si a esto se le añade el que enfermedades emergentes y reemergentes provocadas por patógenos multihuesped y compartidas entre la vida silvestre y los animales domésticos, se están extendiendo continuamente a nuevas áreas geográficas, como ha ocurrido recientemente en la región de Asturias donde se ha detectado la muerte provocada por el virus del moquillo canino en cuatro especies carnívoras incluyendo tres mustélidos (*Meles meles*, *Martes martes* y *Mustela putorius*) (Oleaga et al., 2022), se debe realizar un control lo más exhaustivo posible de las enfermedades que pudieran ayudar a la desaparición de esta especie.

El objetivo del presente estudio ha sido conocer la prevalencia de infección por *L. infantum* en el visón europeo y ver si existe relación entre la seropositividad y signos de enfermedad de los sujetos estudiados, como se detectó en un estudio previo en el que se asoció la seropositividad a *L. infantum* con una mala condición corporal del visón (Tsakmakidis et al., 2019).

Este estudio se incluye en el proyecto LIFE, aprobado por la Comisión Europea para la conservación del visón europeo (00NAT/E/7299, 00NAT/E/7335 y 00NAT/E/7331). El manejo de los visones incluidos en este estudio se llevó a cabo de acuerdo con la Política de Protección Animal española (RD 53/2013), que cumple con la Directiva de la Unión Europea 2010/63 sobre la protección de los animales utilizados para experimentación y/u otros fines científicos.

Entre 2014 y 2020 se examinaron un total de 181 animales (139 visones americanos y 42 visones europeos). Se realizó una extracción sanguínea a todos los individuos incluidos en el estudio y una exploración física completa incluyendo sexo, peso y valoración de la condición corporal a todos los visones europeos. Los sueros obtenidos se evaluaron mediante la técnica ELISA tal y como se describe previamente en la publicación del primer caso clínico de leishmaniosis en hurones domésticos (Giner et al., 2020), con algunas modificaciones. Como control positivo se utilizó suero de un hurón infectado por *L. infantum* con cuadro clínico (Giner et al., 2020) y como control negativo, suero de un hurón sano no infectado. Se obtuvieron unos valores de seroprevalencia de la infección por *L. infantum* en el visón americano del 45,3% (IC 95 %, 34–52,4) y del 52,4 % (IC 95 %, 36,4– 66.6) en el visón europeo. Por otro lado, no se encontró una asociación significativa ($p > 0.05$) entre la seropositividad a anticuerpos anti-*Leishmania* y las variables estudiadas como cuenca del río donde se capturo al animal, el sexo o la condición corporal.

Debido al estado "en peligro crítico de extinción" en el que se encuentra el visón europeo en España, cualquier factor que afecte negativamente a la población puede tener graves repercusiones sobre la conservación de la especie.

La alta incidencia de la infección por *L. infantum* observada en visones americanos en este trabajo sugiere la necesidad de realizar más estudios como un análisis sobre prevalencia detectada mediante PCR o el xenodiagnóstico en estas especies para poder desarrollar un conocimiento más profundo con el fin de evitar un posible riesgo potencial añadido a la conservación del visón europeo.

Del mismo modo, a raíz de los resultados detectados en este estudio, se considera necesario realizar estudios de seroprevalencia tanto en visones americanos criados en cautividad en granjas cercanas a poblaciones de visón europeo, como en mamíferos silvestres capturados dentro de los proyectos de conservación de la vida silvestre que pueden jugar un papel epidemiológico como reservorios secundarios de la enfermedad.

III. Caracterización de la evolución de los anticuerpos anti-*Leishmania infantum* en hurones seropositivos en una zona endémica de la enfermedad.

Los flebótomos, vectores responsables de la transmisión de la leishmaniosis canina, presentan unos patrones estacionales de actividad que son de gran importancia para los estudios epidemiológicos y preventivos de la leishmaniosis, así como para los estudios clínicos relacionados con la enfermedad. Se ha confirmado que la temperatura, cuya magnitud se correlaciona negativamente con la latitud, es un determinante importante para el inicio de la actividad de los vectores de la leishmaniosis, por lo que el mayor riesgo potencial de transmisión de *L. infantum* en la región Mediterránea puede detectarse durante los meses de junio a octubre (Alten et al., 2016), siendo entre los meses de abril y noviembre cuando los reservorios de la enfermedad, principalmente los perros, se encuentran potencialmente más expuestos a la una posible infección por *L. infantum* (Vlkova et al., 2011). Aunque los títulos de anticuerpos anti-*Leishmania* son de suma importancia, tanto en el diagnóstico como en el tratamiento y seguimiento de la leishmaniosis canina, existen pocos datos que comparen estos títulos de anticuerpos con la estacionalidad de los vectores de transmisión. Recientemente, un estudio ha demostrado que existe una variación en los títulos de anticuerpos anti-*L. infantum*, en perros, entre los periodos de transmisión y de no transmisión (Cavalera et al., 2021). Por ello, en áreas donde los flebótomos tienen una clara estacionalidad en sus patrones de actividad, esta posible variación en la titulación de anticuerpos contra *L. infantum* debe ser tenida en cuenta a la hora de interpretar los resultados en perros asintomáticos de cara a tomar decisiones sobre la estadificación clínica y el tratamiento (Cavalera et al., 2021).

Tras la detección de los primeros casos clínicos de leishmaniosis en mustélidos, se decidió evaluar las variaciones estacionales de las titulaciones de anticuerpos anti-*Leishmania infantum* en hurones de un área hiperendémica para leishmaniosis canina y comprobar si existía la misma variación encontrada en la especie canina. Para ello, se incluyó un grupo de 21 hurones seropositivos, clínicamente sanos, procedentes de la Provincia de Valencia. A cada hurón se le

realizaron dos extracciones sanguíneas de forma aséptica de la vena cava craneal, en dos periodos diferentes, uno durante el periodo de transmisión del parásito por parte del vector (abril-octubre 2020) y otro durante el periodo de no transmisión del parásito por parte del flebótomo responsable (diciembre de 2020-febrero de 2021). Se detectó una reducción significativa en los anticuerpos anti-*Leishmania* durante el período de no transmisión en comparación con el período de transmisión. Durante el período de transmisión 17 hurones presentaron valores positivos bajos, 3 valores de anticuerpos positivos medios y un hurón un valor positivo alto. Por otro lado, durante el período de no transmisión, se observó una disminución de la titulación de anticuerpos en los que, siete hurones se volvieron seronegativos, 11 hurones tuvieron un positivo bajo y finalmente tres hurones tuvieron un título positivo medio.

Durante el período de no transmisión, los niveles de anticuerpos anti-*Leishmania* disminuyeron en 12 de 21 (57,14 %) hurones. Los anticuerpos anti-*Leishmania* aumentaron en cuatro hurones (19 %). Dos de ellos aumentaron desde niveles bajos a niveles medios, mientras que las titulaciones en los otros dos hurones aumentaron ligeramente, aunque los animales fueron clasificados como positivos bajos. Cinco hurones (23,8 %) mantuvieron una titulación de anticuerpos anti-*Leishmania* similar durante ambos periodos.

Este estudio describe, por primera vez, una variación estacional en los anticuerpos anti-*Leishmania* detectados en hurones domésticos, después de la exposición natural en un área mediterránea donde *L. infantum* está presente de forma endémica.

IV. Caracterización clínica de los hurones seropositivos a *L. infantum*.

La caracterización clínica de leishmaniosis en hurones seropositivos se plasma en la publicación de diversos trabajos incluidos en la tesis doctoral, describiendo las alteraciones clinicopatológicas detectadas en hurones hasta la fecha.

i. Manifestaciones clínicas de leishmaniosis provocada por *L.infantum* en hurones y otros mustélidos:

Las manifestaciones clínicas más relevantes detectadas en los hurones domésticos son lesiones en la piel. En el **primer caso clínico** de leishmaniosis en hurones, la única lesión dermatológica que se evidenció fue una lesión papular eritematosa, edematosa y no dolorosa de cinco milímetros de diámetro localizada en el pabellón auricular derecho, compatible con una dermatitis papulo-nodular asociada al punto de inoculación del parásito; lesión que en la especie caninaaparece inicialmente como una pápula elevada en zonas con poco pelo, como la carainterna del pabellón auricular, los párpados, el área dorso nasal, los labios y el abdomen caudal. Se realizó una citología de la lesión dermatológica a partir de una muestra tomada por aspiración con aguja y teñida con Diff-Quick®, donde se observó la presencia de un infiltrado piogranulomatoso. Durante el examen físico, el hurón se encontraba en buen estado, activo y alerta, normotérmico y adecuadamente hidratado. La auscultación cardíaca estaba dentro de los límites normales, los sonidos respiratorios también eran normales y no había evidencia de linfadenomegalia (Giner at al., 2020). Por otro lado, una nutria euroasiática mostró epistaxis bilateral, anorexia, apatía y pérdida de peso junto a linfadenomegalia mesentérica (Cantos-Barreda et al., 2020).

En esta tesis se presenta los signos clínicos del **segundo caso clínico** descrito de leishmaniosis en un hurón doméstico, que fue llevado a consulta debido a la presencia de una lesión inflamatoria no pruriginosa en la dermis de la zona del mentón. El paciente presentaba una lesión eritematosa, ulcerativa, edematosa y dolorosa en el margen del labio inferior derecho; linfadenomegalia locorregional y esplenomegalia, sin otros signos clínicos aparentes. En la exploración el hurón se encontraba en una buena condición física, activo, alerta, normotérmico y bien hidratado. Al igual que en el primer caso clínico, se realizó una citología de la lesión

mediante aspiración con aguja fina y posterior tinción con Diff-Quick®. Se observó la presencia de un infiltrado piogranulomatoso.

Las lesiones piogranulomatosas se observan con frecuencia en hurones, y pueden ser provocadas por diferentes patógenos tales como coronavirus sistémico, *Pseudomona luteola*, *Criptococcus* spp o dermatofitos (Lucas et al., 2000; Garner et al., 2008; Morera et al., 2014; Baum et al., 2015, Giner et al., 2018; Giner et al., 2022). Por ello, la leshmaniosis se debe incluir en el diagnostico diferencial de lesiones piogranulomatosas en esta especie.

Por otro lado, un **tercer caso clínico** de leishmaniosis en hurones se presentó en el 5th International Conference on Avian, Herpetological and Exotic Mammal Medicine (ICARE) (Giner et al., 2022) que presentaba pérdida de peso progresiva, anorexia parcial, poliuria y polidipsia. En la exploración física, se detectó una marcada esplenomegalia, confirmada por ecografía abdominal, en la que también se detectó un aumento del tamaño de los linfonodos mesentéricos.

ii. Alteraciones laboratoriales detectadas en hurones y otros mustélidos con leishmaniosis clínica:

La alteración laboratorial más característica detectada en todos los casos de leishmaniosis en mustélidos es la alteración de las proteínas séricas (detectando hiperproteinemia) en los tres casos descritos.

En el primer caso clínico se detectó una gammapatía policlonal con elevación de la fracción de las alfa-2 globulinas y de las gammaglobulinas. En el caso clínico de la nutria afectada clínicamente por *L. infantum* se detectó hiperglubulinemia con aumento de la haptoglobulina y ferritina. En el segundo caso de leishmaniosis en hurón se detectó de nuevo una hiperproteinemia con gammapatía policlonal en la electroforesis sérica de las proteínas. Por último, el tercer caso de leishmaniosis clínica presentado en el ICARE 2022 reveló una hiperglobulinemia con un aumento de las fracciones beta y gamma en el proteinograma realizado al paciente.

Por ello, la leishmaniosis se debe incluir en el diagnóstico diferencial de la hiperglobulinemia en hurones domésticos. La hiperglobulinemia es una

anormalidad clínico-patológica generalmente asociada con una variedad de infecciones crónicas en esta especie, incluyendo micosis sistémicas, virus (coronavirus sistémico, enfermedad aleutiana o virus del moquillo canino principalmente) y determinadas neoplasias. En la leishmaniosis canina, la electroforesis sérica de proteínas revela igualmente un aumento de proteínas totales y globulinas, así como una gammapatía típicamente policlonal. En este sentido, las alteraciones detectadas en el proteinograma de los hurones con infección clínica por *L. infantum* fue muy similar a la electroforesis de proteína sérica típica en perros (Maia y Campino, 2018) y gatos (Pennisi et al., 2013) con leishmaniosis clínica, pero no es específica para una sola enfermedad infecciosa en hurones.

Otras alteraciones laboratoriales detectadas en estos pacientes fueron la elevación de la actividad de las enzimas séricas alanino-amino tranferasa, gamma-glutamil transferasa y fosfatasa alcalina. No obstante, no pudo demostrarse que la causa fuera debida a la leishmaniosis como ocurre en determinadas ocasiones en perros (Villanueva-Saz et al., 2020), ya que no se tomaron muestras de tejido hepático para su posterior estudio. En la nutria afectada también se detectaron alteraciones en la hematología, que reveló disminución del recuento de glóbulos blancos y disminución del número de plaquetas. La trombocitopenia pudo estar relacionada con la leishmaniosis clínica del paciente como ocurre en perros con alteraciones hematológicas debido a la infección por *L. infantum* (Solano-Gallego et al., 2011). Igualmente se detectó disminución de la densidad urinaria y proteinuria, pero no se demostró mediante biopsia renal la posible implicación de la leishmaniosis en estas alteraciones. No obstante, en el cuarto caso clínico de leishmaniosis en un hurón, se detectó una elevación de los valores de nitrógeno ureico en sangre y creatinina sérica junto a hipoalbuminemia. En el urianalisis se observó una baja densidad urinaria y una marcada proteinuria, con aumento del cociente proteína/creatinina en orina. Los hallazgos histopatológicos de una sección de riñón de este hurón revelaron una glomerulonefritis crónica grave similar a las lesiones glomerulares por el depósito de los complejos inmunes formados, detectadas en perros infectados con organismos de *L. infantum* (Roura et al., 2021).

iii. Tratamiento de la infección por *L. infantum* en mustélidos con enfermedad clínica:

Se han descrito diferentes protocolos de tratamiento con un tiempo de supervivencia variable tanto en perros como en gatos con leishmaniosis clínica: alopurinol solo, una combinación de antimoniato de meglumina más alopurinol, una combinación de miltefosina más alopurinol, y finalmente antimoniato de meglumina solo con un tiempo de supervivencia muy variable entre los tratamientos anti-*Leishmania* (Oliva et al, 2010; Solano-Gallego et al., 2011; Mana et al., 2015; Fernández-Gallego et al., 2020; Garcia-Torres et al., 2022).

Hasta el momento, no existe información científica sobre un tratamiento farmacológico anti-*Leishmania* que puede aplicarse en hurones enfermos, puesto que no existen fármacos autorizados para el tratamiento de esta enfermedad en esta especie. En las publicaciones incluidas en esta tesis doctoral se describen por primera vez dos protocolos terapéuticos utilizados en los primeros casos clínicos descritos de leishmaniosis en hurones.

En el primer caso de leishmaniosis se utilizó un protocolo terapéutico combinado basado en alopurinol a 10 mg/kg, vía oral, dos veces al día, *sine die* y antimoniato de meglumina durante tres semanas en dosis crecientes cada semana para controlar los posibles efectos adversos de los medicamentos. Se inició con una dosis de 25 mg/kg, vía subcutánea, cada 12 horas la primera semana y se fue incrementando hasta 50 mg/kg, cada 12 horas vía subcutánea la tercera semana. El tratamiento a base de alopurinol y antimoniato de meglumina fue bien tolerado en este paciente y demostró clara mejoría clínica, desapareciendo la dermatitis papular en las primeras semanas después de iniciado el tratamiento, que se acompañó de una reducción de los anticuerpos anti-*Leishmania*. Del mismo modo, las alteraciones detectadas en la electroforesis de las proteínas séricas iniciales, se resolvieron parcialmente hasta que se detectó una infección por *Cryptosporidium spp.* a los siete meses de iniciado el tratamiento. Igualmente se detectó una disminución de los anticuerpos anti-*Leishmania* durante los meses en los que se llevó a cabo la combinación terapéutica. No obstante, en este paciente apareció una xantinuria en un control a los seis meses de iniciado el tratamiento con alopurinol, que se resolvió tras la suspensión temporal de la administración de dicho fármaco.

Este caso clínico representa la primera descripción del tratamiento y seguimiento de la leishmaniosis clínica en un hurón doméstico mediante monitorización mensual a lo largo de un año con examen físico de rutina, y pruebas de laboratorio incluyendo hematología, bioquímica sérica, urianálisis, medición de anticuerpos anti-*Leishmania* mediante serología y PCR.

En el segundo caso clínico publicado sobre leishmaniosis clínica en un hurón doméstico e incluido igualmente en esta tesis doctoral, se estableció un protocolo terapéutico anti-*Leishmania* a base de miltefosina a 2 mg/kg una vez al día durante 28 días por vía oral y alopurinol a 10 mg/kg dos veces al día *sine die* con resultados satisfactorios y bien tolerado por el paciente. Se encontró una mejoría clínica con resolución a las pocas semanas de tratamiento tanto de la dermatitis piogranulomatosa observada como de la esplenomegalia y la linfadenomegalia detectada. Por otro lado, se observó una reducción de los niveles de anticuerpos anti-*Leishmania* a lo largo del tiempo.

En este caso, tras un año con un tratamiento continuado con alopurinol, no se observó xantinuria en el sedimento de orina en ninguno de los controles periódicos realizados. Este hallazgo sugiere que el efecto adverso que produce el tratamiento continuado con alopurinol en esta especie es variable y aparecerá dependiendo de la respuesta individual de cada paciente, como ocurre en perros tratados con alopurinol de forma continuada.

En el caso clínico de la nutria euroasiática publicado recientemente, se administró un protocolo terapéutico a base de alopurinol como único fármaco frente a la leishmaniosis durante 3 meses, con resultados satisfactorios dada la completa desaparición de los signos clínicos.

En el último caso descrito de leishmaniosis en un hurón presentado en el Congreso Internacional ICARE (International Conference on Avian, Herpetological and Exotic Mammal Medicine) 2022, el protocolo terapéutico llevado a cabo frente a *L. infantum* consistió también, al igual que el segundo caso descrito, en miltefosina a 2 mg/kg una vez al día durante 28 días por vía oral y alopurinol a 10 mg/kg dos veces al día de forma continuada. Dos meses después, las alteraciones séricas para evaluar la función renal detectadas en sangre se normalizaron y hubo una

disminución tanto de los niveles de globulinas séricas como de los niveles de anticuerpos anti-*Leishmania*. No obstante, cinco meses tras el inicio de la terapia, el paciente fue sometido a eutanasia tras acudir al centro veterinario con azotemia severa, presencia de signos clínicos relacionados con la pérdida de función renal y de manifestaciones extrarrenales de la enfermedad. Tras la negativa por parte del propietario a instaurar un tratamiento médico, se llevó a cabo la eutanasia humanitaria del paciente.

Pese a que los tratamientos anti-*Leishmania* utilizados en estos casos clínicos han resultado satisfactorios, queremos remarcar la falta de información sobre las características farmacocinéticas y farmacodinámicas de estos fármacos en mustélidos. Tampoco se dispone de estudios sobre su seguridad y esto debe ser tenido en cuenta a la hora de instaurar uno de estos protocolos, cuando aparezca un caso de leishmaniosis clínica en mustélidos, realizando seguimientos clínicos adecuados para detectar posibles efectos secundarios de los fármacos utilizados, como la xantinuria asociada a la administración de alopurinol, detectada en el primer hurón doméstico tratado frente a esta enfermedad.

5. Conclusiones:

De los estudios realizados que componen esta tesis doctoral hemos obtenido las siguientes conclusiones:

Primera: Nuestro trabajo demuestra por primera vez las tasas de seroprevalencia y prevalencia de *L. infantum* en mustélidos en dos áreas geográficas de España.

Segunda: Los resultados serológicos obtenidos en este estudio indican que los hurones domésticos, mantenidos como animales de compañía, procedentes de una zona endémica de leishmaniosis canina (provincia de Valencia), se encuentran expuestos al parásito de *L. infantum*, presentando una tasa de seroprevalencia del 28,4% (IC 95%; 20,6-37,9%).

Tercera: En nuestro estudio, el visón europeo y el visón americano de la zona norte de la Península Ibérica presentan una alta seroprevalencia de la infección por *L. infantum*, con una tasa del 52,4 % (IC 95 %, 36,4– 66,6) en el visón europeo y del 45,3% (IC 95 %, 34– 52,4) en el visón americano.

Cuarta: No se ha observado asociación entre nivel de positividad a la exposición del parásito *L. infantum* y signos clínicos o patológicos de la enfermedad en la mayor parte de los animales analizados (hurones domésticos, visones europeos y visones americanos).

Quinta: De nuestro estudio se desprende que existe variación de anticuerpos anti-*Leishmania* entre el período de transmisión y el período de no transmisión, similar a lo descrito en la especie canina.

Sexta: En este trabajo se describe el segundo caso de leishmaniosis clínica en hurones, así como el tratamiento de los dos primeros hurones domésticos diagnosticados.

Séptima: Para el diagnóstico de la leishmaniosis en hurones hemos requerido de la integración del cuadro clínico, las alteraciones laboratoriales (hemograma completo, perfil bioquímico completo, análisis de orina y electroforesis de proteínas séricas), y de un resultado positivo en cualquiera de las técnicas confirmatorias descritas (observación parasitológica, moleculares o serológicas incluyendo ELISA, IFI, WB).

Octava: Los dos protocolos terapéuticos utilizados en este trabajo para el tratamiento de los hurones con leishmaniosis clínica (antimoniato de meglumina combinado con alopurinol o miltefosina combinada con alopurinol) han resultado efectivos.

Novena: En el seguimiento del tratamiento de los hurones afectados de leishmaniosis clínica de nuestro trabajo, no se han presentado efectos secundarios o reacciones adversas con los protocolos terapéuticos utilizados, con la excepción de la aparición de xantinuria en uno de los pacientes.

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