Universitat Autònoma de Barcelona

Clinical and epidemiological aspects of feline and canine leishmaniosis in Venezuela

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“Clinical and epidemiological aspects of feline and canine leishmaniosis in Venezuela”

“Aspectos clínicos y epidemiológicos de la leishmaniosis canina y felina en Venezuela”

TESIS DOCTORAL

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

INFORMA:

Que el treball de tesi doctoral titulat

"Clinical and epidemiological aspects of feline and canine leishmaniosis in Venezuela"

del que és autora la llicenciada en veterinària

Aruanai Kalú Rivas Estanga

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

I per què així consti, signo el present informe a Bellaterra, 25 de setembre de 2018.

Dra. Laia Maria Solano Gallego — Directora

Aruanai Kalú Rivas Estanga — Doctoranda

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ABREVIACIONES

ACL: American human cutaneous leishmaniosis

A non-template control (NTC)

Confidence interval (CI) deoxyribonucleic acid (DNA)

DAB: diaminobenzidine

ELISA: enzyme-linked immunosorbent assay, ensayo por inmunoabsorción ligado a enzimas

EDTA: ethylenediaminetetraacetic acid

EU: ELISA units

FIV: feline immunodeficiency virus, virus de la inmunodeficiencia felina

FeL: feline leishmaniosis

FeLV: feline leukemia virus, virus de la leucemia felina

Fe-SODe: iron superoxide dismutase

HE: hematoxylin and eosin

HRM: high resolution melting

IHC: immunohistochemistry

IHQ: inmunohistoquímica

IFAT: indirect fluorescent antibody test

IFA: inmunofluorescencia indirecta

ITS1: internal transcribed spacer 1

Kd: kilodalton

LV: Leishmaniosis visceral

LC: Leishmaniosis cutánea

LM: Forma mucocutánea

PBS: phosphate-buffered saline

PCR: polymerase chain reaction, reacción en cadena de polimerasa

RT-PCR: real-time polymerase chain reaction, PCR en tiempo real

qPCR: quantitative polymerase chain reaction

WB: Western blot

RESUMEN

Venezuela es un área endémica de leishmaniosis en humanos, así como también en perros y gatos, lo que constituye un problema de salud pública. La información científica en esta región acerca de la clínica y epidemiología de la enfermedad en perros y gatos es limitada. El objetivo principal de la presente tesis doctoral fue determinar los aspectos clínicos, diagnósticos y epidemiológicos de la infección por *Leishmania* spp. en perros y gatos de Venezuela.

Para ello se desarrollaron tres estudios, el primer estudio tuvo como fin diagnosticar gatos enfermos con lesiones altamente sugestivas de infección por *Leishmania* mediante el uso de técnicas de diagnóstico directo como citología, biopsia e inmunohistoquímica, y otras pruebas tales como, las serológicas y moleculares.

El objetivo específico del segundo estudio fue el de comparar técnicas serológicas para la detección de anticuerpos frente *L. infantum* y *L. braziliensis* en perros de diferentes regiones y el tercer estudio permitió determinar la tasa de infección de *Leishmania* mediante técnicas serológicas y moleculares en perros que viven en zonas endémicas de leishmaniosis humana cutánea y visceral en Venezuela.

En conclusión, esta tesis doctoral demuestra nuevos hallazgos clínicos en la leishmaniosis cutánea felina en Venezuela, el uso práctico de técnicas serológicas como ELISA y Western blot para identificar infección temprana en perro y gato además de discriminar entre especies de *Leishmania*, y estudios moleculares que permitieron

identificar la infección de *L. infantum* en perros así como *L. mexicana* o especies muy relacionadas en gatos con lesiones cutáneas sugestivas de estas infecciones.

SUMMARY

Venezuela is an endemic area for human leishmaniosis, as well as for dogs and cats, which causes a public health problem. The scientific information about the clinic and epidemiological data of the disease is limited for dogs and cats. The main purpose of this doctoral thesis was to determine the clinical, epidemiological and diagnostic aspects of the *Leishmania* infection in dogs and cats from Venezuela. For this reason, three studies were developed.

The first study aimed to diagnose ill cats with highly suggestive lesions of *Leishmania* infections by means of direct diagnostic techniques, such as cytology, biopsy and immunohistochemistry, and other serological and molecular tests.

The second study specifically aimed to compare serological techniques to detect antibodies of *L. infantum* and *L.braziliensis* in dogs from different regions and the third study permitted to determine the *Leishmania* infection rate through molecular and serological techniques in dogs living in endemic areas of cutaneous and visceral human leishmaniosis in Venezuela.

Ultimately, this doctoral thesis demonstrates new clinical findings in cutaneous feline leishmaniosis in Venezuela, the practical use of serological techniques such as ELISA and Western blot to identify early infection in dogs and cats along with discrimination between *Leishmania* species, and molecular studies enabling to identify *L. infantum* infection in dogs and *L. mexicana* or *a* closely related species infection incats suffering from cutaneous lesions compatible with these infections.

RESUM

Veneçuela és una àrea endèmica de leishmaniosi en humans, així com també en gossos i gats, el que constitueix un problema de salut pública. La informació científica en aquesta regió sobre la clínica i epidemiologia de la malaltia en gossos i gats és limitada. L'objectiu principal d'aquesta tesi va ser determinar els aspectes clínics, diagnòstics i epidemiològics de la infecció per *Leishmania* spp. en gossos i gats de Veneçuela.

Per aquest motiu es van desenvolupar tres estudis. El primer estudi tenia com a fi diagnosticar gats malalts amb lesions altament suggestives d'infecció per *Leishmania* mitjançant l'ús de tècniques de diagnòstic directe com citologia, biòpsia i immunohistoquímica, i altres proves com les serològiques i moleculars.

El objectiu específic del segon estudi va ser el de comparar tècniques serològiques per la detecció d'anticossos enfront *L. infantum* i *L. braziliensis* en gossos de diferents regions, i el tercer estudi va permetre determinar la taxa d'infecció de *Leishmania* mitjançant tècniques serològiques i moleculars en gossos que viuen en zones endèmiques de leishmaniosi humana cutània i visceral a Veneçuela.

En conclusió, aquesta tesi doctoral demostra noves troballes clíniques sobre la leishmaniosi cutània felina a Veneçuela, l'ús pràctic de tècniques serològiques com l'ELISA i Western blot per identificar la infecció primerenca en el gos o el gat a més de discriminar entre espècies de *Leishmania*, i estudis moleculars que van permetre identificar la infecció de *L. infantum* en gossos i, i *L. mexicana* o espècies molt relacionades en gats amb lesions cutànies compatibles amb aquestes infeccions.

CAPÍTULO 1

- **INTRODUCCIÓN**

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CAPÍTULO 1

INTRODUCCIÓN

El objetivo principal de esta introducción es realizar una revisión sobre los aspectos clínicos, diagnósticos y epidemiológicos de la leishmaniosis canina y felina debida a la infección por *Leishmania* spp. con especial atención a las especies *Leishmania braziliensis* y *Leishmania infantum* , tanto enperros y como gatos de Venezuela y Sudamérica en base a los estudios ofrecidos por la literatura médica. Además, describir recomendaciones sobre el diagnóstico, tratamiento, monitorización, pronóstico y prevención. A continuación se presentan diferentes preguntas de interés relacionadas con el tema:

1. ¿Qué es leishmaniosis?

La leishmaniosis es una enfermedad protozoaria producida por unas 20 especies diferentes pertenecientes al género *Leishmania.* Estas infecciones se transmiten al ser humano por la picadura de insectos de la familia *Psychodidae*, género *Phlebotomus* en Europa, Medio Oriente, Asia y África, y por el género *Lutzomyia* en América (Oletta et al., 2011). Algunas especies causan una enfermedad zoonótica y otras antropozoonoticas, estando ampliamente distribuidas en todo el mundo (Alvar et al., 2012).

Además, dependiendo del ciclo de transmisión de la infección, diferentes reservorios juegan un papel importante, entre ellos el perro, pequeños roedores, lagomorfos, cánidos y félidos salvajes, e incluso el ser humano (Pace, 2014).

2. ¿Cuáles son las manifestaciones clínicas de leishmaniosis en humanos?

Las distintas formas clínicas que puede presentar la enfermedad son: cutánea (localizada o difusa), mucocutánea y visceral. La leishmaniosis visceral (LV), o kala-azar, es altamente prioritaria debido a que en ausencia de tratamiento es potencialmente fatal (Rosilved, 2008). Los principales signos clínicos de leishmaniosis visceral son: malnutrición, pérdida de peso, esplenomegalia, fiebre crónica, vómito, diarrea y linfadenomegalia (Diro et al., 2015).

La leishmaniosis cutánea (LC) localizada se manifiesta con úlceras dérmicas delimitadas. La forma mucocutánea (LM) comienza con una lesión cutánea ulcerosa y se extiende hacia el tabique nasal, la laringe y la mucosa oral, y las manifestaciones difusas con lesiones nodulares cutáneas que aparecen en la piel y diseminan (De Lima et al., 2010).

3. ¿Cuál es el ciclo de transmisión del parásito?

Para que el ciclo de transmisión se lleve a cabo es condición *sine qua non* que una hembra del vector contenga formas infectantes del parásito (promastigote metacíclico)y se alimente de la sangre de un mamífero (figura 1.1), depositando así al promastigote en la dermis del hospedador (Desjeux, 2004). Una vez que el promastigote se encuentra dentro del hospedador mamífero, múltiples células del sistema inmunitario, principalmente macrófagos y neutrófilos migrarán hacia el foco inflamatorio fagocitando a los parásitos presentes. Los parásitos se transformarán en amastigotes en el interior de las células inflamatorias (Moradin and Descoteaux, 2012).

En el interior del macrófago, se formará una vacuola parasitófora, con la finalidad de eliminar al parásito mediante la síntesis y liberación de diversas moléculas con capacidad leishmanicida como el óxido nítrico (NO) entre otras (Zafra et al., 2008). En el caso de que la respuesta fagocítica no sea efectiva, el parásito pondrá en marcha diversos mecanismos de evasión (Alvar et al., 2012) .

La ruptura de los macrófagos infectados libera amastigotes, quienes son fagocitados por nuevos macrófagos, de esta forma se propaga la infección. Los amastigotes ingeridos por nuevos insectos que chupan sangre de un hospedero infectado, se transforman en promastigotes en el tracto digestivo del insecto vector, donde permanecen de cuatro a siete días, se diferencian a infectivos, migran hacia la válvula cardíaca y bloquean la proboscis del insecto (Oletta et al., 2011).

Otros mecanismos de transmisión que todavía no se han demostrado incluyen la transmisión a través de otros vectores como pulgas y garrapatas (Dantas-Torres, 2011; Morais et al., 2013).

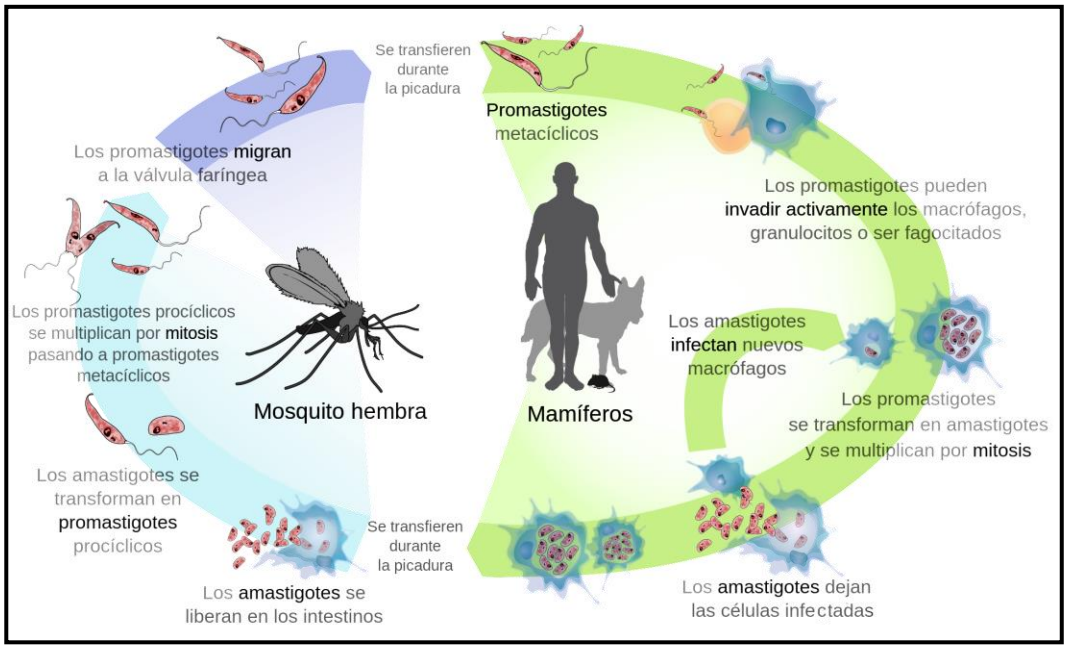

Figura 1.1 Ciclo de transmisión del parásito (Oletta et al., 2011).

4. ¿Existen mecanismos de transmisión no vectorial?

Otros modos de transmisión no vectorial demostrados incluyen la infección a través de transfusiones de sangre procedentes de donantes infectados (De Freitas et al., 2006), la transmisión vertical (da Silva et al., 2009; Rosypal et al., 2005) y venérea (Silva et al., 2009). Se ha detectado también la presencia de ADN de *Leishmania* en el semen, epidídimo, glande y prepucio de perros enfermos infectados por vía natural (Diniz et al., 2005), la transmisión directa de perro a perro (Daval et al., 2016) y por mordeduras y heridas en regiones no endémicas donde el vector no está presente (Shaw et al., 2009).

5. ¿Cuál es el rol epidemiológico del perro y gato en la transmisión de la leishmaniosis humana?

El perro juega un papel fundamental en la epidemiología de la infección por *Leishmania infantum*, debido a que es el principal reservorio peridoméstico para el hombre (Gramiccia and Gradoni, 2005).

Para *Leishmania braziliensis*, el perro es un huésped incidental y su papel en el ciclo zoonótico de transmisión no es significativo, excepto en los focos de transmisión peridomésticos y domésticos, donde no solo los perros sino también los animales domésticos en general tienen un papel en la epidemiología de la leishmaniosis tegumentaria americana (Dantas-Torres, 2007).

La leishmaniosis se considera poco frecuente en los gatos, pero se han reportado varios casos en todo el mundo en los últimos años (Mattos et al., 2005). No obstante, la susceptibilidad real de los gatos a la infección por *Leishmania* spp. y el resultado de la leishmaniosis en estos animales es poco conocido y su papel como reservorios aún no está claro (Rougeron et al., 2011).

Sin embargo, se han informado casos de enfermedad clínica sistémica e infección subclínica debido a *L. infantum* y otras especies (Shaw et al., 2001). En los gatos, la enfermedad y la infección pueden persistir durante periodos muy largos, por lo tanto, pueden desempeñar algún papel en la transmisión de *L. infantum* en regiones donde muchos gatos están infectados (Maia and Campino, 2011).

De acuerdo con el conocimiento actual, algunos autores sostienen que los gatos pueden desempeñar un papel como reservorio adicional de *L. infantum*, por lo que recomiendantomadar medidas preventivas (Pennisi and Persichetti, 2018). Sin embargo, no es compatible con la infección persistente en un entorno natural. Si el reservorio primario está ausente, el gato solo no es responsable de la infección persistente (Maia and Campino, 2011; Pennisi et al., 2015). El rol epidemiológico del gato en el mantenimiento de la infección por *L. infantum* debería ser investigado (Maia and Campino, 2011).

6. ¿Cuáles son los aspectos inmunológicos relevantes en el perro y en el gato?

En el perro, se ha comprobado que las principales citocinas sintetizadas por los linfocitos con inmunofenotipo CD4+ Th1 se asocian con la producción de IL-2, IFN-γ y TNF-α, por el contrario, en los linfocitos con inmunofenotipo CD4+ Th2, las citocinas más representativas son IL-4, IL-5, IL-10 e IL-13. En aquellos perros con una respuesta inmunitaria protectora frente al parásito predominará un inmunofenotipo CD4+ Th1, promoviendo un ambiente inmunológico donde las citocinas IFN-γ, IL-2 y TNF-α son más abundantes, estas citocinas en la activan la acción macrofágica frente al parásito.

Sin embargo, los perros enfermos se caracterizarán por un perfil mixto Th1/Th2 donde predomina una exagerada respuesta humoral y una reducción de la respuesta celular, en las que el control de la replicación del parásito, la progresión de la enfermedad o la recuperación están determinadas por el equilibrio entre estas dos direcciones dicotómicas (Baneth et al., 2017).

La activación de linfocitos T reguladores va a inducir la secreción de diferentes tipos de citocinas como la IL-10, limitando los efectos perjudiciales y lesiones asociados a

una respuesta mediada por los linfocitos CD4+ Th1 (respuesta de tipo celular), permitiendo de este modo la persistencia de la infección en el perro y por tanto, la existencia de animales infectados aparentemente sanos (Day, 2011).

Con respecto a los gatos, la respuesta inmunitaria, es principalmente de tipo celular, siendo lo suficientemente efectiva como para controlar la infección y conferir un cierto grado de resistencia natural, si no hay eventos inmunosupresores tales como enfermedad viral, bacteriana, rickettsial, fúngica o protozoaria (Solano-Gallego et al., 2007). Sin embargo, existen pocos estudios sobre los aspectos inmunológicos de la leishmaniosis felina (Pennisi and Persichetti, 2018).

7. ¿Qué importancia tiene la leishmaniosis animal desde el punto de vista de salud pública?

Situación en las Américas (Oletta et al., 2011):

- ✓ Las leishmaniosis son enfermedades de impacto en la Región.
- ✓ La incidencia de la LV se han incrementado en los años recientes.
- ✓ Los sistemas de vigilancia son deficientes.
- ✓ Existe una falta de recursos humanos capacitados para actividades de diagnóstico, tratamiento y medidas de control.
- ✓ Falta de medicamentos para el tratamiento en forma oportuna.

En las Américas se observó un aumento del 5% de los casos debido al mayor registro de la enfermedad en Colombia, Perú y Nicaragua, mientras que en Brasil, se presentó una reducción de cerca de 34% de los casos de leishmaniosis cutánea. En

particular, en Nicaragua se registró la mayor tasa de incidencia de leishmaniosis en la región representando un incremento del 157%. A partir del año 2014, los datos de LV muestran una discreta reducción de casos en la región (Organización Panamericana de la Salud., 2018).

Existe un aumento del 32% en el porcentaje de casos de coinfección de LV y el virus de inmunodeficiencia humana (VIH) comparado con el año anterior. Este incremento fue observado principalmente en Paraguay, el cual pudo estar asociado a un mejor diagnóstico dado a la disponibilidad de pruebas rápidas para la detección temprana del VIH en personas con LV.

Además, se observó una mejora en la proporción de casos de LV diagnosticados por el criterio de laboratorio y la información de mejora clínica. La letalidad por LV en la región sigue siendo un reto debido al progresivo aumento observado desde 2014, llegando en este último año a su mayor tasa ya registrada (Organización Panamericana de la Salud., 2018) .

8. ¿Cómo están distribuidas geográficamente las áreas endémicas de leishmaniosis en personas, perros y gatos en Sudamérica?

En humanos, la leishmaniosis se distribuye en Argentina, Belice, Bolivia, Brasil, Colombia, Ecuador, Costa Rica, Guatemala, Honduras, Nicaragua, Panamá, Paraguay, Perú, y Venezuela (Grimaldi and Tesh, 1993)(figura 2).

La LV sigue presentando una amplia distribución geográfica de casos humanos en Brasil, donde se destaca las regiones Noreste, Sudeste y Centro-Oeste. Asimismo, la

dispersión geográfica sigue ocurriendo en Paraguay y Argentina, fronteras con Brasil y Uruguay. En 2016, también se pudo observar esa dispersión en Roraima, Norte del Brasil, donde fueron registrados casos en las áreas de frontera con Venezuela, lo que requiere una mayor atención y fortalecimiento de la vigilancia en los municipios de esos dos países (Organización Panamericana de la Salud., 2018).

Leishmania braziliensis y L. infantum son las especies más extendidas que infectan perros en América del Sur y su distribución es probablemente más amplia de lo que realmente se concibe, presentándose casos en los países endémicos a leishmaniosis en humanos (Dantas-Torres, 2009) (Tabla 1.1).

En gatos, han sido reportados casos en Guayana francesa (Rougeron et al., 2011), Venezuela (Bonfante-Garrido et al., 1991), Brasil y Paraguay (Oliveira et al., 2015; Soares et al., 2015).

Figura 1.2. Áreas endémicas de leishmaniosis en Sudamérica. Modificado de (Dantas-Torres, 2009)

Tabla 1.1 Especies de *Leishmania* que infectan perros y gatos en Sudamérica (Dantas-Torres, 2009; Pennisi et al., 2015)

Especies ***Leishmania***	**Presentación clínica perros**	**Presentación clínica gatos**	**Distribución geográfica**
L. amazonensis	Visceral	Cutánea	Brasil
L. braziliensis	Cutánea	Cutánea	Argentina, Bolivia,** Brasil, Colombia, Perú, Venezuela, *. Guayana Francesa
L. colombiensis	Visceral	-	Argentina, Bolivia, Brasil, Colombia, Venezuela
L. infantum	Visceral, mucocutánea, cutánea	Cutánea, visceral	Argentina, Bolivia, ^** Brasil, Colombia, Guayana Francesa, Venezuela, *Paraguay
L. mexicana	Cutánea	-	Ecuador
L. panamensis	Cutánea	-	Colombia, Ecuador
L. peruviana	Cutánea	-	Perú
L. pifanoi	Cutánea	-	Ecuador
L. venezuelensis	-	Cutánea	Venezuela

*Área endémica de leishmaniosis en gatos, ** Área endémica de leishmaniosis en perros y gatos.

9. ¿Cómo están distribuidas geográficamente las áreas endémicas de leishmaniosis humana en Venezuela?

En Venezuela la tasa de incidencia de casos de LV anual ha oscilado desde 0,08/100.000 habitantes en el año 1990 a 0,22/100.000 habitantes durante el año 2005. Según la tasa de incidencia anual en el país para el año 2005, el estado Nueva Esparta presentó la tasa más alta: 5,65/100.000 habitantes, seguida por 0,72 casos/100.000 habitantes en los estados Guárico y Anzoátegui y 0,70 casos/100.000 habitantes en el estado Lara (Rosilved, 2008) (Figura 1.3).

Figura 1.3. Distribución geográfica de la leishmaniosis visceral humana en Venezuela. Modificado de (Rosilved, 2008).

Han sido reportados casos de leishmaniosis cutánea americana en todas las entidades federales, el cálculo de las tasas por estado permite dividir al país en cuatro grupos o regiones de riesgo de acuerdo con la tasa de incidencia promedio del periodo de 20 años en estudio. Un primer grupo conformado por los estados Trujillo y Mérida con tasas superiores a 30 por 100.000 habitantes, un segundo grupo de estados (Lara, Sucre, Táchira, Cojedes) con tasas de 20 o más pero menores de 30, en tercer lugar los estados Yaracuy, Miranda y Anzoátegui con tasas que son de 10 o más y menores de 20, y finalmente el resto de los estados (quince en total, incluido el Distrito Capital) con tasas inferiores a 10 por 100.000 habitantes (De Lima et al., 2010), (Figura 1.4).

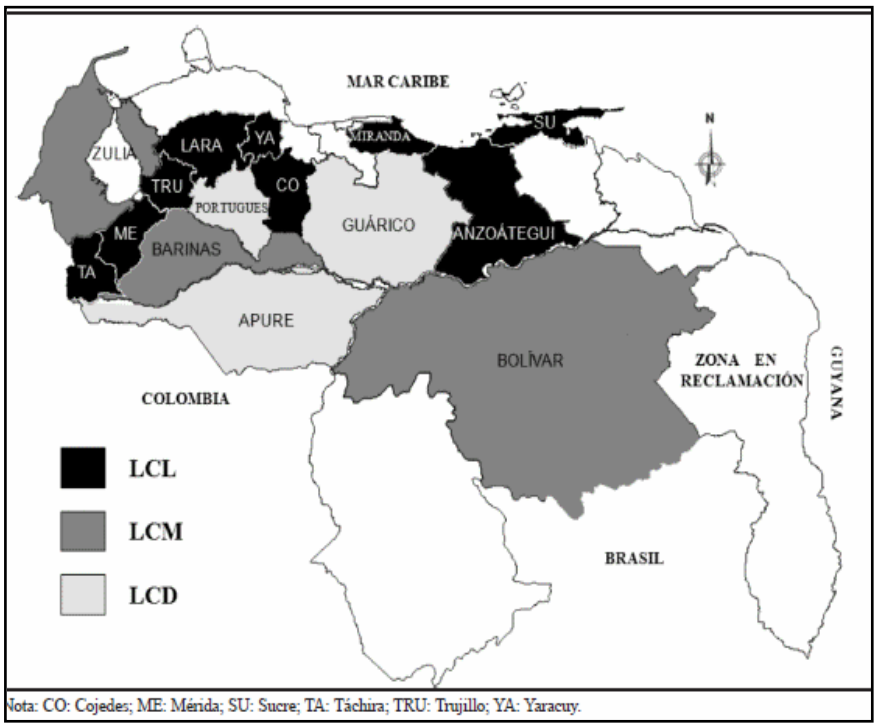

Figura 1.4. Tasa de incidencia de leishmaniosis cutánea según entidad federal, Venezuela (De Lima et al., 2010), LCL: leishmaniosis cutánea localizada, LCM: Leishmaniosis mucocutánea, LCD: leishmaniosis cutánea difusa.

10. ¿Cuáles son las manifestaciones clínicas y diagnósticos diferenciales de leishmaniosis en perros y gatos?

Los signos clínicos causados por *L. infantum* en perros son principalmente dermatológicos tales como: dermatitis exfoliativa, alopecia focal-multifocal no pruriginosa, dermatitis erosivo-ulcerativa, onicogrifosis, paroniquia, dermatitis pustular estéril (Koutinas et al., 1992) y dermatitis papular (Ordeix et al., 2005).

Se pueden presentar signos inespecíficos pero de evolución progresiva en el tiempo, siendo los más frecuentemente detectados por los propietarios tales como debilidad generalizada, problemas dermatológicos, pérdida de peso y anorexia, así como intolerancia al ejercicio. En el examen clínico, es posible detectar linfadenomegalia localizada o generalizada, esplenomegalia, manifestaciones oculares, atrofia muscular, que suele afectar con mayor frecuencia a los músculos masticadores (maseteros), poliartritis, falla renal crónica y/o estomatitis ulcerativa (Koutinas et al., 1999).

En el caso del gato, los signos clínicos más comunes por *L. infantum* incluyen lesiones cutáneas o mucocutáneas, nódulos de tamaño variable que pueden estar ulcerados, localizados en cabeza, párpado, nariz, labio, zona distal de los miembros o mucosa anal, sin dolor o prurito. La infección en gatos puede ser promovida por procesos inmunosupresores como el virus de la inmunodeficiencia felina (FIV) o el virus de la leucemia felina (FeLV) (Sobrinho et al., 2012). En algunos casos hay alteraciones sistémicas pero la mayoría está relacionada a infecciones concomitantes como FIV y FeLV (Pennisi et al., 2015)

Los signos clínicos de leishmaniosis causada por *L. braziliensis* en perros y gatos incluyen lesiones cutáneas que comienzan con la formación de un nódulo en el lugar donde ocurrió la picadura del mosquito. Este nódulo aumenta de tamaño y en ocasiones presenta ulceración, así como también pueden observarse placas. Las lesiones generalmente se presentan en la nariz, orejas y la región perineal que son las regiones del cuerpo donde los animales tienen menos pelo, en estos casos no hay evidencia de alteraciones sistémicas (Mattos et al., 2005; Shaw et al., 2001; Velez et al., 2012)

Diagnóstico diferencial en el perro:

- Dermatitis exfoliativa: foliculitis bacteriana, dermatofitosis, demodicosis, seborrea, linfoma epiteliotropo, adenitis sebácea (Koutinas et al., 1992).
- Ulceración: dermatitis acral, granuloma infeccioso, úlcera por decúbito, neoplasia cutánea, pénfigo vulgaris (Koutinas et al., 1992), esporotricosis (dos Santos et al., 2007).
- Nódulos: neoplasia cutánea, granuloma micobacterial, piogranuloma, granuloma estéril (Koutinas et al., 1992; Santoro et al., 2008)

Diagnóstico diferencial en el gato:

- Forma nodular: criptococosis, esporotricosis, histoplasmosis, granuloma estéril/eosinofílico, micobacteriosis, neoplasia cutánea (Pennisi et al., 2015).

- Forma ulcerativa: neoplasia cutánea, úlcera indolente, hipersensibilidad a la picadura de mosquito, lepra felina, vasculitis cutánea, dermatitis ulcerativa idiopática (Pennisi et al., 2015).

10. ¿Existe predisposición de especie, raza, sexo o edad?

Se han realizado estudios en perros con infección por *L. infantum* en los cuales se evidencia predisposición racial para presentar enfermedad tales como Rottweiller, Pastor Alemán, Boxer y Cocker spaniel (França-silva et al., 2003).

Por otro lado, en relación al sexo, en la mayoría de los estudios epidemiológicos no se observan diferencias, y, en relación a la edad de los animales, se sigue una distribución bimodal, de forma que es posible agrupar la mayor parte de los casos en perros con una edad inferior a los 3 años. Además, entre los 8-10 años de edad, se encuentra un segundo pico de casos clínicos (Miranda et al., 2008).

Las evidencias científicas que evalúen estos factores en el gato son limitadas, así como también con otras especies de *Leishmania* (Pennisi et al., 2015). Estudios previos, no han encontrado una asociación clara entre la infección por *L. infantum* en gatos y otros factores como la edad, el género, la raza y las condiciones de vida(Cardoso et al., 2010; Dedola et al., 2018).

11. ¿Cuáles son los hallazgos clínico-patológicos en el perro y en el gato?

Existen diferentes grados de severidad de la enfermedad causada por *L. infantum*, los perros con leishmaniosis clínica pueden ser clasificados en cuatro estadios diferentes

(enfermedad leve, enfermedad moderada, enfermedad severa y finalmente enfermedad muy severa) en función de los signos clínicos presentes, alteraciones de laboratorios detectadas y nivel de anticuerpos anti-*Leishmania* (tabla 2). El pronóstico del animal dependerá del estadio en que se encuentra en el momento de su evaluación y clasificación. Además, el tipo de tratamiento también diferirá dependiendo del estadio de enfermedad (Solano-Gallego et al., 2009), (Tabla 1.2).

En la infección por *L. braziliensis* en el perro no se ha demostrado que existan hallazgos clínico-patológicos relevantes (Carvalho et al., 2015; Figueredo et al., 2012).

Se dispone de información limitada sobre anomalías clínico-patológicas en gatos con infección por *L. infantum*, solo se basa en informes de casos. La anemia normocítica normocromica moderada a grave no regenerativa es la anomalía hematológica más frecuente notificada en casos clínicos (Pennisi et al., 2015). Desafortunadamente, no hay disponible estudios donde demuestren las anomalías clínico-patológicas en gatos con *L. braziliensis.*

Tabla 1.2. Estadio clínico de la leishmaniosis canina por *L.infantum*, basada en la serología, signos clínicos y hallazgos de laboratorio (Solano-Gallego et al., 2009).

Estadio clínico	Serología	Signos clínicos	Hallazgos de laboratorio
I **Enfermedad leve**	Negativo o positivo bajo	Linfadenomegalia periférica y/o dermatitis papular	No presenta anormalidades clínico-patológicas.
II **Enfermedad moderada**	Positivo bajo o positivo alto	Signos del estadio I Dermatitis ulcerativa, onicogrifosis, ulceración, anorexia, pérdida de peso, fiebre y epistaxis.	Anemia no regenerativa, hipergammaglobulinemia, hipoalbuminemia , de perfil renal normal a leve proteinuria
III **Enfermedad severa**	Positivo medio o positivo alto	Signos del estadio I – II Vasculitis, artritis, uveítis, glomerulonefritis	Anormalidades del estadio II y enfermedad renal crónica
IV **Enfermedad grave**	Positivo medio o positivo alto	Signos del estadio III Tromboembolismo pulmonar, síndrome nefrótico y enfermedad renal	Anormalidades del estadio II-III, síndrome nefrótico, marcada proteinuria

12. ¿Cuáles son los métodos para el diagnóstico de la infección por *Leishmania*?

Dentro del amplio abanico de pruebas diagnósticas disponibles para confirmar la infección, éstas pueden ser clasificadas en: pruebas basadas en el diagnóstico

parasitológico, pruebas basadas en el diagnóstico serológico y aquellas relacionadas con el diagnóstico molecular (Solano-Gallego et al., 2011), (figura1. 5).

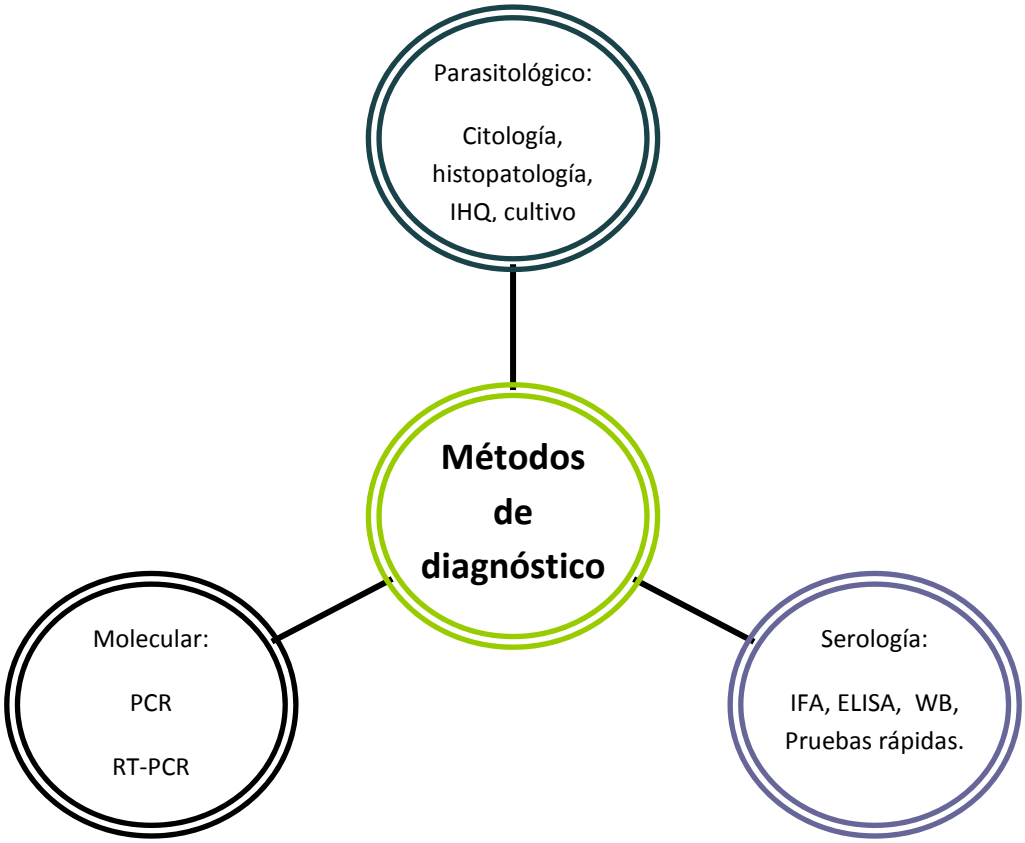

Figura 1.5.Métodos de diagnóstico más frecuentes utilizados en la leishmaniosis canina. (Solano-Gallego et al., 2011). IFA: inmunofluorescencia indirecta, ELISA: ensayo por inmunoabsorción ligado a enzimas, WB: Western blot, PCR: reacción en cadena de polimerasa, RT-PCR: PCR en tiempo real, IHQ: inmunohistoquímica.

13. ¿Cuáles son las pruebas para el diagnóstico parasitológico?

Para la confirmación de la infección por *Leishmania s*e puede realizar la visualización de parásitos por microscopía en un frotis de tejido, como aspirado esplénico, médula ósea o biopsia hepática para LV, y raspados, líquido o biopsia de lesiones cutáneas en el caso de LC (Stockdale and Newton, 2013). Un resultado positivo mediante la detección directa del parásito en un animal sospechoso confirma el diagnóstico de leishmaniosis canina, no obstante, un resultado negativo no permite descartar la enfermedad. Por otro lado, la sensibilidad de esta técnica disminuye considerablemente en perros sin signos clínicos de enfermedad (Solano-Gallego et al., 2011).

Otra prueba de diagnóstico parasitológico es el estudio histopatológico. Estudios previos sobre la infección por *L. infantum* en caninos describen que la piel es un tejido importante donde pueden encontrarse parásitos en perros sanos y enfermos infectados por *Leishmania.* La imagen histopatológica de tales lesiones cutáneas comúnmente consiste en una reacción inflamatoria granulomatosa difusa con números variables de células plasmáticas y parásitos en la dermis (Ordeix et al., 2005; Solano-Gallego et al., 2004).

En ocasiones, el patrón histológico observado se corresponde con el producido por *Leishmania*; sin embargo, en los casos que no es posible detectar ningún amastigote en las preparaciones, la combinación del estudio histopatológico junto con técnicas de inmunohistoquímica permiten incrementar la sensibilidad del diagnóstico (Solano-Gallego et al., 2004).

Los métodos inmunohistoquímicos son una herramienta poderosa para caracterizar las respuestas del huésped a la infección. Éstos también se han usado en otras enfermedades granulomatosas que comparten algunas, aunque no todas, las propiedades con LV, además, estas técnicas pueden utilizarse para evaluar el mecanismo celular de nuevas vacunas, medicamentos y regímenes de tratamiento (Salguero et al., 2018).

14. ¿Qué técnicas serológicas se pueden emplear para el diagnóstico?

Las técnicas serológicas cuantitativas permiten la cuantificación de los niveles de anticuerpos anti-*Leishmania* circulantes. En el caso de la técnica de inmunofluorescencia indirecta (IFI), ésta permite dar un título de anticuerpos, aunque su sensibilidad puede disminuir considerablemente en perros infectados subclínicos (Solano-Gallego et al., 2014).

La técnica de ensayo por inmunoabsorción ligado a enzimas (ELISA), trabaja con densidades ópticas, de forma que puede ser utilizada como referencia para la clasificación de los niveles de anticuerpos anti-*Leishmania.* Generalmente, en aquellos perros en los que se detecte altos niveles de anticuerpos anti-*Leishmania* (considerado un valor 3-4 veces superior al punto de corte de la prueba serológica cuantitativa), este resultado será concluyente para confirmar el diagnóstico de leishmaniosis canina (Solano-Gallego et al., 2009).

En el gato, las técnicas de detección de anticuerpos específico más usadas son ELISA e IFAT. En general, los anticuerpos anti-*leishmania* deberían ser siempre evaluados por laboratorios usando métodos serológicos validados para gatos (Pennisi et al., 2015).

Además, se ha demostrado que la técnica Western blot tiene ventajas sobre otras pruebas serológicas por su capacidad de detectar infecciones tempranas o subclínicas tanto en perros como en gatos (Aisa et al., 1998; Persichetti et al., 2017; Trevisan et al., 2015).

15. ¿Cuál es la utilidad de la PCR?

Esta técnica presenta una buena sensibilidad y especificidad (Rennó et al., 2014), es capaz de detectar la infección antes que tenga lugar la seroconversión y, por tanto, la detección de los anticuerpos anti-*Leishmania* mediante las técnicas serológicas (Oliva et al., 2006). Asimismo, también permite determinar la especie de *Leishmania* involucrada (Carvalho Ferreira et al., 2014; Paulo, 2015).

La detección del ADN puede basarse del ADN procedente del kinetoplasto o bien del propio ADN genómico de *Leishmania*, aunque la detección del ADN del kinetoplasto puede resultar en un incremento de la sensibilidad de dicha prueba cuando se compara con la detección del ADN genómico (Solano-Gallego et al., 2009).

16. ¿Qué tipo de muestras se pueden utilizar para el diagnóstico molecular?

La sensibilidad de la técnica varía dependiendo del tipo de muestra analizada, siendo los tejidos más sensibles y específicos para la detección de ADN del parásito la

médula ósea, el bazo y la piel. Por el contrario, la extracción de ADN procedente de la sangre da como resultado una menor sensibilidad de la prueba (Rennó et al., 2014)

17. ¿Cuál es el tratamiento de la leishmaniosis canina y felina?

El tratamiento más comúnmente utilizado en perros enfermos con *L. infantum* se basa en la combinación de dos fármacos tales como: opción 1: el antimoniato de meglumina a la dosis de 75-100 mg/kg cada 24 horas vía subcutánea por 4-8 semanas + alopurinol 10 mg/kg/ cada 12 horas vía oral por 6-12 meses; u opción 2: miltefosine 2mg/kg vía oral, cada 24 horas por 4 semanas + alopurinol a la dosis anteriormente mencionada. Estos fármacos pueden administrarse durante un tiempo más prolongado de acuerdo al caso (Solano-Gallego et al., 2009).

Otras alternativas terapéuticas son las siguientes: anfotericina B a la dosis de 0.5-0.8 mg/kg, vía intravenosa, cada 24 horas, dos veces por semana por dos meses, metronidazol 25mg/kg cada 24 horas + espiramicina 150000 Unidades cada 24 horas, vía oral por tres meses, marbofloxacina 2 mg/kg por vía oral cada 24 horas (Solano-Gallego et al., 2009).

En los gatos con infección por *L. infantum*, la información disponible sobre el tratamiento se basa principalmente en informes de casos únicos, no siempre con un seguimiento adecuado. La administración a largo plazo de alopurinol (10-20 mg/kg cada 12 horas o cada 24 horas) suele ser clínicamente efectiva, incluso en gatos FIV-positivos. Sin embargo, la infección no desaparece y los signos clínicos pueden reaparecer después de suspender el tratamiento (Pennisi et al., 2013).

Puede realizarse combinación de fármacos similar al perro con una dosis de antimoniato de meglumina de 50 mg/kg una vez al día, vía subcutánea, por 30 días + alopurinol 10 mg/kg cada 12 horas por vía oral a largo plazo (Basso et al., 2016).

No existe suficiente evidencia científica con respecto al tratamiento de la infección por *L. braziliensis* en el perro (Velez et al., 2012) y en el gato (Pennisi and Persichetti, 2018).

18. ¿Cómo se realiza el seguimiento durante el tratamiento?

Los parámetros clínico-patológicos a seguir durante el tratamiento dependerán de las anormalidades individuales. Sin embargo, generalmente se recomienda realizar hemograma completo, perfil bioquímico y urianálisis, incluida la proporción proteína/creatinina en orina en perros proteinúricos. La frecuencia del seguimiento de los parámetros clinicopatológicos es diferente en cada paciente, pero los parámetros clinicopatológicos deberían, en la mayoría de los casos, ser seguidos con mayor frecuencia inicialmente, es decir, después del primer mes de tratamiento y luego cada 3-4 meses. Más adelante, si el perro o gato se recupera completamente clínicamente con tratamiento, se recomienda una nueva revisión cada 6 meses o una vez al año (Pennisi et al., 2013; Solano-Gallego et al., 2009).

20. ¿Cuáles son las medidas de prevención de la leishmaniosis en el perro y en el gato?

Control vectorial:

- Mantener los animales en el interior durante la temporada del vector, desde el atardecer hasta el amanecer.

- Reducir los microhábitats favorables a los mosquitos en las proximidades de la casa o en lugares donde el perro o gato pasa tiempo.

- Uso del tratamiento insecticida ambiental.

- Uso de insecticidas tópicos.

Los principales compuestos de eficacia comprobada para su utilización tópica en el perro son los piretroides sintéticos, dada su efectividad y baja toxicidad para el perro se distribuyen a través del estrato córneo de la epidermis. La forma de presentación puede ser tanto en forma de collar, en el cual el principio activo se libera lentamente, como en pipeta y pulverizador (Solano-Gallego et al., 2009).

En cuanto a la leishmaniosis felina, se ha realizado un primer estudio en el cual se evaluó una estrategia preventiva contra la infección felina por *Leishmania*, y se demostró que el collar, cuyo principio activo es 10% de imidacloprid + 4.5% de flumetrina, redujo significativamente el riesgo de infección por *L. infantum* en gatos. Estos hallazgos cierran un vacío en la medicina veterinaria, ya que confirman este collar como una herramienta, reduciendo el riesgo de infección por *Leishmania* en gatos y podría contribuir a la reducción del riesgo de la enfermedad en animales y en poblaciones humanas cuando se incluye en programas integrados de control de leishmaniosis (Brianti et al., 2017)

Inmunomoduladores:

Se ha registrado una molécula inmunomoduladora, la domperidona, demostrándose que es capaz de activar a las poblaciones celulares fagocíticas que constituyen la respuesta innata, favoreciendo la respuesta Th1. La domperidona, siendo utilizada como medida preventiva, disminuye el riesgo de desarrollo de leishmaniosis clínica.

También es utilizada para prevenir el progreso clínico de la enfermedad combinada con los fármacos anti-*Leishmania*, aunque su eficacia es controversial debido a que depende de la capacidad del sistema inmune del animal para responder a la infección y cambiar el curso de la enfermedad. Además, se ha demostrado una eficacia limitada cuando la carga parasitaria es alta (Miró et al., 2017).

Inmunoprofilaxis:

Un estudio realizado en una zona altamente endémica a *L. infantum* en Brasil evaluó la eficacia de la vacuna comercial Leish-Tec®. Leish-Tec® es una vacuna basada en un antígeno recombinante de amastigote de *Leishmania*.

Este estudio demostró que la vacunación realizada en tres dosis vía subcutánea a intervalo de 21 días, seguida de una revacunación anual, era bien tolerada por perros. La eficacia de la vacuna incluye una protección clínica satisfactoria pero no tiene impacto en la disminución de la incidencia de la infección (Grimaldi et al., 2017).

Otro estudio realizado para evaluar la eficacia de dos collares con diferentes principios activos:uno con 10% imidacloprid + 4.5% flumetrina (seresto) y el otro 4% de

deltametrina (scalibor); para el tratamiento y la prevención de infestaciones por pulgas, además de evaluar el efecto de estos collares sobre la incidencia de la infección por *Leishmania infantum* en comparación con un grupo de perros vacunados con la vacuna comercial Canileish®, basada en un antígeno de promastigote excretado por *L. infantum*.

Esta investigación obtuvo como resultado que ambos collares proveían una protección efectiva, pero no se encontraron diferencias significativas en la frecuencia de infección entre perros vacunados con Canileish® y aquéllos protegidos con collares (Brianti et al., 2016).

Eutanasia:

La eutanasia de perros seropositivos es una práctica de obligado cumplimiento en Brasil y, en Latinoamérica, se realiza con el fin de controlar el reservorio (Romero and Boelaert, 2010) pero se ha demostrado que es una estrategia infructuosa para interrumpir la transmisión de la enfermedad (Miró et al., 2017).

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OBJETIVOS

CAPÍTULO 2

OBJETIVOS

Venezuela es un área endémica de leishmaniosis en humanos causada por diferentes especies, así como también en perros y gatos, lo que constituye un problema de salud pública. La información científica en esta región acerca de la clínica y epidemiología de estas infecciones en perros y gatos es limitada, así como también el desarrollo de técnicas para el diagnóstico que además permitan discriminar entre especies de *Leishmania,* la identificación de infecciones subclínicas, e interpretación y análisis de los signos clínicos.

El desarrollo de esta tesis doctoral responde a un conocimiento sólido de los contextos en los problemas actuales de salud pública en Venezuela, obtenidos a través de la interacción con la comunidad afectada. Esta tesis doctoral proporciona un avance en la clínica y epidemiología de la leishmaniosis canina y felina, además de extender la investigación clínica vinculada a la realidad práctica.

El objetivo principal de este estudio fue determinar los aspectos clínicos, diagnósticos y epidemiológicos de la infección por *Leishmania* spp. en perros y gatos de Venezuela.

Los objetivos específicos fueron los siguientes:

1. Describir y diagnosticar gatos enfermos de Venezuela con lesiones altamente sugestivas de infección por *Leishmania*.

2. Determinar la tasa de infección de *Leishmania* mediante técnicas serológicas y moleculares en gatos y perros que viven en zonas endémicas de leishmaniosis humana cutánea y visceral en Venezuela.
3. Puesta a punto y comparación de técnicas serológicas para la detección de anticuerpos frente a antígenos de *L. infantum* y *L. braziliensis* en perros y gatos.

CAPÍTULO 3

❖ **ESTUDIO 1**

Clinical and diagnostic aspects of feline cutaneous leishmaniosis in Venezuela

CAPÍTULO 3

Clinical and diagnostic aspects of feline cutaneous leishmaniosis in Venezuela

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ABSTRACT

Venezuela is an endemic area for human and canine leishmaniosis due to *Leishmania infantum* and parasites of the *Leishmania braziliensis* and *L. mexicana* complexes. Limited data are available on feline leishmaniosis (FeL) in this region. The aim of this study was to describe clinical and diagnostic aspects of FeL in Venezuela. Thirty-one domestic cats from urban areas of Lara State in Venezuela were enrolled. Twenty-five were healthy. Six other cats had solitary or multiple nodular lesions, which were located on the nose; ears; ears and nose; and nose, ears, tail and lower limbs. Cutaneous lesions were characterized by diffuse pyogranulomatous infiltrate in all sick cats with numerous intracellular and extracellular amastigotes, and immunohistochemistry was positive for *Leishmania* in five sick cats. All healthy cats were seronegative for *L. infantum* and *L. braziliensis* antigens by ELISA. Two out of five sick cats yielded a positive ELISA result to both *Leishmania* antigens with higher antibody levels to *L. braziliensis* compared to *L. infantum*. Significantly higher antibody levels by ELISA as well as a higher number of bands by Western blot (WB) were found for *L. braziliensis* when compared to *L. infantum* antigens in all sera from Venezuelan sick and healthy cats. All healthy cats were blood *Leishmania* spp. qPCR negative while three out of six sick cats were blood qPCR positive. All paraffin-embedded skin biopsies (n = 4) as well as cutaneous cytology (n = 3) were positive by *Leishmania* spp. qPCR in sick cats. *Leishmania* speciation was obtained only from the cutaneous lesion samples from cytological preparations of two out of three sick cats which were identified as infected with *L. mexicana* or a closely related specie.

FeL should be included in the differential diagnosis list of nodular-ulcerative lesions. The most reliable diagnostic technique in sick cats is cytological or histopathological examination along with immunohistochemistry, since blood PCR and serology by ELISA might be negative. WB appears to be more sensitive in detecting infection. Cats with leishmaniosis from Venezuela are most likely infected with species of *L. mexicana* or a closely related species or the *L. braziliensis* species complex and not with *L. infantum*.

Keywords: *Leishmania mexicana*, *Leishmania infantum*, *Leishmania braziliensis*, Cats, Nodular-ulcerative lesions, ELISA, qPCR, Immunohistochemistry, Venezuela

1. INTRODUCTION

Leishmaniosis is a parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) (Solano-Gallego and Baneth, 2006). In humans, it is manifested clinically in multiple forms including the cutaneous, mucosal and visceral leishmaniosis (Bailey and Lockwood, 2007). The cutaneous form of leishmaniosis in the Eastern Hemisphere is caused by *Leishmania tropica*, *Leishmania major* and *Leishmania aethiopica*, as well as *Leishmania infantum* and *Leishmania donovani*. The *Leishmania* species found in America (the Western Hemisphere) are either in the subgenus *Leishmania* represented by the *L. mexicana* species complex (*L. mexicana*, *L. amazonensis*, *L. pifanoi*, *L. garnhami*, *L. aristidesi* and *L. venezuelensis*) or in the subgenus *Viannia* represented by the *L. braziliensis* species complex [*L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) panamensis,* and *L. (V.) peruviana*)] (Martins et al., 2014).

Cutaneous lesions are the most common clinical sign of leishmaniosis both in human (Scorza et al., 2017) and canine patients (Saridomichelakis and Koutinas, 2014) for certain *Leishmania* species such as *L. infantum* and the *L. mexicana* and *L. braziliensis* species complexes. Over 200,000 people develop dermal and mucosal leishmaniosis annually in Central and South America (Alvar et al., 2012; Hepburn, 2003). Venezuela is an endemic area of American human cutaneous leishmaniosis (ACL). A wide distribution of ACL has been observed, but the localized clinical form tends to concentrate in states with mountainous geography especially in the Andes (Trujillo, Mérida, Lara and Táchira). It is interesting to note that Lara and Mérida states are characterized by a fairly stable endemic situation that represents almost a third of all national cases (De Lima H et al., 2010). Additionally, cutaneous lesions have been reported in association with *L. venezuelensis*, in the endemic focus of ACL both in humans and domestic animals such as cats in Barquisimeto, Lara State, Venezuela (Bonfante R et al., 1996).

Moreover, description of cutaneous leishmaniosis caused by *L. infantum* is also documented (De Lima et al., 2009). However, *Viannia* is the most relevant subgenus in this territory and is also responsible for metastatic mucosal leishmaniosis, the severe form of tegumentary disease (Blum et al., 2012; Weigle and Saravia, 1996).

Feline leishmaniosis has been described with both visceral and cutaneous forms by demonstration of the presence of the parasite in cats. Several cases in domestic cats have been globally reported, also in America and especially in endemic areas of Central America (Trainor et al., 2010,), South America such as Brazil (Sobrinho et al., 2012; Vides et al., 2011). and Paraguay (Velázquez A et al., 2011) and also in the

Mediterranean basin (Solano-Gallego et al., 2007) and the Middle East (Akhtardanesh et al., 2017).

Nonetheless, the real susceptibility of cats to infection by *Leishmania* spp., their role as reservoir hosts, and the outcome of leishmaniosis in these animals, are poorly understood (Simoes-Mattos et al., 2005). Several *Leishmania* species such as *L. mexicana* (Barnes et al., 1993), *L. venezuelensis* (Bonfante-Garrido et al., 1991), *L. braziliensis* (Passos et al., 1996; Rougeron et al., 2011; Schubach et al., 2004), *L. amazonensis* (de Souza et al., 2005) and *L. infantum* (Coelho et al., 2010; Coelho et al., 2011; da Silva et al., 2008; Savani et al., 2004; Vides et al., 2011) have been identified to infect cats in Central and South America.

The most frequently described lesions in FeL are ulcerocrusting, nodular dermatitis, alopecia and scaling (Pennisi et al., 2004; Rufenacht et al., 2005; Sherry et al., 2011) while the visceral form of the disease involving the spleen, liver, lymph nodes, bone marrow, eye and kidney is less commonly diagnosed (Hervas et al., 2001; Leiva et al., 2005). Although clinical cases of leishmaniosis have been reported in cats with coinfection of feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV), the true association between FeL and retroviral infections remains unclear (Sherry et al., 2011; Solano-Gallego et al., 2011).

The laboratory tests recommended for diagnosis of FeL due to *L. infantum* include demonstration of the presence of the parasite by direct microscopic examination in stained smears, and/or culture, serological tests [indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and Western blot (WB)], and molecular techniques such as the polymerase chain reaction (PCR) (Pennisi et al., 2015). However, limited information is available regarding the diagnosis of other

species of *Leishmania* such as *L. braziliensis* infection in dogs and cats (Trevisan et al., 2015).

Only very limited data are available on FeL in Venezuela, the identification of clinical manifestations associated with *Leishmania* spp. infection in domestic cats in endemic areas as well as the best diagnostic techniques to be employed are crucial topics. Therefore, the aim of this study was to describe clinical and diagnostic aspects of FeL in an endemic area of American cutaneous leishmaniosis, the state of Lara in Venezuela.

1. MATERIAL AND METHODS

2.1 Cats and sampling

Thirty-one outdoor domestic short hair cats from urban areas (Barquisimeto, Cabudare, Quibor) of Venezuela Lara State were enrolled. A full physical examination was performed, and breed, age and gender were recorded before sampling. Twenty-five cats were apparently healthy, and 6 cats presented cutaneous lesions. Blood samples (*n* = 31) were collected into ethylenediaminetetraacetic acid (EDTA) for DNA extraction and quantitative PCR (qPCR) and into plain tubes to obtain sera samples from 30 cats for Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) and stored at -20 °C before use. Unfortunately, hematological and biochemical profiles were not performed.

2.1.1 Diagnosis of *Leishmania* infection based on cytology, histopathology and immunohistochemistry of cutaneous lesions

Impression smears and fine needle aspirates from ulcerative nodular lesions from 6 sick cats were sampled and stained with a Romanowsky stain variant (Diff-Quick).

Macroscopical skin lesions of solitary or multiple nodular and ulcerative areas were biopsied in 5 cats. Skin biopsies were fixed in 10% neutral buffered formalin. The dermal inflammatory pattern and the cell populations were evaluated histologically in hematoxylin and eosin (HE)-stained sections. A deparaffinization step was performed on the paraffin blocks of skin biopsies before *Leishmania* immunohistochemistry (IHC). Later, a standard staining protocol with AutostainerPlus (Dako Denmark A/S, Glostrup, Denmark) using rabbit polyclonal antibodies to *L. infantum* was followed. Sections were then counterstained with hematoxylin and cover-slipped for their interpretation (Esteve et al., 2015).

2.1.2 Detection of antibodies against *L. infantum* and *L. braziliensis* antigens by quantitative enzyme-linked immunosorbent assay (ELISA)

A *Leishmania infantum* in-house ELISA protocol previously described for cats (Solano-Gallego et al., 2007) was slightly modified and *L. infantum* (MHOM/MON-1/LEM 75) and *L. braziliensis* (MHOM/BR/88/BCN-25) antigens were used in the same ELISA plate. Cat sera were diluted to 1:800 in phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma-Aldrich, St. Louis, Missouri, USA) containing 1% of dry milk and incubated in sonicated crude *L. infantum* and *L. braziliensis* antigen-coated each in half plates (20 µg/ml) for 1 h at 37 °C.

All plates included serum from a sick cat from Cyprus with a confirmed infection with *L. infantum* as a positive control (Attipa et al., 2017) and serum from a healthy cat as a negative control. All samples were analysed in duplicate. The result was quantified as ELISA units (EU) related to a positive feline serum used as a calibrator and arbitrarily set at 100 EU (Solano-Gallego et al., 2014) for both antigens.

The cut-off for *L. infantum* was established at 9.2 EU (mean + 4 SD of values from 80 cats from the UK, a non-endemic area). Sera were classified as being positive, when having a value equal or higher than 15.3 EU and negative with 9.2 EU. Values in between were considered doubtful.

The cut-off for *L. braziliensis* was established at 13.8 EU (mean + 4 SD of values from 80 cats from the UK, a non-endemic area). Sera were classified as positive when having a value equal or higher than 21.0 EU and negative with 13.8 EU. Values in between were considered doubtful.

2.1.3 Western blot (WB)

Sera from 25 apparently healthy cats and five cats with lesions compatible with cutaneous leishmaniosis from Lara State in Venezuela were assessed by WB. In addition, sera from 8 cats from the Queen Mother Hospital at the Royal Veterinary College (RVC), University of London, were used as negative controls from cats living in a non-endemic area of leishmaniosis. Sera from 8 cats from Catalonia in Spain, of which 6 cats were seropositive to *L. infantum* by ELISA, one presenting a doubtful result and one being negative, were also evaluated. Cats from Catalonia (a non-endemic area for *L. braziliensis* infection) were included to compare the pattern of WB with cats from Venezuela. The seropositive cats to *L. infantum* from Catalonia were diagnosed with clinical leishmaniosis and used as positive controls for *L. infantum* antigen.

Western blot was performed with *L. infantum* (MHOM/MON-1/LEM 75) and *L. braziliensis* (MHOM/BR/88/BCN-25) promastigotes as antigens (Aisa et al., 1998; Riera et al., 1999). Sera from cats with leishmaniosis that reacted with polypeptides of low molecular mass (< 36 kDa) of *L. braziliensis* or *L. infantum* antigens were considered

positive for WB due to the fact that these antigens are the most specific fractions in diagnosis of FeL (Persichetti et al., 2017; Solano-Gallego et al., 2007).

2.2 DNA extraction from blood, paraffin-embedded skin biopsies and cytology from skin lesions

Blood DNA purification

DNA was extracted using the Gen Elute blood genomic DNA kit (Sigma-Aldrich) from 31 blood samples. Blood from a non-infected clinically healthy cat was included as negative control every time that DNA extraction was performed (Solano-Gallego et al., 2016).

2.2.1 Purification of genomic DNA from formalin-fixed, paraffin-embedded skin biopsies

A deparaffinization step was performed on the paraffin blocks of skin biopsies from 4 sick cats (FeV2, FeV3, FeV5 and FeV6) using buffer (20mM TRIS-HCL Ph 8.5; 1mM EDTA), heating for 10 min at 95 °C and centrifuging for 20 min at 12,000× *g*. Then, DNA extraction was performed using the QIAamp® DNA FFPE (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations.

2.2.2 Purification of genomic DNA from cytological slides from skin lesions

DNA extraction was performed from cutaneous lesions from cytological slides from 3 sick cats (Fev3, Fev4, Fev5) with QIAamp® DNA Mini and Blood (Qiagen) following the manufacturer's instructions. A scalpel (Braun, Tuttlingen, Germany) was used to obtain tissue in a tube from each sample. Twenty microliters of protease, 200 µl of PBS and 200 µl of lysis buffer (Buffer AL) were added and vortexed. Samples were incubated at 56 °C.

***2.2.3 Leishmania* spp. kinetoplast quantitative polymerase chain reaction (qPCR)**

The presence of *Leishmania* spp. DNA in blood samples (*n* = 31), paraffin embedded skin biopsies (*n* = 4) and cytological slides from cutaneous lesions (*n* = 3) was initially analysed by amplification of kinetoplast DNA sequence by a real-time polymerase chain reaction (qPCR). Each amplification was performed in triplicate, in 20 µl reaction, 15 pmol of direct primer (5'-CTT TTC TGG TCC TCC GGG TAG G-3'), 15 pmol of reverse primer (5'-CCA CCC GGC CCT ATT TTA CAC CAA-3'), 50 pmol of the labelled TaqMan probe (FAM-TTT TCG CAG AAC GCC CCT ACC CGC-TAMRA) and 5 µl of sample DNA. Amplification and detection were performed in the ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA.) in two-step temperature (94 and 55 °C) cycling over 45 cycles. Positive controls (DNA from *L. infantum* MHOM ⁄ES ⁄04 ⁄BCN-61) and negative controls were included in each RT-PCR analysis (Martin-Ezquerra et al., 2009).

2.2.4 Internal transcribed spacer 1 (ITS1) restriction fragment length polymorphism (RFLP), quantitative PCR (qPCR), sequencing and phylogenetic analysis

The species identification of the *Leishmania* isolates was performed on DNA from cutaneous lesions (cytological preparations) from 3 sick cats from Venezuela (FeV3, FeV4 and FeV5) and on DNA from cutaneous lesions (skin paraffin-embedded biopsies) from 4 sick cats (FeV2, FeV3, FeV5 and FeV6). Two different techniques were performed.

2.2.5 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of amplified ITS-1 sequences

For the identification of *Leishmania* species, we amplified the ribosomal ITS-1 region with primers LITSR (5'-CTG GAT CAT TTT CCG ATG-3') and L5.8S (5'-TGA TAC CAC TTA TCG CAC TT-3') (Schonian et al., 2003). Amplification reactions were performed in

volumes of 50 ml containing 3 µl of isolated DNA, 5 ml of 10× buffer (BIOTAQ DNA Polymerase, Bioline, London, UK), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 mM of each primer and 1.5 units of Taq polymerase (BIOTAQ DNA Polymerase, Bioline).

A denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturing for 20 s at 95 °C, annealing for 30 s at 53 °C, and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 1 h was carried out in thermal cycler (MJ Research PTC-200 DNA Engine, Alameda, CA, USA). DNA samples extracted from promastigote cell cultures of *L. infantum*, *L. tropica*, *L. major* and *L. braziliensis* were used as positive controls. A non-template control with the same reagents described above but without DNA was added to PCR to rule out contamination.

The PCR products, previously digested with the restriction enzyme *Bsu*RI (*Hae*III), were separated by electrophoresis in 2% wide-range agarose (Sigma) at 150 V in SGTB 1× buffer (GRISP LDA, Research Solutions, Porto, Portugal). A solution of SYBR safe DNA gel stain (Invitrogen Ltd., Paisley, UK) was used to visualize the separated DNA fragments under UV light (Tomas-Perez et al., 2013).

2.2.6 *Quantitative PCR high-resolution melting (qPCR-HRM) Leishmania genotyping based on a ITS1, sequencing and phylogenetic analysis*

A fragment of ITS1 region of the leishmanial ribosomal RNA operon was amplified (265–288 bp) by real-time PCR using primers ITS-219F (5'-AGC TGG ATC ATT TTC CGA TG-3') and ITS-219R (5'-ATC GCG ACA CGT TAT GTG AG-3') and then evaluated by high resolution melting (HRM) analysis as previously reported (Talmi-Frank et al., 2010).

DNA samples extracted from promastigote cell cultures of *L. infantum*, *Leishmania tropica* and *Leishmania major* were used as positive controls for each

corresponding PCR reaction and DNA from colony-bred dogs negative by PCR for vector-borne pathogens was used as a negative control. A non-template control (NTC) with the same reagents described above but without DNA was added to each PCR to rule out contamination.

All positive PCR products were sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), at the Center for Genomic Technologies, Hebrew University of Jerusalem, Israel. DNA sequences were evaluated with the ChromasPro software version 2.1.1 (Technelysium Pty Ltd., South Brisbane, Australia) and compared for similarity with sequences available on the GenBank, using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis was performed by MEGA6 (Tamura et al., 2013) using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Initial phylogenetic trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985) and branches corresponding to partitions reproduced in less than 60% bootstrap replicates were collapsed.

1.2.7 Detection of FeLV antigen and FIV antibody

In order to evaluate retroviral infections to rule out concomitant infections the same 30 cats from Venezuela described above (5 sick cats and 25 apparently healthy cats) were tested serologically for FeLV antigen and FIV antibody. Detection of FeLV p27 antigen and anti-FIV antibodies was performed by a commercial ELISA (INGEZIM

FeLV and INGEZIM FIV®, Ingenasa, Madrid, Spain) according to the manufacturer's protocol.

2. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 17.0 software for Windows (SPSS Inc., Chicago, USA). A non-parametric Mann-Whitney *U*-test was used to compare groups. A non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. Differences were considered significant with a 5% significance level ($P < 0.05$. The descriptive statistical analysis was conducted with R project software (2017).

3. RESULTS

4.1 Cats

Physical examination of all cats included in this study determined that 24 were adults, 5 were old cats and 2 were young cats. Moreover, the distribution according to gender was 13 females and 18 males. The majority of cats did not present any systemic clinical sign or dermatological lesions compatible with leishmaniosis and were classified as apparently healthy (25/31, 80.6%). All apparently healthy cats were owned cats from Barquisimeto and Cabudare cities from Lara State. They were 9 females (*n* = 2, old cats; *n* = 7, adults) and 16 males (*n* = 3, old cats; *n* = 12, adults; and *n* = 1 young cat).

On the other hand, 6 of the 31 cats (6/31, 19.3%) presented dermatological clinical signs. Signalment, geographical location and clinical description are summarized in Table 3.1. Sick cats were all stray cats living in cat colonies. They were 4 females and 2 males all adults, except for one 8-month-old young male. Skin lesions consisted of solitary or multiple nodular lesions (Fig.3. 1) which were located on the

nose (*n* = 3) (Fig.3.1 d-f); ears (*n* = 1) (Fig. 3.1c); ears and nose (*n* = 1) (Fig. 3.1a, b); and nose, ears, tail and lower limbs (*n* = 1). Cats did not show other clinical signs. Those cats did not receive any treatment and were humanely euthanized.

4.1.1 Cytology, histopathology and immunohistochemistry

Cytology was performed from cutaneous lesions in 4 sick cats. In the majority of cases, mixed inflammation with predominance of macrophages and neutrophils was found. Numerous intracellullar and extracellular *Leishmania* amastigotes were also noted (Fig. 3.2a, b). Histologically, cutaneous lesions from 4 sick cats were characterized by epidermal hyperplasia and hyperkeratosis.

Diffuse infiltrate with predominance of macrophages and plasma cells with numerous intracellular and extracellular amastigotes were observed (Fig. 3.2c). Occasionally, mast cells, lymphocytes and eosinophils were also encountered. Additionally, one sick cat presented crusting and necrosis, and presence of amastigotes was not observed. Immunohistochemistry was positive for *Leishmania* spp. in the 5 biopsied cats (Fig. 3.2d).

4.1.2 ELISA

All apparently healthy cats (*n* = 25) were negative by ELISA for *L. braziliensis* (mean ± SD = 4.5 ± 2.6 EU) and most of them (*n* = 22) were negative for *L. infantum* (mean ± SD = 2.5 ± 1.5 EU) antigens. Interestingly, when all sera samples (*n* = 30) were analysed, statistically significant higher antibody levels were found for *L. braziliensis* (mean ± SD = 7.0 ± 9.8 EU) when compared to *L. infantum* (mean ± SD = 4.5 ± 7.6 EU) antigen (Wilcoxon signed-rank test: $Z = -4.679$, $P < 0.0001$). Additionally, 3 out of 25 apparently healthy cats and one sick cat presented doubtful results for *L. infantum* antigen.

There were no animals with doubtful results for *L. braziliensis* antigen. Specific antibody response was significantly higher in sick cats when compared to healthy cats to both *L. braziliensis* (Mann-Whitney U-test: $Z = -2.47$, $P = 0.01$) and *L. infantum* (Mann-Whitney U-test: $Z = -2.69$, $P = 0.05$) antigens.

Two out of 5 sick cats yielded positive ELISA result to both *Leishmania* antigens (*L. infantum*: mean ± SD = 30.2 ± 10.9 EU and *L. braziliensis*: mean ± SD = 39.9 ± 17.9 EU) while the rest were seronegative (Table 1). Also, higher antibody levels were found for *L. braziliensis* (mean ± SD = 19.9 ± 20.3 EU) antigen than to *L. infantum* (mean ± SD = 14.7 ± 15.2 EU) antigen when all sick cats were evaluated for ELISA (Wilcoxon signed-rank test: $Z = -2.023$, $P = 0.043$).

The cats that were seropositive to *L. infantum* antigen from Catalonia were diagnosed with clinical leishmaniosis. When those animals were tested serologically with *L. braziliensis* antigen, 5 out of 8 presented negative ELISA results, one had a doubtful result and two showed positive results.

3 Western blot

As expected, sera from cats from the UK did not react with any polypeptides from both antigens. Bands recognized for *L. braziliensis* and *L. infantum* antigens by cat sera from Venezuela and Catalonia are described in Table 3.2. In the case of the Venezuelan samples, the highest sensitivity for *L. braziliensis* antigen was found in the following fractions: 70, 65, 52, 50, 46, 42, 36, 34, 30, 28, 18 and 16 kD. The highest sensitivity for *L. infantum* antigen in Venezuelan cats was found in the following fractions: 70, 65, 46, 34, 30, 28, 24, 18 and 16 kDa (Table 3.2). In contrast, Catalonian samples recognized a higher number of bands for *L. infantum* antigen (70, 65, 52, 46,

28, 24, 20, 18, 16 and 14 kDa) when compared to *L. braziliensis* antigen (70, 68, 65, 16 and 14 kDa).

The majority of sick cat sera from Venezuela recognized variable patterns of polypeptides with molecular masses ranging between 14–70 kDa for *L. braziliensis* antigen while they recognized polypeptide from *L. infantum* antigen less frequently (Table 3.1).

There was a statistically significant predominance of bands specific for *L. braziliensis* antigen in cats from Venezuela when compared to Catalonian cats (Table 3.2) (Mann-Whitney U-test: $Z = -4.03$, $P = 0.0001$). Also, when sick Venezuelan cats were compared to seropositive cats from Catalonia, a high number of bands for *L. braziliensis* antigen was observed (Mann-Whitney U-test: $Z = -2.55$, $P = 0.01$) (Table 3.3).

The Venezuelan cats showed a significantly higher number of bands for *L. braziliensis* antigen when compared to *L. infantum* antigen (Wilcoxon signed-rank test: $Z = -3.15$, $P = 0.02$) (Table 3.2). Additionally, a higher number of bands for *L. brazilienis* antigen were also found when compared with *L. infantum* antigen in Venezuelan sick cats (Wilcoxon signed-rank test: $Z = -3.58$, $P = 0.0001$) (Table 3). No statistical differences were observed within Catalonian cats when *L. braziliensis* and *L. infantum* bands were compared.

Four out of five sick cats from Venezuela resulted positive for *L. braziliensis* [low molecular mass (< 36 kDa)]. In addition, positive results to *L. braziliensis* WB were found in 6 out of 25 (24%) apparently healthy cats from Venezuela and also in 2 sick cats from Catalonia. In the case of cats from Venezuela, 3 sick cats and 7 apparently healthy cats resulted positive for *L. infantum* WB. Five out of eight Catalonian cats

presented compatible results for *L. infantum* antigens. In general, the intensity of bands in sick cats increased with an increase in the antibody level.

4.1.4 Blood and cutaneous lesions *Leishmania* kinetoplast qPCR, ITS1 RFLP and qPCR for speciation, sequencing and phylogenetic analysis

All twenty-five clinically healthy cats were blood qPCR negative (25/31 cats, 90.3%). Additionally, when blood from sick cats was analysed, 3 out of 6 cats were *Leishmania* kinetoplast qPCR positive (3/31 cats, 9.7%) while 3 were negative, including 1 seropositive sick cat by ELISA. DNA extraction was performed from paraffin-embedded skin biopsies of 4 sick cats and all samples were positive by kinetoplast qPCR (Table3.1). There was no correlation between the pattern of bands recognized by WB and PCR results.

DNA samples from cytological preparations of cutaneous lesions from 3 cats was extracted and all samples were positive by kinetoplast qPCR (Table 3.1). Positive DNA samples from cutaneous lesions (paraffin-embedded skin biopsies [(n = 4) and cytological preparations (n = 3)] were submitted to parasite species identification by PCR amplifying a fragment of the ITS1 region.

Only samples from cytological preparations of 2 cats were positive by ITS1 qPCR-HRM (FeV3 and FeV4) while samples from FeV5 were negative. In addition, the same DNA from the cytological preparation from FeV3 was also confirmed as positive by PCR-RFLP, but FeV4 and FeV5 were PCR-RFLP-negative (Table 3.1). All DNA samples from paraffin-embedded skin biopsies were negative by ITS1 qPCR-HRM (Table 3.1) and PCR-RFLP.

The DNA sequence of cat FeV3 was 100% identical to a partial *18S* rRNA ITS1 sequence of *L. mexicana* (GenBank: AB558250.1) over 210 bp, as found by BLAST

analysis. In contrast, the DNA sequence of cat FeV4 was only 93% identical to a partial *18S* rRNA ITS1 sequence of *L. mexicana* (GenBank: AB558250.1). A phylogenetic tree with the two Venezuelan cats results is presented in Fig. 3.3; in this tree, the DNA sequences from cats FeV3 and FeV4 clustered together with *L. mexicana* sequences from other sources deposited in GenBank.

4.1.5 Detection of FeLV antigen and FIV antibodies and relationship with *Leishmania* infection

All cats tested (*n* = 30) were negative for FeLV antigenemia. FIV-specific antibodies were found in 2 out of 25 apparently healthy cats tested (6.6%), both of them seronegative for *L. infantum* and *L. braziliensis*-specific antibodies based on ELISA and also negative by kinetoplast qPCR, but when WB positivity was studied, both cats resulted positive to *L. braziliensis*-specific bands and one was positive to *L. infantum*-specific bands.

4. DISCUSSION

The present study describes a clinical case series of solitary or multiple ulcerative nodular dermatitis due to *Leishmania* spp. in cats from Lara State, Venezuela. Unfortunately, so far, *Leishmania* identification has only been possible from the skin lesion of two cats and identified as *L. mexicana*. To the best knowledge of the authors, we report the first feline case of cutaneous lesions due to *L. mexicana* infection from Lara State, Venezuela. Interestingly, so far cats have only been described to be infected with *L. mexicana* in Texas in the USA (Barnes et al., 1993; Craig et al., 1986; Trainor et al., 2010). In agreement with our findings, *L. mexicana* infection was previously diagnosed in humans with cutaneous leishmaniosis from Lara State, Venezuela (Kato et

al., 2011) as well as in pools of *Lutzomyia* sand flies from Sucre State in Venezuela (Jorquera et al., 2005).

Unfortunately, *Leishmania* identification was not obtained from the remaining sick cats from Venezuela described in the present study. Since formalin-fixation of histological specimens causes partial DNA destruction, which may hamper diagnostic PCR analysis (Muller et al., 2003; Webster et al., 2010), we believe that there was inhibition of DNA PCR-based analyses of histological specimens.

Therefore, the amount of DNA amplified was reduced and this did not enable the identification of *Leishmania*. However, based on clinical and serological findings and the geographical distribution of FeL (Pennisi et al., 2015), it is likely that species of *L. mexicana* and/or *L. braziliensis* complexes were the cause of infection of the remaining cats described here. As with the cases presented here, clinical disease in cats caused by natural infection with species other than *L. infantum* is typically reported as nodular or ulcerative dermatitis with no systemic clinical signs. Skin lesions are often single but they can metastasize (Pennisi et al., 2015).

Solitary cutaneous lesions have been reported in association with *L. venezuelensis* infection, in the endemic focus of ACL both in humans and domestic animals in Barquisimeto, Lara State, Venezuela (Bonfante R et al., 1996; Kato et al., 2011). Interestingly, in this previous study, four cats were observed with cutaneous nodules on the nose and smaller nodules on the ears, and diffuse nodular lesions on the tail and legs (Bonfante R et al., 1996).

This clinical presentation was similar with the findings obtained in the present study from cats from the cities of Quíbor, Cabudare and Barquisimeto in Lara State, Venezuela. It is also important to highlight that feline cutaneous lesions described in

the present study are similar to the ones described also in humans. It is likely that cats might only be an accidental host of *L. venezuelensis* infection (Pennisi et al., 2015). It is also important to remark that the grouping of *L. mexicana* species complex is still controversial (Kato et al., 2011).

Leishmania venezuelensis was originally described on the basis of distinguishable multilocus enzyme electrophoresis (MLEE) patterns as a species independent of other members of the *L. mexicana* complex (Bonfante-Garrido, 1983). Furthermore, a monoclonal antibody specific for *L. venezuelensis* was developed for identification using immunological methods (Hanham et al., 1990). However, there are limited molecular data regarding *L. venezuelensis* (Berzunza-Cruz et al., 2002) and some authors strongly suggested that *L. venezuelensis* is a variant of *L. mexicana* (Kato et al., 2011).

A study carried out in Cojedes State of Venezuela revealed human, dog and equine populations with ulcers, other active lesions, skin scars and mucosal alterations, due to *Leishmania* (*Viannia*) *braziliensis* characterized by zymodeme and serodeme typing (Aguilar et al., 1989). In addition, natural (Rougeron et al., 2011; Schubach et al., 2004) and experimental (Simoes-Mattos et al., 2005).

L. braziliensis infections have been described in domestic cats. *Leishmania braziliensis* natural infection in cats has been described in Brazil (Aguilar et al., 1989), French Guiana (Rougeron et al., 2011) and in northern Argentina (Ruiz et al., 2015). Cutaneous lesions previously described were also similar to the ones reported in the present study. However, the finding of cats with cutaneous leishmaniosis does not reflect an important role of these domestic animals in the natural transmission of the disease in these areas, and these animals probably represent accidental hosts.

In the present study, cats presented ulcerative nodular dermatitis mainly on the face. Cutaneous lesions in cats naturally infected with *Leishmania* spp. occur mainly on the nose, followed by the ears or at both sites (Bailey and Lockwood, 2007) and also occasionally on the limbs. The skin alterations in FeL are unspecific and can be associated with other clinical conditions (de Souza et al., 2005).

The commonly seen cutaneous nodular form in FeL cases should be distinguished from nodules caused in cats with sterile or eosinophilic granuloma, cryptococcosis, sporotrichosis, histoplasmosis, mycobacterioses, and a wide variety of cutaneous neoplasms, e.g. feline sarcoids, mast cell tumor, fibrosarcoma, basal cell carcinoma, bowenoid *in situ* carcinoma and lymphoma (Pennisi et al., 2015).

The main differentials of the ulcerative lesions include squamous cell carcinoma, idiopathic ulcerative dermatitis, herpes virus dermatitis, mosquito-bite dermatitis, atypical mycobacteriosis and feline leprosy, cutaneous vasculitis, erythema multiforme and cold-agglutinin disease. Interestingly, squamous cell carcinoma may co-exist with *Leishmania* infections as clinical case reports due to *L. infantum* have been documented in cats in Europe (Grevot et al., 2005; Pocholle et al., 2012). In the present study, concomitant disease was not diagnosed in the sick cats studied.

Leishmaniosis is diagnosed by demonstration of the parasite by direct microscopic examination in stained smears, and/or culture of skin lesions, lymph node aspirates, peripheral blood, bone marrow aspirates or indirectly by serological techniques (Riera et al., 1999). A clinical form characterized by a very low number of intralesional parasites can be detected by an immunohistochemical technique. This technique is a highly sensitive and specific tool for the diagnosis of canine and feline leishmaniosis (Ordeix et al., 2005; Pennisi et al., 2015).

In this study, different techniques were used in the cats with cutaneous lesions, such as cytology, skin biopsy and immunohistochemistry, by which the diagnosis of infection by *Leishmania* spp. was made. Besides that, serological and molecular diagnostic techniques were also used in sick and apparently healthy cats. One sick cat and 25 clinically healthy cats were negative by serology and blood qPCR. The sick cats did not always give a positive result to blood qPCR and serology. The present findings might indicate that clinically healthy cats are not carriers of *Leishmania* spp. present in Venezuela.

Moreover, the ELISA and qPCR discordant results can be attributed to the inherent differences between serological testing and molecular methods. PCR is a very sensitive technique. However, intermittent parasitemias are very likely in cats as described in dogs (Manna et al., 2008) and therefore, PCR from blood might not be very sensitive. The parasite load in blood was quite variable in the cats studied and poorly correlated with the degree of antibody levels (Solano-Gallego et al., 2007). The present findings suggest that more than one technique should be used for detection of feline cutaneous leishmaniosis in South America.

Leishmania species such as *L. infantum* and *L. braziliensis* among others co-exist in South America and both species can infect cats (Pennisi et al., 2015). However, there are limited studies regarding serological tests that will distinguish between *L. infantum*- and *L. braziliensis*-specific feline antibodies in these regions. Here, we report an ELISA that combines both antigens. Interestingly, higher antibody levels were found using *L. braziliensis* antigen in Venezuelan cats when compared with *L. infantum* antigen.

This quantitative in-house ELISA appears to help establishing what *Leishmania* species or closely related *Leishmania* species are most likely infecting cats in the respective endemic areas. These findings are extremely important in areas where several parasite species might co-exist, therefore, this type of ELISA should be used to determine the most likely *Leishmania* parasite infecting dogs and cats in South America. Unfortunately, we did not have antigen of *L. mexicana* to perform WB.

Furthermore, the WB analysis also revealed that Venezuelan cat sera antigen. In addition, the intensity of bands increased with an increase in the antibody level. There was no correlation between the pattern of bands recognized and PCR results. Antigens of low molecular weight (12–14 and 14–18 kDa), seem to be very specific and their recognition in the immunoblot is highly sensitive in the diagnosis of subclinical *Leishmania* infection in dogs and cats (Persichetti et al., 2017). In the cats analysed, similar results were observed. In experimentally infected dogs, antibodies specific for low molecular weight fractions are the first to appear following infection (Riera et al., 1999).

Based on the results of this study, it appears that the WB enables detection of early phases of infection in apparently healthy cats with negative antibody levels by ELISA or PCR results. It also important to highlight that WB seems to be the best serological technique to be used when testing sick and apparently healthy cats from Venezuela as previously reported for European cats (Persichetti et al., 2017).

In agreement with the present study, another study reveals that the use of WB with whole antigen or antigenic Fe-SODe (iron superoxide dismutase) fraction was an optimal method for the detection of FeL (Longoni et al., 2012). The use of antigenic fractions of cultures of *L. mexicana*, *L. braziliensis* and *L. infantum* showed satisfactory

results with high sensitivity, specificity and efficacy for the detection of this disease in cats (Longoni et al., 2012). Therefore, WB should be widely used in the clinical setting for diagnosis clinical FeL as well as for detection of subclinical infections.

5. CONCLUSIONS

We conclude that leishmaniosis should be included in the differential diagnosis list of nodular-ulcerative lesions in cats, mainly on the nose and ears. In addition, to the best of our knowledge, we described for the first time, cutaneous lesion associated with *L. mexicana* infection from two Venezuelan cats.

The most reliable diagnostic technique in sick cats is cytological or histopathological examination along with immunohistochemistry, since blood PCR and serology by ELISA might be negative. However, WB appears to be more sensitive in detecting infected cats. Based on molecular and serological findings, cats from Venezuela are most likely infected with species of *L. mexicana* or *L. braziliensis* species complexes rather than *L. infantum*.

Finally, the present findings might indicate that clinically healthy cats are not carriers of *Leishmania* spp. present in Venezuela.

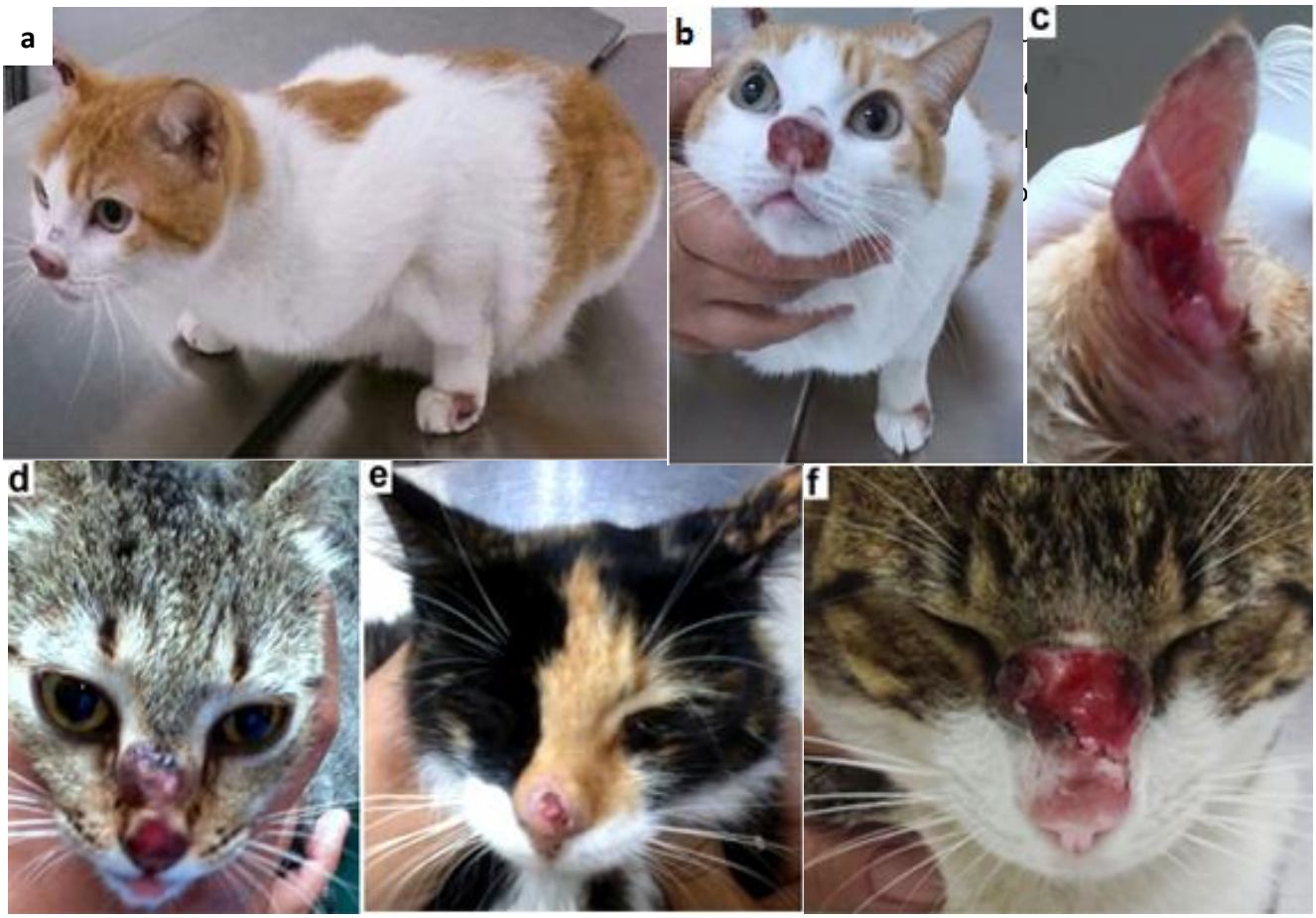

Fig. 3.1 Cats with cutaneous leishmaniosis: **a** Adult female cat with ulcerative nodular lesions in the nose and pinna (ID: FeV2). **b** Close up of the same cat (ID: FeV2). **c** Adult female cat, ulcerative lesion in the pinna (ID: FeV4). **d** Adult female cat with a nasal ulcer (ID: FeV6). **e** Adult female cat with an ulcerative nodular lesion on the nose (ID: FeV5); **f** 8-month-old male cat with an ulcerated nodule on the nose (ID: FeV3)

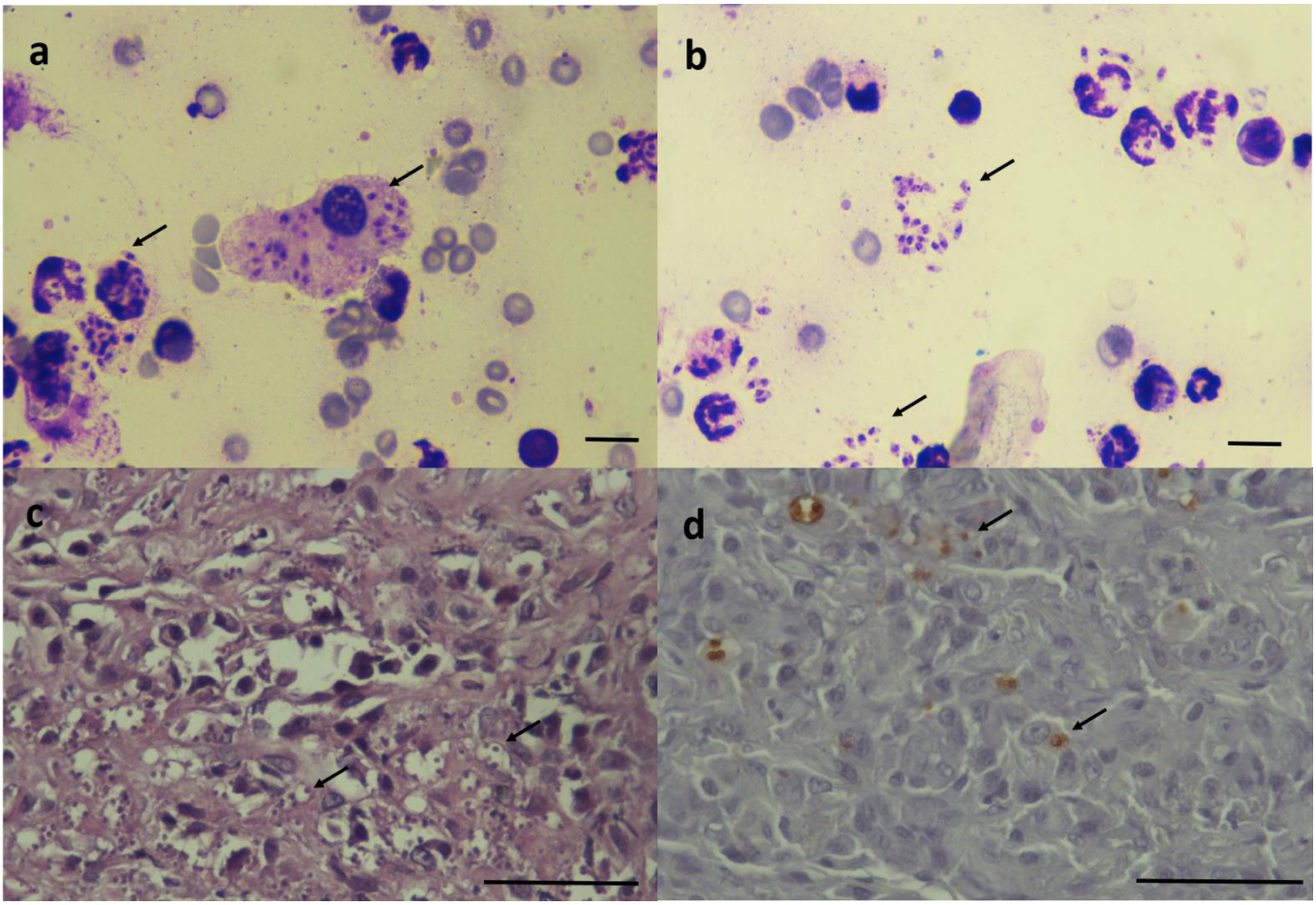

Fig. 3.2 a, **b** Cytology from cutaneous lesions from cat ID Fev3 with macrophagic-neutrophilic inflammation, showing numerous intracellular and extracellular *Leishmania* amastigotes (arrows) (diff quick stain 1000×). *Scale-bars*: 50 μm. **c** Diffuse pyogranulomatous inflammatory infiltrate from cat ID Fev5 with numerous *Leishmania* amastigotes (arrows) (H&E 400×). **d** Positive immunohistochemistry for *Leishmania* amastigotes from cat Fev5 (brown dots are indicated with arrows) (400×). *Scale-bars*: 10 μm

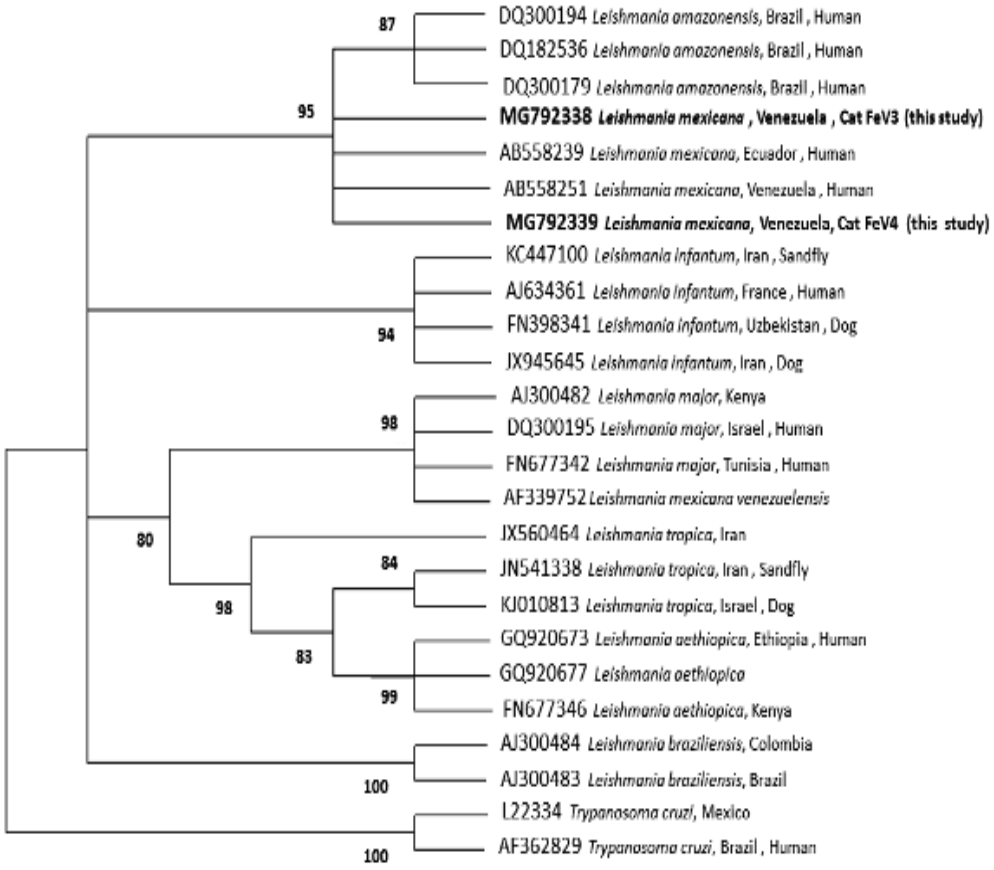

Fig.3.3 Phylogenetic analysis constructed based on 210bp DNA sequences of the ITS1 locus of Venezuelan cats (FeV3 and FeV4). Sequences from this study were compared to other sequences deposited in GenBank. Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model. The number of bootstrap replicates are 1000 and branches corresponding to less than 60% bootstrap replicates are collapsed. Bootstrap values higher than 60% are indicated next to the branches. GenBank accession numbers, the strain, country of origin and host from which the sequences were derived are included for each sequence.

Table 3.1 Summary of signalment, clinical findings and diagnostics tests results in six cats with cutaneous leishmaniosis.

Cat ID	Signalment and clinical description (city)	ELISA (EU)		Western blot (bands kDa)		Microscopical observations			*Leishmania* spp. kinetoplast qPCR			*Leishmania* spp. ITS1 qPCR	
		L.i	*L. b*	*L. i*	*L. b*	Cytology	Biopsy H/E	IHC	Blood	Skin lesions		Skin lesions	
										Biopsy	Cytology	Biopsy	Cytology
FeV1	Adult, male, ulcerative nodular lesion on the nose (Cabudare)	– 4.5	– 7.1	–	+ (14, 18, 22, 38)	–	–	+	–	np	np	np	np
FeV2	Adult, female, ulcerative nodular lesion in the nose pinna and interdigital area (Cabudare)	– 2.4	– 3.7	–	–	–	+	+	–	+	np	–	np
FeV3	8 months male ulcerated nodule on the nose (Quíbor)	+ 22.5	+ 27.2	+ (16–20, 24–30, 46)	+ (14–20, 24–36, 42, 46–52)	+	+	+	–	+	+	–	+[b]
FeV4[a]	Adult, female, ulcerative lesion in the pinna (Quíbor)	np	np	np	np	+	np	np	+	np	+	np	+[c]
FeV5	Adult, female, ulcerative	– 6.2	– 9.0	–	+ (18, 24, 28,	+	+	+	+	+	+	–	–

	nodular lesion in the nose (Barquisimeto)				65, 70)								
FeV6	Adult, female nasal ulcer (Barquisimeto)	+ 38.0	+ 52.5	–	+ (16, 28, 30–36, 42, 46, 70)	+	+	+	+	+	np	–	np

Abbreviations: np, not performed; +, positive; –, negative, *L.i, L.infantum, L.b, L.braziliensis*

[a]Serum was not available

[b]100% identity with *L. mexicana* ITS1. Identified also as *L. mexicana* by RFLP (GenBank: AB558250.1)

[c]93% identity with *L. mexicana* ITS1 (GenBank: AB558250.1)

Table 3.2 Antibody recognition of *L. infantum* and *L. braziliensis* antigens by WB in era of cats from Venezuela and Catalonia (Spain).

WB band (kDa)	*Leishmania braziliensis* antigen						*Leishmania infantum* antigen					
	Total no. of cats (*n* = 38)		**Endemic area Catalonia (*n* = 8)**		**Endemic area Venezuela (*n* = 30)**		**Total no. of cats (*n* = 38)**		**Endemic area Catalonia (*n* = 8)**		**Endemic area Venezuela (*n* = 30)**	
	n	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***N***	**%**	***n***	**%**
70	11	12	2	10	9	13	5	9	1	4	4	11
68	2	2	2	10	2	3	0	0	0	0	0	0
65	7	8	2	10	5	7	3	5	1	4	3	8
58	1	1	0	0	1	1	0	0	0	0	0	0
56	2	2	0	0	2	3	0	0	0	0	0	0
52	5	5	0	0	5	7	1	2	1	4	0	0
50	3	3	0	0	3	4	0	0	0	0	0	0
48	2	2	0	0	2	3	0	0	0	0	0	0
46	6	7	1	5	5	7	3	5	1	4	2	6
44	2	2	2	10	0	0	1	2	0	0	1	3
42	2	2	0	0	2	3	0	0	0	0	0	0
40	1	1	1	5	0	0	0	0	0	0	0	0
38	1	1	0	0	1	1	0	0	0	0	0	0
36	3	3	1	5	2	3	0	0	0	0	0	0
34	4	4	1	5	3	4	7	12	3	13	4	11
30	6	7	1	5	5	7	5	9	3	13	2	6
28	7	8	1	5	6	8	6	10	1	4	5	14
24	3	3	1	5	2	3	5	9	2	9	3	8
22	1	1	0	0	1	1	0	0	0	0	0	0
20	3	3	0	0	3	4	3	5	2	9	1	3
18	6	7	1	5	5	7	5	9	2	9	3	8
16	8	9	2	10	4	6	11	19	4	17	7	19
14	5	5	2	10	3	4	3	5	2	9	1	3
Total no. of bands	91		20		71		58		23		36	
Mean no. of bands	3.9		0.8		3.0		2.5		1.5		1	

Note: Statistical results: *L. braziliensis* bands: Venezuelan cats > Catalonian cats (Mann-Whitney U-test: $Z = -4.03$, $P = 0.0001$). Venezuelan cats: *L. braziliensis* > *L. infantum* (Wilcoxon signed-rank test: $Z = -3.15$, $P = 0.02$).

Table 3.3 Antibody recognition of *L. braziliensis* and *L. infantum* antigens by WB in seropositive and sick cats from Venezuela and Catalonia (Spain).

WB band (kDa)	*Leishmania braziliensis* antigen						*Leishmania infantum* antigen					
	Total no. of cats *n* = 10		**Endemic area Catalonia (*n* = 5)**		**Endemic area Venezuela (*n* = 5)**		**Total no. of cats (*n* = 10)**		**Endemic area Catalonia (*n* = 5)**		**Endemic area Venezuela (*n* = 5)**	
	n	**%**	***N***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**
70	5		2	10	3	8	3	8	1	0	2	13
68	3	5	2	10	1	3	0	0	0	0	0	0
65	4	7	2	10	2	6	3	8	1	0	2	13
58	1	2	0	0	1	3	0	0	0	0	0	0
56	1	2	0	0	1	3	0	0	0	0	0	0
52	1	2	0	0	1	3	1	3	1	0	0	0
50	1	2	0	0	1	3	0	0	0	0	0	0
48	1	2	0	0	1	3	0	0	0	0	0	0
46	3	5	1	5	2	6	3	8	1	0	2	13
44	2	4	2	10	0	0	1	3	0	0	1	7
42	2	4	0	0	2	6	0	0	0	0	0	0
40	1	2	1	5	0	0	0	0	0	0	0	0
38	1	2	0	0	1	3	0	0	0	0	0	0
36	3	5	1	5	2	6	0	0	0	0	0	0
34	3	5	1	5	2	6	3	8	3	1	0	0
30	3	5	1	5	2	6	4	11	3	1	1	7
28	4	7	1	5	3	8	4	11	1	0	3	20
24	3	5	1	5	2	6	3	8	2	0	1	7
22	1	2	0	0	1	3	0	0	0	0	0	0
20	1	2	0	0	1	3	3	8	2	0	1	7
18	4	7	1	5	3	8	3	8	2	0	1	7
16	4	7	2	10	2	6	5	13	4	1	1	7
14	4	7	2	10	2	6	2	5	2	0	0	0
Total no. of bands	56		20		36		38		23		15	
Mean no. of bands	2		1		2		2		1		1	2

Note: Statistical results: *L. braziliensis*-specific bands: Venezuelan sick cats > Catalonian sick cats (Mann-Whitney U-test: $Z = -2.55$, $P = 0.011$). Venezuelan sick cats: *L. braziliensis* > *L. infantum* (Wilcoxon signed-rank test: $Z = -3.58$, $P = 0.0001$).

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CAPÍTULO 4

❖ **ESTUDIO 2**

ELISA and Western blot to discriminate between specific *Leishmania infantum* and *Leishmania braziliensis* canine antibodies

CAPÍTULO 4

ELISA and Western blot to discriminate between specific *Leishmania infantum* and *Leishmania braziliensis* canine antibodies

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ABSTRACT

Leishmania species that infect dogs such as *Leishmania infantum* and *Leishmania braziliensis* coexist in South America. However, there are limited studies regarding serological tests that distinguish between *L. infantum* and *L. braziliensis* specific canine antibodies. The aim of this study was to develop quantitative Enzyme linked immunosorbent assay (ELISA) and Western blot (WB) tests with *L. infantum* and *L. braziliensis* antigens to evaluate specific canine antibodies and to discriminate between antibodies responses to both antigens in dogs living in endemic areas of canine leishmaniosis (CanL) in South America (Venezuela) and in Europe (Catalonia). ELISA and WB with slight modifications were performed with *L. infantum* and *L. braziliensis* antigens. Sera were obtained from 40 dogs from Catalonia (Spain) in different clinical stages of CanL and 20 clinically healthy dogs from Yaracuy and Lara state (Venezuela). Additionally, ten dog sera samples from the UK, were used as negative controls from a non-endemic area of leishmaniosis for WB. Sick dogs from Catalonia were 87.5% seropositive to *L. infantum* antigen (n=35), and all of them (n=40) were also positive to *L. braziliensis* antigen by ELISA. In addition, *L. infantum* specific antibody levels were significantly higher ($p<0.001$) when compared with *L. braziliensis* specific antibody levels. Catalonian samples recognized a significantly higher number of bands for *L. infantum* antigen when compared to *L. braziliensis* antigen ($p=0.050$). Apparently healthy dogs from Venezuela (n = 20) were 10% seropositive (n=2) to *L. infantum* antigen and 35% (n = 7) were seropositive to *L. braziliensis* antigen. Venezuelan samples recognized significantly higher number of bands

for *L. braziliensis* antigen than for *L. infantum* antigen (p=0.001). The results of ELISA, and WB presented here, appear to be a useful tool for establishing the *Leishmania* species or related *Leishmania* species that are most likely infecting dogs in endemic areas. These findings are extremely important in areas where these two parasites might co-exist. Therefore, both ELISA and WB should be used to determine the most likely *Leishmania* parasite infecting dogs in South America.

Keywords: *Leishmania infantum*, *Leishmania braziliensis,* dog, ELISA, WB, Venezuela, Catalonia.

1. INTRODUCTION

Leishmania are protozoan parasites of great significance in human and veterinary medicine, which are transmitted to a susceptible host by phlebotomine sand flies of the genera *Phlebotomus* and *Lutzomyia* in the Old and New Worlds (Dantas-Torres, 2007; Dantas-torres et al., 2012).

Human leishmaniasis due to *Leishmania* spp. is a zoonosis widely distributed, which includes three different clinical forms: cutaneous (CL), muco-cutaneous (ML) and visceral (VL). VL causes higher mortality rates in humans (Reithinger and Davies, 1999). Specifically, American cutaneous leishmaniasis (ACL) are a group of diseases mainly caused by *Leishmania* (*Viannia*) spp. of which *Leishmania braziliensis* is the most widespread (Reithinger and Davies, 1999). ACL is characterized by a spectrum of clinical

manifestations, ranging from localized dermal ulcers to mucocutaneous lesions (Stockdale and Newton, 2013).

Canine leishmaniosis (CanL) is endemic in more than 70 countries worldwide, including regions of southern Europe, Africa, Asia, South and Central America (Baneth et al., 2008). *Leishmania* species coexist in South America and most cases of CanL are caused by *Leishmania infantum* (syn. *Leishmania chagasi*) and *Leishmania braziliensis* (Dantas-Torres, 2009), while in the Mediterranean basin, *L. infantum* is the major species infecting dogs (Dantas-torres et al., 2012).

It is well known that the dog plays an important role in the zoonotic cycle of transmission of *L. infantum*; in the case of *L. braziliensis*, the dog is most likely an incidental host and its role in the zoonotic cycle of transmission is probably negligible (Dantas-Torres, 2007; Dantas-torres et al., 2012).

In endemic areas, the prevalence of *L. infantum* infection in dogs is greater than the seroprevalence and the prevalence of clinical disease. CanL is a good example of a disease in which infection does not equal clinical illness due to the high prevalence of persistent sub-clinical infection (Baneth et al., 2008). There is no diagnostic test with 100% sensitivity and 100% specificity for detection of *L. infantum* infection and therefore it is essential to know the terms and limitations of each diagnostic test, and to select the best tests for the purpose of the diagnosis (Solano-Gallego et al., 2009).

Serological methods such as IFAT, ELISA and rapid tests are the most common diagnostic techniques employed in clinical and research studies on canine *L. infantum* infection. For both IFAT and ELISA, quantification using antibody titer or optical density allows classification of antibody levels against *L. infantum* antigen. The IFAT technique has traditionally been considered a gold standard for the serological diagnosis of *L. infantum* infection, with optimal performance measures concerning sensitivity and specificity. The ELISA has a good diagnostic performance with a medium to high sensitivity and high specificity that increases when multiple antigens are used (Solano-Gallego et al., 2017).

Western blot (WB) analysis is a sensitive and specific technique that has improved diagnostic capabilities for CanL (Aisa et al., 1998). Another study revealed that WB sensitivity was 90.9% and specificity was 100% with a better diagnostic performance than ELISA (López-Céspedes et al., 2012). It appears that the use of antigenic fractions by WB technique to determine the presence of antibodies is much more sensitive for the detection of leishmaniosis (Trevisan et al., 2015).

Additionally, there is a limited number of studies regarding serological tests that distinguish between *L. infantum* and *L. braziliensis* specific canine antibodies (Trevisan et al., 2015). Therefore, the aim of this study was to develop an in-house quantitative ELISA and WB with *L. infantum* and *L. braziliensis* antigens to evaluate specific canine antibodies and to discriminate between antibody responses to both antigens in dogs living in endemic areas of leishmaniosis in South America (Venezuela) and in Europe (Catalonia).

2. MATERIALS AND METHODS

2.1. Study area and dogs

The subjects involved in the study were seventy dogs.

2.1.1. Sick dogs from Catalonia (Spain)

Sera from 40 sick dogs from Catalonia (Spain) in different clinical stages of leishmaniosis, stage I (n=10), stage II (n=20), stage III (n=8), stage IV (n=2) (Solano-Gallego et al., 2009)were studied by means of, ELISA for *L. infantum* and *L. braziliensis* antigens and ten dogs of this group were selected to also perform WB.

Both sexes were represented with 16 females and 24 males. Median of age was 5.5 years old with a range from 5 months to 13 years. Dogs belonging to the following breeds were studied: German shepherd (n=2), Siberian husky (n=1), Dalmatian (n=1), Boxer (n=2), Doberman (n=1), Breton (n=1), German braco (n=3), Teckel (n=1), Podenco (n=6), Labrador retriever (n=2), Galgo (n=1), Setter (n=3), Beagle (n=1), American Staffordshire terrier (n=1), Chihuahua (n=1), Akita inu (n=1). The rest of dogs (n=12) were mixed-breed.

2.1.2. Clinically healthy dogs from Yaracuy and Lara state (Venezuela)

After a physical examination, 20 sera samples from clinically healthy dogs from Yaracuy and Lara state (Venezuela) were collected and evaluated by ELISA and WB for both antigens. Both sexes were represented with 4 females and 16 males. Median of age was 3.5 years old with a range from 5 months to 13 years. Dogs belonging to the following

breeds were enrolled: German shepherd (n=1), Siberian husky (n=2), Mastiff (n=1), Pitbull (n=2), Poodle (n=1). The remaining dogs (n=13) were mixed-breed.

2.1.3. Control dogs

Ten dog sera from the Queen Mother Hospital at the Royal Veterinary College (RVC, University of London) were used as negative controls for WB for both antigens.

2.2. In-house Enzyme-Linked Immunosorbent Assay (ELISA)

A previously described *L. infantum* in house ELISA protocol (Solano-Gallego et al., 2014) was slightly modified and *L. infantum* (MHOM/MON-1/LEM 75) and *L. braziliensis* (MHOM/BR/88/BCN-25) antigens were used in the same ELISA plate as recently described for cats (Rivas et al., 2018). Dog sera were diluted to 1:800 in phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma-Aldrich, St. Louis Missouri, USA) containing 1% of dry milk and incubated in sonicated crude *L. infantum* and *L. braziliensis* antigen-coated half plates (20 µg/mL) for 1 hour at 37°C.

All plates included serum from a sick dog with confirmed *L. infantum* infection as a positive control and serum from a healthy dog as a negative control. The result was quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and arbitrarily set at 100 EU for both antigens. All samples were analyzed in duplicate.The plates were developed by adding the substrate solution ortho-phenylene-diamine (SIGMAFAST OPD, Sigma Aldrich, St. Louis Missouri, USA). The reaction was stopped with

50 µL of 2.5M H_2SO_4. Absorbance values were read at 492 nm in an automatic micro ELISA reader (Anthos 2020, Cambridge, UK).

Dogs that presented positive results with an optical density (OD) equal or higher than three were studied using a two-fold serial dilution, endpoint quantitative ELISA protocol previously described for dogs (Solano-Gallego et al., 2016).

Sera were classified for *L. infantum* and *L. braziliensis* ELISAs as positive when having a value equal or higher than 35 EU and 8.5 EU (mean + 4 SD of values from 80 dogs from non- endemic area), respectively.

2.2.1. WESTERN BLOT

Western Blot was performed with *L. infantum* (MHOM/MON-1/LEM 75) and *L. braziliensis* (MHOM/BR/88/BCN-25) promastigotes as previously described (Rivas et al., 2018). Antigen for immunoblot was obtained from promastigotes from culture in Schneider's medium (S Sigma Aldrich, St. Louis Missouri, USA) containing 20% fetal calf serum at the exponential growth phase.

Antigen electrophoresis on 0.1% SDS-15% polyacrylamide gels together with molecular mass proteins standards (Standard Low Range; Bio-Rad) was performed on a Mini- Gel AE 6400 Dual Mini Slab Kit (Atto, Bunkyo-ku, Japan).

Gels were run at 100 V for 1hr-1.30 hr at room temperature. Polypeptides were transblotted onto nitrocellulose sheets (0.45- mm pore size, HAWP 304 FO; Millipore,

Bedford, MA). The sheets were blocked with 20 mM Tris, 0.13 mM NaCl, and pH 7.6 in Tris-buffered saline (TS), 5% milk overnight at 4 º C.

The sheets were washed in TS and introduced into a multiscreen apparatus (Mini Protean II, Multiscreen Apparatus; Bio-Rad). Sera were diluted 1:200 in TS-1% milk, 0.2% Tween 20, and 500 ml of each sample was introduced into each channel of the multiscreen apparatus and incubated for 2 hr at 37°C. Bound immunoglobulins were developed by incubation with a 1:1,000 dilution of Protein A peroxidase conjugate (Sigma Aldrich, St. Louis Missouri, USA) for 1 hr.

After the sheets were washed three times with TST and a final time with TS, the color was developed with 4-chloro-1-naphthol (Sigma Aldrich, St. Louis Missouri, USA) and H_2O_2 and the reaction was stopped with tap water after 30 min. Sera from dogs with leishmaniosis that reacted with polypeptides of low molecular mass (< 36 kDa) of *L. braziliensis* or *L. infantum* antigens were considered positive for WB (Aisa et al., 1998).

2.3. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 17.0 software for Windows (SPSS Inc., Chicago, USA). A Non-parametric Mann-Whitney U-test was used to compare groups. A non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. Differences were considered significant with a 5% significance level ($p < 0.05$).

3. RESULTS

3.1. ELISA

Two out of 20 apparently healthy dogs from Venezuela were seropositive (10%) to *L. infantum* antigen with a mean ± standard deviation (SD) of 118.3± 63.8 EU. Interestingly, seven dogs (35%) from Venezuela were seropositive to *L. braziliensis* antigen with a mean± SD of 18.3± 15.6 EU which were significantly lower when compared to *L. infantum* antigen (Wilcoxon signed-rank test, $p<0.001$, Table 4.1).

Thirty-five out of 40 sick dogs from Catalonia were seropositive (85%) to *L. infantum* antigen and all of them were seropositive (100%) to *L. braziliensis* antigen. *Leishmania infantum* specific antibody levels were significantly higher [mean± SD of 2738 ± 6134 EU] Wilcoxon signed-rank test, $p<0.001$ when compared with *L. braziliensis* specific antibody levels [mean±SD of 622±1184 EU](Table 4.1).

The specific antibody response was significantly higher to both *L. infantum* and *L. braziliensis* antigens (Mann-Whitney U-test, $p = 0.001$) in Catalonian seropositive dogs when compared to apparently healthy dogs from Venezuela.

3.2. WESTERN BLOT

As expected, UK dog sera samples did not react to any polypeptides of *L. braziliensis* antigen. Interestingly, two dogs from the UK recognized *L. infantum* bands (Tables 4.1 and 4.3 and figure 4.1).

However, these two dogs presented very weak bands when compared with Catalonian and Venezuelan dogs. UK samples recognized a significantly higher number of bands for *L. infantum* antigen (46, 44, 28, 24, 20, 18 and 16 kDa) when compared to *L.*

braziliensis antigen (without diagnostic bands)) (Wilcoxon signed-rank test, *p*=0.014) (Tables 4.1 and 4.3).

In the case of the Venezuelan samples, the highest sensitivity for *L. braziliensis* antigen was found in the following fractions: 70, 68, 48, 46, 34, 30, 28, 18, 16 and 14 kDa (Tables 4.1 and 4.2 and figure 4.2). The highest sensitivity for *L. infantum* antigen in Venezuelan dogs was found in the following fractions: 65, 46, 30, 28, and 18 kDa (Tables 4.1 and 4.3).

Venezuelan samples recognized statistically higher number of bands for *L. braziliensis* antigen (70,68,48,46,38, 36,34,30, 28, 24,22, 20,18 16 and 14 kDa) than for *L. infantum* antigen (65,56,48,46,30,28,24,18,16 and 14 kDa) (Wilcoxon signed-rank test, *p*=0.001).

There was statistically significant predominance of bands specific for *L. braziliensis* antigen in dogs from Venezuela when compared to UK dogs (Mann-Whitney U-test, *p* = 0.001).

Catalonian samples recognized a statistically higher number of bands for *L. infantum* antigen (70, 65, 48, 46, 44, 42, 40, 34, 30, 28, 24, 20, 18, 16 and 14 kDa) when compared to *L. braziliensis* antigen (70, 65, 34, 28, 20, 16 and 14 kDa) (Wilcoxon signed-rank test, *p*=0.05) (Tables 4.1, 4.2 and 4.3).

Catalonian seropositive dogs presented a statistically significant predominance of specific bands to *L. infantum* (Mann-Whitney U-test, p = 0.001) and *L. braziliensis* p = 0.001 antigens when compared to dogs from UK (Tables 4.1, 4.2 and 4.3).

There was not statistically significant predominance of bands specific for *L. braziliensis* antigen in dogs from Venezuela when compared to Catalonian dogs (Mann-Whitney U-test, p = 0.236)(Table 4.2).

4. DISCUSSION

Canine leishmaniosis is widespread in rural and urban areas of South America. *Leishmania* species such as *L. infantum* and *L. braziliensis* among others co-exist in Venezuela and South America in general and both species can infect dogs (Dantas-Torres, 2009). Furthermore, their distribution is probably wider than is actually conceived-(Dantas-Torres, 2009). However, there are limited studies regarding serological tests that will distinguish between *L. infantum*- and *L. braziliensis*-specific canine antibodies in these regions (Trevisan et al., 2015). Here, we report ELISA and WB techniques that test for two antigens simultaneously.

Interestingly, in the present study, a higher percentage of seroreactive dogs was found with *L. braziliensis* antigen in Venezuelan healthy dogs when compared with *L. infantum* antigen by ELISA as recently demonstrated in sick and healthy cats from Venezuela (Rivas et al., 2018). In agreement with the present findings, the detection of IgG by ELISA using *L. braziliensis* antigen presents a good diagnostic performance for

cutaneous leishmaniosis due to *L. braziliensis* or related *Leishmania* species in dogs (Ribeiro et al., 2007).

In contrast, higher antibody levels were found using *L. infantum* antigen in Catalonian sick dogs when compared with *L. braziliensis* antigen by ELISA. The quantitative ELISA appears to help establishing what *Leishmania* species or closely related *Leishmania* species are most likely infecting dogs in the respective endemic areas. These findings are extremely important in areas where several parasite species might co-exist. Therefore, this type of ELISA could be used to determine the most likely *Leishmania* parasite infecting dogs in South America. In disagreement with the present results, a study performed in Israel using an ELISA serology with crude promastigote antigen from *L. infantum*, *L. tropica* and *L. major* was not found to be distinctive between *Leishmania* species in dogs as there was no significant association between the *Leishmania* species infecting the dogs and their seroreactivity to its antigen when compared to antigen of other species (Baneth et al., 2017).

We believe that the main difference between studies was that we used and endpoint ELISA (Solano-Gallego et al., 2016) while the study from Israel used a single sera dilution of 1:100. Therefore, it is likely that the effect of saturation of optical density at only one dilution in dogs with high antibody levels did not permit to discriminate between antigens (Baneth et al., 2017). Recently, a study identified *L. infantum*, *L. braziliensis* and *Leishmania amazonensis* by molecular techniques detected in dogs and humans from outbreaks of VL in Colombia (Herrera et al., 2018). In this study, IFAT tests were not able

to discriminate between *Leishmania* species and only molecular tests were capable to do so (Herrera et al., 2018).

Furthermore, the WB analysis also revealed that Venezuelan dog sera recognized a significantly higher number of *L. braziliensis* polypeptides when compared to *L. infantum* antigen as recently demonstrated in cats from Venezuela (Rivas et al., 2018). Previous investigations performed on canine *Leishmania* infection in Mexico reported that the use of iron-superoxide dismutase excreted (Fe-SODe) by the parasites as the antigen fraction on ELISA and WB tests allowed to confirm the presence of at least three species of *Leishmania* (*L. mexicana, L. braziliensis*, and *L. panamensis*)(López-Céspedes et al., 2012).

In addition and as expected, Catalonian dog sera recognized a significantly higher number of *L. infantum* polypeptides when compared to *L. braziliensis* antigen. The pattern of recognition of *L. infantum* polypeptides in dogs from Catalonia is similar to other studies (Aisa et al., 1998; Talmi-Frank et al., 2006). The use of Fe-SODe antigen fraction in ELISA and WB appears to also have a good diagnostic performance in the diagnosis of *L. infantum* infection in dogs from Spain and Italy (Longoni et al., 2012).

Antigens of low molecular weight (12–14 and 14–18 kDa), seem to be very specific and their recognition in the immunoblot is highly sensitive in the diagnosis of subclinical *Leishmania* infection in dogs and cats (Aisa et al., 1998; Rivas et al., 2018). Previous investigations performed by our group in feline sample from Venezuela and Catalonia reveals that the WB enables detection of early phases of infection in apparently healthy cats with negative antibody levels by ELISA or PCR results (Rivas et al., 2018).

In dog experimentally infected with *L. infantum*, antibodies specific for low molecular weight fractions are the first to appear following infection (Riera and Enric, 1999). Based on the results of this study, it appears that WB enables detection of early phases of infection in apparently healthy Venezuelan dogs with negative antibody levels by ELISA mainly for *L. braziliensis* antigen. It also important to highlight that WB seems to be the best serological technique to be used when testing apparently healthy dogs from Venezuela as recently reported for Venezuelan (Rivas et al., 2018) and European cats (Persichetti et al., 2017).

Therefore, WB should be widely used in the clinical setting for diagnosis of clinical CanL as well as for detection of *Leishmania* subclinical infections in South America. One potential field of application of the WB method is the discrimination between subclinical infection and disease (Solano-Gallego et al., 2011).

The search toward the establishment of novel serological tests for the diagnosis of animal and human leishmaniosis and proper differential diagnosis may represent one alternative to the invasive parasitological methods currently used to identify infected animals and humans. The development of new serological tests that will discriminate between *Leishmania* species and related species such as *Trypanosoma* in animals and humans are highly recommended. A study performed in humans demonstrated the usefulness of an ELISA format with exoantigens from insect trypanosomatids (*Leptomonas seymouri* and *Crithidia fasciculata*) to develop a rapid, accurate and sensitive diagnostic procedure for differentiating between human VL and ACL (Kesper et al., 2017).

Another study investigated the potential use of recombinant peroxidoxin of *Leishmania (Viannia) braziliensis* as a potential antigen for the immunodiagnosis by ELISA of human cutaneous and visceral leishmaniosis and CanL due to *L. infantum*. This assay demonstrated the detection of high antibodies in patients with cutaneous leishmaniosis but also in human and canine leishmaniosis due to *L. infantum*. However, the peroxidoxin ELISA showed a greater ability to discriminate between vaccinated and *L. infantum* infected dogs (Menezes-Souza et al., 2014).

Furthermore, a study identified putative secreted *L. amazonensis, L. braziliensis*, and *L. infantum* proteins that have differential reactivity to hyperimmune canine serum and suggest that these antigens can be used as target to antigens detection in several *Leishmania* spp. infections (Lima et al., 2016). Another important question to be addressed will be to discriminate also between serocrossreaction with other species apart of *Leishmania* such as *Trypanosoma* spp. or *Sporothrix* spp. as some serological techniques have achieved in canines (Ribeiro et al., 2007).

In Brazil, VL is caused by *L. infantum*, whose main vector is *Lutzomyia longipalpis* and the reservoir in domestic and peridomestic environment is the domestic dog (*Canis familiaris*). The Brazilian Ministry of Health recommends euthanasia of seroreactive dogs. Unfortunately, especially in endemic areas, dogs were unnecessarily killed when the decision was based on a single serological test and the combination of different laboratory tests could not confirm the infection (Silva et al., 2011). Studies have demonstrated the misdiagnosis made by serological tests in Brazil and the euthanasia of dogs infected with

L. braziliensis (Morgado et al., 2016) or *Trypanosoma caninum* (Silva et al., 2011). It is important for veterinarians to promote a differential diagnosis for co-infected dogs, with an emphasis on defining the *Leishmania* species involved, because animals infected by *L. braziliensis* (Morgado et al., 2016) or *Trypanosoma* spp (Silva et al., 2011) were unnecessarily euthanized.

We consider that the present serological tests studied allowed to discriminate between specific *L. infantum* and *L. braziliensis* canine antibodies, and might help avoiding unnecessary euthanasia in *L. braziliensis* or *Leishmania* related species as well as *Trypanosoma* endemic areas.

5. CONCLUSIONS

ELISA and WB might help establishing which *Leishmania* species or related *Leishmania* species are most likely infecting dogs in endemic areas. Furthermore, WB could be a diagnostic option for early stages or subclinical infection in dogs.

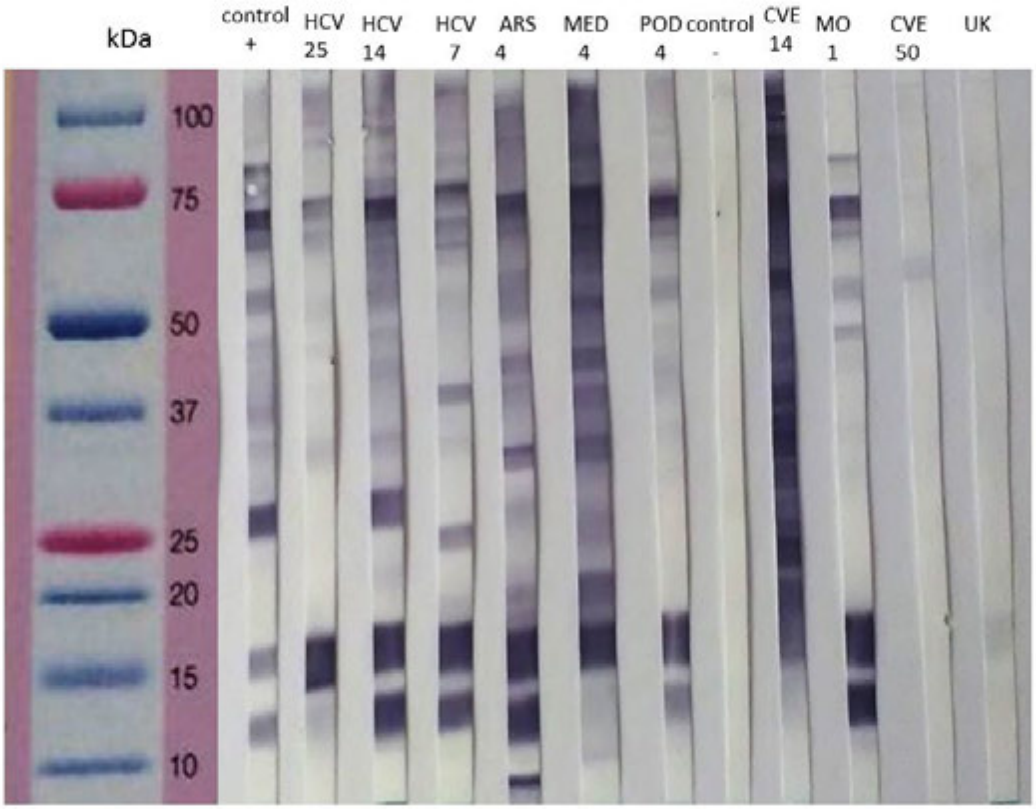

Figure 4. 1. Antibody recognition of *L. infantum* antigen by WB in sera of dogs from Venezuela and Spain. Dogs from Spain: hcv 25, hcv 14, hcv 7, ars4, med 4, pod4, mo1; dogs from Venezuela: cve 14, cve 50; dogs fromUK: UK, + positive, - negative.

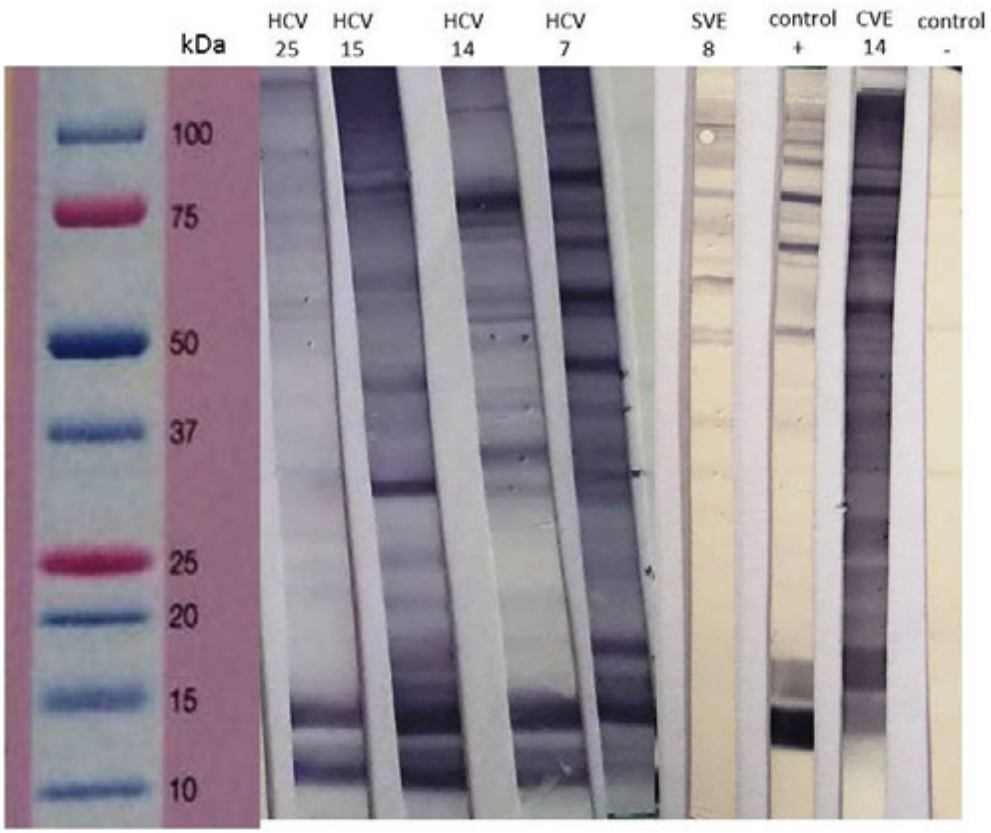

Figure 4.2. Antibody recognition of *L. braziliensis* antigen by WB in sera of dogs from Venezuela and Spain. Dogs fromSpain: hcv 25, hcv 15, hcv 14, hcv 7; dogs from Venezuela: cve 14, sve 8,

; + positive, - negative.

Table 4.1 Summary of diagnostics tests results from all dogs studied with both ELISA and WB

Country	ID dog	ELISA (EU)		Western blot (bands kDa)	
		L. infantum	*L. braziliensis*	*L. infantum*	*L. braziliensis*
Venezuela	Cve50	7.4 -	4.4-	65	16,18,46,70
	Yve7	30.1 -	8.96+	16,65	14,16,18,70
	Yve35	10.2 -	8.4 -	-	16,24,30,46
	Yve43	21.2-	10.6+	30,65	14,16,18,24,28,30,34,70
	Yve2	7.2-	4.3-	18	16,36,46,70
	Yve13	3.8-	2.5-	-	-
	Cve7	4.9 -	4.6-	-	-
	Cve14	3.5 -	3.6-	14,18,28,30,46,65	14,16,18,24,28,30,46,70
	Cve48	1.7 -	3.9-	-	-
	Cve 60	3.6 -	4.4-	-	46,70
	Sve18	34.6-	14.2+	65	34,46,65,70
	Sve19	17.2 -	12.0+	-	14,16,18,24,28
	Sve 20	15.3 -	10.3+	-	-
	Sve3	4.4 -	3.7-	-	46,70
	Sve4	11.6 -	4.9-	28,46	14,16,18,24,28,30,36,46,70
	Tve8	2.3 -	0-	-	24,46,70
	Tve12	73.2 +	19.3+	28,30,46,65	16,18,28,34,46,70
	Tve11	14.4 -	8.1 -	28,46	16,28,30
	Tve4	163.4 +	52.6+	24,28,36,44,65	14,16,24,28,30,34,36,70
	Tve19	25.5 -	0.6-	-	16,46,65,70

Spain	Hcv-14	239.2+	128.9+	14,16,18,24,28,30,34,36,40,52,70	14,16,20,28,34,52,65,70
	Hcv-7	135.6+	87.1+	14,16,18,28,30,46,65,70	14,16,28,34,52,65,70
	Hcv-15	1354.4+	340.0+	14,16,18,28,30,36,46,65,70	14,16,20,28,34,52,65,70
	Hcv-25	152.2+	74.0+	14,16,28,46,65,70	14,16,20,30,34,52,65,70
	Ars-4	24687.9+	5225.0+	14,16,18,28,30,34,44,46,65,70	14,16,18,30,36,46,70
	Mo-1	92.3+	24.1+	14,16,28,30,46,65	14,16,46,70
	Ars-8	68.5+	24.4+	28,30,65,70	24,28,36,70
	Med-4	300.0+	259.6+	14,16,18,28,30,34,44,46	14,16,18,30,38,44,50,65,70
	Ars-7	251.2+	126.6+	14,16,18,28,30,36,44,46,65,70	14,16,44,65,70
	Pod-4	77.4+	44.0+	14,16,24,28,30,34,44	14,16,28,30,36,46,70
UK	UK21522	23.3-	0.7-	18,20,28	-
	uk219499	16.01-	2.5-	16,18,24,44,46	-

Positive +, negative -. ELISA *L. infantum* cutoff at 35 U, *L. braziliensis* at 8.5 EU

Table 4.2 Antibody recognition of *L. braziliensis* antigens by WB in sera of dogs from Venezuela and Spain.

WB band (kDa)	***Leishmania braziliensis* antigen**					
	Total dogs*		Catalonia		Venezuela	
	N=40		N= 10		N=20	
	N° bands	%	N° bands	%	N° bands	%
70	23	12.4	10	12.9	13	12.2
68	8	4.3	3	3.9	5	4.7
65	8	4.3	6	7.7	2	1.9
58	1	0.5	1	1.3	0	-
56	7	3.8	3	3.9	4	3.8
52	3	1.6	3	3.9	0	-
50	2	1.1	1	1.3	1	1.0
48	5	2.7	0	-	5	4.7
46	14	7.6	3	3.9	11	10.3
44	2	1.1	2	2.6	0	-
42	4	2.2	0	-	4	3.8
40	0	-	0	-	0	-
38	3	1.6	1	1.3	2	1.9
36	6	3.2	3	3.9	3	2.8

34	10	5.4	5	6.4	5	4.7
30	9	4.9	3	3.9	6	5.6
28	14	7.6	7	9.0	7	6.6
24	9	4.9	2	2.6	7	6.6
22	3	1.6	0	-	3	2.8
20	8	4.3	5	6.4	3	2.8
18	9	4.9	2	2.6	7	6.5
16	21	11.4	9	11.5	12	11.2
14	16	8.7	9	11.5	7	6.5
Total number of bands	185		78		107	
Mean number of bands	8.1		3.4		4.7	

*No bands were observed from UK dogs

Table 4.3 Antibody recognition of *L. infantum* antigens by WB in sera of dogs from Spain, Venezuela and the UK.

WB band (kDa)	*Leishmania infantum* antigen							
	Total dogs		Catalonia		Venezuela		UK	
	N=40		N= 10		N=20		N= 10	
	N° bands	%	N° bands	%	N° bands	%	N° bands	%
70	7	4.8	7	6.7	0	-	0	-
68	0	-	0	-	0	-	0	-
65	14	9.5	7	6.7	7	24.1	0	-
58	0	-	0	-	0	-	0	-
56	3	2.0	2	1.9	1	3.4	0	-
52	1	0.7	1	1.0	0	-	0	-
50	0	-	0	-	0	-	0	-
48	6		4		2		0	

		4.1		3.9		7.0		-
46	18	12.2	7	6.7	4	13.8	7	50.0
44	6	4.1	4	3.9	1	3.5	1	7.1
42	5	3.4	5	4.8	0	-	0	-
40	4	2.7	4	3.9	0	-	0	-
38	3	2.0	3	2.9	0	-	0	-
36	4	2.7	3	2.9	1	3.5	0	-
34	4	2.7	4	3.9	0	-	0	-
30	12	8.2	9	8.7	3	10.3	0	-
28	16	10.9	10	9.6	5	17.2	1	7.1
24	6	4.0	4	3.9	1	3.5	1	7.1
22	1		1		0		0	

		0.7		1.0		-		-
20	6	4.0	5	4.8	0	-	1	7.1
18	10	6.8	6	5.8	2	7.0	2	14.3
16	11	7.5	9	8.7	1	3.5	1	7.1
14	10	6.8	9	8.7	1	3.5	0	-
Total number of bands	147		104		29		14	
Mean number of bands	12.3		4.4		2.4		1.2	

Catalonian samples recognized a significantly higher number of bands for *L. infantum* antigen when compared to *L. braziliensis* antigen (Wilcoxon signed-rank test: Z= -1,964 , p=0.050).

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CAPÍTULO 5

❖ **ESTUDIO 3**

Serological and molecular survey of *Leishmania* infection in dogs from Venezuela

CAPÍTULO 5

Serological and molecular survey of *Leishmania* infection in dogs from Venezuela

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ABSTRACT

Venezuela is an endemic country for human and canine leishmaniosis due to *Leishmania infantum*, *Leishmania braziliensis* and other *Leishmania* species. However, only limited data is available on canine *Leishmania* infection in Venezuela. The aim of this cross-sectional study was to evaluate the rate of *Leishmania* infection in dogs (n=152) from the states of Lara (n=91) and Yaracuy (n=61) from Venezuela by means of serological and molecular methods. Physical examination was performed and blood samples were collected from all dogs. Serology for antibodies reactive with *Leishmania infantum* and *L. braziliensis* antigens was assessed by the enzyme-linked immunosorbent assay (ELISA) and detection of *Leishmania* DNA from blood samples was evaluated by kinetoplast *Leishmania* real-time polymerase chain reaction (RT-PCR). In addition, *Leishmania* internal transcribed spacer (ITS-1) RT-PCR was performed on the samples positive by kinetoplast RT-PCR.

The rate of *Leishmania* infection based on serological and/or molecular techniques was 11.8%. The seropositivity rates for *L. infantum* and *L. braziliensis* antigens were 2.1% (3/144) and 8.3% (12/144), respectively. All dogs from the state of Yaracuy were serologically negative to *L. infantum* while 4.6% (4/86) of the dogs were reactive with *L. braziliensis* antigen. Fourteen percent (8/58) of the dogs from the state of Lara were positive to *L. infantum* and 5.2% (3/58) to *L. braziliensis* antigen. Three dogs were positive to both *Leishmania* antigens.

By PCR, 6.5% (4/61) and 4.4% (4/91) of the dog were positive for infection in the states of Lara and Yaracuy, respectively. The PCR product of one dog from the state of Yaracuy was

sequenced revealing a 100% identity with *L. infantum*. However, all PCR-positive dogs were seronegative to both *Leishmania* antigens.

In conclusion, the rates of positivity for *Leishmania* spp. infections observed indicate that dogs are frequently infected by *L. infantum*, *L. braziliensis* or related *Leishmania* spp. in Venezuela.

Keywords: *Leishmania infantum*, *Leishmania braziliensis,* canine, ELISA, PCR, Venezuela, South America.

1. INTRODUCTION

Canine leishmaniosis is widespread in South America and is among the most important canine vector-borne diseases in this region, mainly because of its major zoonotic relevance (Dantas-Torres et al., 2012). Leishmaniosis constitutes a significant public health in many areas of tropical and subtropical Latin America (Grimaldi and Tesh, 1993).

The primary mode of transmission of *Leishmania* parasites from dog to dog is through the bite of an infected phlebotomine sandfly. In South America, the sandfly vectors of *Leishmania* spp. belong to the genus *Lutzomyia* (López-Céspedes et al., 2012). Moreover, secondary modes of transmission might be involved and could be relevant for the establishment of new foci of canine leishmaniosis in non-endemic areas (Dantas-Torres, 2009).

Several *Leishmania* spp. have been isolated or molecularly characterized from dogs in South America: *Leishmania amazonensis.* (Tolezano et al., 2007), *Leishmania braziliensis* (Falqueto et al., 1991), *Leishmania colombiensis* (Pace, 2014), *Leishmania infantum* (syn. *Leishmania chagasi*) (De Andrade et al., 2006), *Leishmania mexicana* (Hashiguchi et al., 2018), *Leishmania panamensis* (Pace, 2014), *Leishmania peruviana* (Hashiguchi et al., 2018) and *Leishmania pifanoi* (Pace, 2014).

Leishmania braziliensis and *L. infantum* are the most widespread species responsible for infecting dogs in South America. The distribution of both species is probably wider than actually reported (Dantas-Torres, 2009). Importantly, there is considerable epidemiological and experimental evidence indicating that dogs are the main reservoir hosts for human infection with *L. infantum* in Latin American countries (Dantas-Torres, 2009; Reithinger and Davies, 1999).

Therefore, the domestic dog plays an important role in the epidemiology of zoonotic human visceral leishmaniosis due to *L. infantum* in these countries (Dantas-Torres, 2007). However, the dog is most likely only an incidental host for *L. braziliensis* and its role in the zoonotic cycle of its transmission is probably negligible (Dantas-Torres, 2007).

Most information on the prevalence of *L. infantum* and *L. braziliensis* infections among dogs in South America is from serological and molecular surveys conducted in Brazil (Carvalho et al., 2015; Marquez et al., 2017; Segatto et al., 2012) while a small number of studies originates from other countries such as Argentina (Barroso et al., 2015),

Colombia (Ramírez et al., 2016) and Venezuela (Jorquera et al., 2005; Panamerican Health Organization., 2018)

In Venezuela, cutaneous human leishmaniosis is most abundant in rural and suburban areas and is mostly prevalent in people with scarce economic resources such as farmers. The disease is widely distributed in the whole country, especially in the Andean region of Venezuela from which about 60% of the total cases are reported by the National Registry of Health (De Lima et al., 2002).

The *Leishmania* species that can cause tegumentary leishmaniosis in humans in Venezuela are *L. amazonensis, L. garnhami, L. pifanoi, L. venezuelensis, L. braziliensis, L. panamensis* and *L. colombiensis* (Oletta et al., 2011). Human visceral leishmaniosis is an important potentially fatal disease caused by *L. infantum*. *Leishmania amazonensis* in Venezuela which has been reported from various disease foci in humans and sporadically with visceral involvement (Zerpa et al., 2005).

However, limited data is available regarding *Leishmania* infections in canines in Venezuela (Aguilar et al., 1984; Feliciangeli et al., 2005). The aim of this cross-sectional study was to evaluate the rate of *Leishmania* infection in dogs from the states of Lara and Yaracuy in Venezuela by means of serological and molecular techniques.

2. MATERIAL AND METHODS

2.1. Study area and sample and data collection

Dogs

Dogs from urban areas in the states of Yaracuy (n=91) and Lara (n=61) in Venezuela that were admitted to veterinary clinics for routine health evaluation and vaccination from

March 2014 to January 2016 were included in the study. A full physical examination was performed before blood sampling by venipuncture from the dogs (n=152... Clinical status, breed, age, sex and full clinical history were also recorded.

The blood samples (n=152) were collected into ethylenediaminetetraacetic acid (EDTA) for DNA extraction and quantitative PCR (qPCR) and into plain tubes centrifuged to obtain sera samples (n=144) for the enzyme-linked immunosorbent assay (ELISA). All samples were stored at -20 °C before use. The geographical distribution (provinces and cities) of the dogs sampled is depicted in Figure 5.1.

Dogs that did not present evidence of systemic clinical signs or dermatological lesions were classified as apparently healthy (n=134). Dogs with evidence of systemic clinical signs (lymphadenomegaly, anorexia, pale membranes mucous; n=2) or dermatological lesions (exfoliative, ulcerative or pustular dermatitis, alopecia and erythema of scrotum; n=16) were classified as sick (n=18). Hematological and biochemical profiles were not performed due to the unavailability of reliable testing for dogs in the surveyed areas.

Dogs were classified according to age into two groups: young dogs if aged equal or less than 12 months, and adults if older than 12 months of age. In Yaracuy state, both sexes were represented with 30 females [young (n=15) and adult (n=15)] and 61 males [young (n=40) and adult (n=21)]. Dog breeds were represented by German shepherd (n=2), Siberian husky (n=5), Pitbull (n=6), Poodle (n=5), Rottweiler (n=3), Beagle (n=1), Dachshund (n=2), Dalmatian (n=2), Golden retriever (n=1), Cocker spaniel (n=1), Pinscher (n=5), Basset hound (n=1), Doberman (n=1) and Mastiff (n=1). Fifty-six were mongrel.

In Lara state, both sexes were represented with 16 females [young (n=4) and adult (n=12)] and 45 males [young (n=18) and adult (n=27)]. Dog breeds were represented by German shepherd (n=2), Pitbull (n=3), Poodle (n=3), Rottweiler (n=3), Dachshund (n=2), Dalmatian (n=3), Golden retriever (n=2), Weimaraner (n=1) and Mastiff (n=1). The rest were mixed-breed (n=40).

2.2. DIAGNOSTIC TECHNIQUES

2.2.1. Detection of antibodies against *L. infantum* and *L. braziliensis* antigens by the quantitative enzyme-linked immunosorbent assay (ELISA)

Serology was based on an *L. infantum* in house ELISA protocol previously described for dogs (Solano-Gallego et al., 2014) which was slightly modified. *Leishmania infantum* (MHOM/MON-1/LEM 75) and *L. braziliensis* (MHOM/BR/88/BCN-25) antigens were used in different wells in the same ELISA plate as recently described for cats (Rivas et al., 2018).

Dog sera were diluted to 1:800 in phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma Aldrich, St. Louis Missouri, USA) containing 1% dry milk and incubated for 1 hour at 37°C with sonicated crude *L. infantum* and *L. braziliensis* antigens (20 µg/mL) in plates divided so that half of the wells contained one antigen while the other half contained the other antigen .

The plates were then washed with 0.05% Tween 20 in PBS (PBSTween 20) and incubated with Protein A conjugated to horseradish peroxidase 1:30.000 dilution for 1 hour at 37°C.Plates were then washed again with 0.05% PBSTween20 and developed by

adding the substrate solution ortho-phenylene-diamine and stable peroxide substrate buffer (SIGMAFAST OPD, Sigma Aldrich, St. Louis Missouri, USA). The reaction was stopped with 50 µl of 2.5MH2SO4 and absorbance values were read at 492 nm by an automatic microELISA reader (ELISA Reader Anthos 2020, Cambridge, UK).

All plates included the serum from a sick dog with confirmed *L. infantum* infection as a positive control and serum from a healthy dog confirmed by serology and PCR as a negative control. All samples were analyzed in duplicate. The results were quantified in ELISA units (EU) related to a positive canine serum used as a calibrator and arbitrarily set at 100EU (Solano-Gallego et al., 2014).

The cut-off value was established at 35 EU (mean + 4 SD of values from 80 dogs from the United Kingdom, a non- endemic area) for *L. infantum*. Sera were classified as positive; when the results were equal or higher than 35 EU and negative when lower than 35 EU. The cut-off for *L. braziliensis* was established at 8.5U (mean + 4 SD of values of 80 dogs from the United Kingdom). Sera were classified as positive for *L. braziliensis* antigen when equal or higher than 8.5 EU and negative when lower than 8.5 EU.

2.2.2. BLOOD DNA EXTRACTION AND *LEISHMANIA* KINETOPLAST REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

DNA was extracted using the Gen Elute blood genomic DNA kit (Sigma Aldrich, Missouri, USA) and following the manufacturer's instructions with a minor modification. Forty µl of proteinase K solution were added to all samples. Four hundred µl of whole blood were used for DNA extraction from the blood samples. The other steps were

performed as described in the manufacturer's protocol. Blood from a clinically healthy non-infected dog was used as a control for DNA contamination in each DNA extraction performed (Solano-Gallego et al., 2016).

The presence of *Leishmania spp*. DNA was analysed by amplification of kinetoplast DNA sequence by a real-time polymerase chain reaction (RT-PCR). Each amplification was performed in triplicate in 20 µl reactions and included 15 pmol of direct primer (CTTTTCTGGTCCTCCGGGTAGG), 15 pmol of reverse primer (CCACCCGGCCCTATTTTACACCAA), 50 pmol of the labelled TaqMan probe (FAM-TTTTCGCAGAACGCCCCTACCCGC- TAMRA) and 5 µl of the tested DNA sample.

Amplifications and detection were performed using the ABI Prism 7700 system (Applied Biosystems, Foster City, CA, U.S.A.) in two-step temperature (94 and 55 ^{0}C) over 45 cycles. Positive controls (DNA from *L. infantum* MHOM ⁄ES ⁄04 ⁄BCN-61) and negative controls were included in each RT-PCR analysis (Martín-Ezquerra et al., 2009; Rivas et al., 2018).

2.2.3. *LEISHMANIA* INTERNAL TRANSCRIBED SPACER (ITS-1) RT-PCR BY HIGH RESOLUTION MELTING ANALYSIS AND SEQUENCING.

The identification of *Leishmania* species was performed only for *Leishmania* spp. RT-PCR positive dogs. A 265 bp fragment of the *Leishmania* internal transcribed spacer 1 (ITS1) region of the *L. infantum* rRNA operon was amplified by RT-PCR using primers ITS-219 F (AGCTGGATCATTTTCCGATG) and ITS-219R (ATCGCGACACGTTATGTGAG) and then evaluated by high resolution melting (HRM) analysis as previously described (Talmi-Frank et al., 2010). DNA samples extracted from cell cultures of *L. infantum*, *Leishmania tropica*,

Leishmania major were used as positive controls for each corresponding PCR reaction and DNA from colony-bred dogs negative by PCR for vector-borne pathogens was used as negative control. A non-template control (NTC) with the same reagents described above but without DNA was added to each PCR to rule out contamination.

All positive RT-PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Center for Genomic Technologies, Hebrew University of Jerusalem, Israel. DNA sequences were evaluated with the ChromasPro software version 2. 1.1 (Technelysium Pty Ltd., Australia) and compared for similarity with sequences available in GenBank®, using BLAST program.

2.3 STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 17.0 software for Windows (SPSS Inc., Chicago, USA). A non-parametric Mann-Whitney U-test was used to compare continuous variables among different groups. The non-parametric Wilcoxon signed-rank test was used to compare paired continuous variables. Fisher's exact test was used to compare frequencies among different groups. Mcnemar's test was used to compare frequencies among the same group. Cohen kappa agreement was used to compare diagnostic techniques. Differences were considered significant with a 5% significance level ($p < 0.05$).

3. RESULTS

3.1. ELISA

The results of serological tests performed are summarized in tables 5.1 and 5.2. The total percentages of *L. infantum* and *L. braziliensis* seropositive dogs were 2.1% (3/144) and 8.3% (12/144), respectively (McNemar test, p=0.09). The levels of antibodies reactive with *Leishmania infantum* and *L. braziliensis* antigens had a mean ± standard deviation (SD) of 39.1± 42.5 EU and 15.6±11.6EU, respectively (Wilcoxon signed-rank test, p=0.001).

All dogs from Yaracuy state were serologically negative to *L. infantum* antigen, while 4.6% (4/86) of the dogs were seroreactive with *L. braziliensis* antigen. Of the dogs from Lara state, 13.8% (8/58) were seroreactive with *L. braziliensis* in and 5.2% (3/58) with *L. infantum* antigen. Three dogs were seroreactive with both *Leishmania* antigens. However, higher antibody levels were found for *L. infantum* when compared with *L. braziliensis* (Wilcoxon signed-rank test, p=0.001, see table 5.2).

The antibody response was significantly higher to both *L. infantum* and *L. braziliensis* antigens (Mann-Whitney U-test, p= 0.005 and p = 0.077, respectively) in Lara state (*L. infantum*: mean ± SD of 101.6± 63.7 EU; *L. braziliensis*: mean±SD= 18.1 ± 13.2 EU) when compared to the response of seropositive dogs from Yaracuy state (*L. infantum*: mean±SD= 21.3±8.3 EU; *L. braziliensis*: mean±SD= 11.1±3.8 EU).

A fair agreement (Cohen kappa agreement of 0.35) was found between *L. infantum* and *L. braziliensis* antibody levels.

A significant association was found between *L. infantum* ELISA and being a female (Fisher´s exact test, p=0.0272; Table 5.1). Furthermore, a statistically non-significant trend

was found between *L. infantum* and *L. braziliensis* seropositivity and geographical location (Fisher´s exact test, p=0.0633 and p=0.0670, respectively) with dogs from Lara state having a higher *Leishmania* seropositivity rate than dogs from Yaracuy state. No other significant associations were encountered.

3.2. BLOOD RT-PCR

The PCR results are listed in tables 5.1 and 5.2. The total rate of *Leishmania* infection in blood samples by means of *Leishmania* kinetoplast RT-PCR was 5.3 % (8/152) with 6.5% (4/61) in Lara state and 4.4% (4/91) in Yaracuy state. No significant association was found between positivity by RT-PCR and age, sex, breed, geographical location or clinical status of dogs (Table5.1).

All the dogs positive for *Leishmania* by kinetoplast RT-PCR were seronegative by ELISA serology to both *Leishmania* antigens (Table 5.2).

The identification of *Leishmania* species was attempted by ITS-1 RT-PCR in for all dogs positive by *Leishmania* kinetoplast RT-PCR. However, the DNA sample from only one adult male dog from Yaracuy state resulted positive by the ITS-1 RT-PCR. The amplicon from this dog (ID:Cve50) was sequenced and 100% identity was found with *L. infantum* isolate MCAN/IL/2010/NT1 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence (ID GenBank®: KM677128®) (Yasur-Landau et al., 2016).

The overall results based on serological and molecular techniques are displayed in table 1. The total rate of *Leishmania* infection based on serological and/or molecular techniques was 11.8% (95% confidence interval of 7.5-18.0). Interestingly, adult dogs

appeared to have a higher rate of *Leishmania* spp. infection when compared with young dogs (Fisher´s exact test, p=0.0893). No other significant associations were found.

4. DISCUSSION

This study evaluated infection with *Leishmania* spp. in dogs in areas that are endemic for human leishmaniosis in Venezuela. Due to the fact that several *Leishmania* species co-exist in Venezuela, an ELISA with two *Leishmania* antigens (*L. infantum* and *L. braziliensis*) was used for the detection of antibodies reactive with these antigens (Rivas et al., 2017, see chapter 2). To the best knowledge of the authors, this is the first serological investigation of *L. braziliensis* infection in dogs from Venezuela.

In total, 2.1% of the sera reacted with *L. infantum* antigens and 8.3%, with *L. braziliensis* while the rate of *Leishmania* spp.infection detected by PCR in blood samples was 5.3%. However, the PCR-positive dogs were all seronegative by ELISA.

Interestingly, *L. braziliensis* was previously diagnosed in cutaneous lesions from several infected mammal species in Venezuela (Aguilar et al., 1984).

A study carried out in Cojedes State revealed human (16/124, 12,9%), dog (3/43, 7%) and (6/29, 21.4%) donkeys populations with ulcers, and other cutaneous lesions such as papules and nodules, skin and mucosal scars which were positive for *L. braziliensis*. *Leismania braziliensis* infection was diagnosed in that study by Giemsa stained cytological preparations. Cutaneous and mucosal lesions that were negative by cytological examination were triturated and injected subcutaneously into the hind paws of hamsters from which parasites were then isolated. The parasites isolated were submitted for

biochemical characterization by means of isoenzyme pattern at the Oswaldo Cruz Institute and they were shown to be *L. braziliensis* (Aguilar et al., 1984).

An epidemiological study performed in Venezuela in humans revealed that *L. braziliensis* it was found in seven states (Bonfante-Garrido et al., 1992): Districto Federal (Caracas); Lara (Barquisimeto, Crespo, Iribarren, Jimenez, Morán, Palavecino, Torres, Urdaneta); Nueva Esparta (Margarita); Portuguesa (Las Cruces, Rio Amarillo); Trujillo (Cuicas); Yaracuy (Agua Fria, Cambural, Guaremal); and Zulia (Zipa-Yare). *Leishmania venezuelensis* which is believed to be a variant of *L. mexicana* was found in the following states: Lara (Barquisimeto, Iribarren, Jimenez, Morán)(Kato et al., 2011); Merida (Zéa); and Yaracuy (Campos Elias). Therefore, *L. venezuelensis* has a much wider geographical distribution than was initially recognized and both species can occur in the same endemic regions (Bonfante-Garrido et al., 1992).

However, *L. venezuelensis* was not diagnosed in the present study in dogs differently from the recent results found in cats in Venezuela where *L. mexicana* was diagnosed (Rivas et al., 2018).

A study on dogs from the city of Ilhéus, north-eastern Brazil, showed a much higher rate of *Leishmania* infection (54.7%) when compared with the present study based on serological and molecular techniques (Carvalho et al., 2015). It is important to highlight that *L. major* antigen was employed for serological testing and not *L. braziliensis* antigen in the study from Brazil (Carvalho et al., 2015).

In this Brazilian study, only *L. braziliensis* was diagnosed in dogs while *L. infantum* infection was not documented (Carvalho et al., 2015). Furthermore, no correlation

between the serological and molecular test results was found in the Brazilian study (Carvalho et al., 2015) in agreement with the current study from Venezuela.

In a study performed in dogs naturally infected by *L. braziliensis* from Paraná in Brazil, the parasite was detected by PCR in the blood, intact skin and internal organs in dogs despite their healthy appearance (Marquez et al., 2017).. This agrees with findings from our study in which 5.3 % of the clinically healthy dogs were positive to *Leishmania* spp. by blood RT-PCR.

Several possible risk factors for *Leishmania* spp. infection were investigated in the present study. These included age, sex, breed, clinical status and geographical location. Interestingly, a significant association was only found between *L. infantum* seropositivity by ELISA and being a female dog. So far, the association of gender with *Leishmania* infection in dogs remains controversial and the majority of studies performed did not find differences between infection rates in female and male dogs in Europe (Cardoso and Solano-Gallego, 2013). As an example, a study performed in Catalonia documented that the development of clinical canine leishmaniosis did not reveal any predilection for sex, age, or breed (Miranda et al., 2008).

Another study performed in Catalonia revealed a higher prevalence among male dogs compared to females, 153 dogs out of the total (n=2110) studied were followed up from three to eight years (mean 4.1 years). (Fisa et al., 1999). However, when the sample was stratified by age in that study, the seroprevalence of leishmaniosis in puppies and adults was the same and differences were found only in old (>7years old) dogs, although the incidence rate remained constant in all age groups (Fisa et al., 1999). The authors of

that study proposed that an increase in female mortality, in which pregnancy and nursing may play an important role could be responsible for the difference in gender distribution of the infection (Fisa et al., 1999). In this sense, the owners of seropositive females repeatedly reported a close relationship between the death of the bitch and pregnancy, delivery and nursing (Fisa et al., 1999).

Studies on risk factors associated with *L. infantum* infection performed in South America are limited in number (Alves et al., 2016) when compared with Europe (Cardoso and Solano-Gallego, 2013). However, findings from those studies are usually similar to those reported from Europe with no strong evidence of sex predisposition to this parasitic infection. In a Brazilian study performed in a highly endemic area of canine *L. braziliensis* infection, no association was found (Carvalho et al., 2015). Similar result were found in another Brazilian study in which the prevalence of positive serology was similar in both sexes and across all ages (Lima et al., 2012) in agreement with the present study.

Although significant differences in infection rates were not encountered between geographical locations in this study, a trend of a higher seropositivity rate for both antigens was evident in dogs from Lara state when compared with dogs from Yaracuy state. A previous study conducted in Lara State (El Brazilar, Curarigua) t described isolation of the parasitefrom dogs and species identification by PCR based on telomeric sequences which detected *L.* i*nfantum* (Feliciangeli et al., 2005). This study concluded that, El Brasilar was an active focus of canine leishmaniosis due to *L. infantum*, mostly children were affected by the disease and the infection appeared to be increasing (Feliciangeli et al., 2005).

In addition, a recent investigation in cats from Lara state based on PCR and serological findings showed that cats were most likely infected with species of *L. mexicana* or related species of *L. mexicana* such as *L. braziliensis* species complexes rather than *L. infantum* (Rivas et al., 2018). However, the only dog infected with *L. infantum* in the current study and confirmed by ITS1 PCR and sequencing was from Yaracuy state. To the author's best knowledge, the present study documented canine *L. infantum* infection in Yaracuy state for the first time. Since dogs are considered the main reservoir for *L. infantum* infection, domestic dogs probably represent the main source of infection in humans also in Venezuela as found in other areas in the world (Sanchez et al., 2004).

The ELISA used in this study for the diagnosis of canine leishmaniosis in Venezuela does not determine the species of *Leishmania* infecting the seropositive dog. It is well recognized that serological cross-reactivity exists between different *Leishmania* species and also between *Leishmania* and *Trypanosoma* spp. exists (Cervantes-Landín et al., 2014).

This supports the recommendation for combined use of serological and/or molecular techniques in canine studies (Trevisan et al., 2015). The application of PCR together with serology not only helps in determining the extension of subclinical infections but also allows estimation of the number of dogs to be targeted for control measures. A greater number of infected dogs can be detected when serological and molecular techniques are employed (Rennó et al., 2014).

PCR was able to detect subclinical canine infection by *L. infantum* in the present study and previous studies (De Andrade et al., 2006), as well as dogs infected sub-clinically with *L. braziliensis* (Carvalho et al., 2015).

In this study, a combination of serological and molecular diagnostic techniques was used. The ELISA and qPCR discordant results can be attributed to the inherent differences between serological testing and molecular methods, and therefore, PCR from blood appears not to have a high sensitivity (Silva et al., 2011).

Another study performed from urban areas of Brazil showed that the prevalence of *L. infantum* infection in dogs determined by PCR and restriction fragment length polymorphism (RFLP) (24.7%) was higher than that detected by serology (15.9%), and demonstrated discordant results between serological and molecular tests (Veloso et al., 2011), in agreement with our study result. Sampling of other tissue such as skin, mucosal areas and lymph nodes was likely to improve the diagnosis of *Leishmania* infection in the present study as described elsewhere (Marquez et al., 2017; Pace, 2014).

Another important point to remark is the fact that significantly high antibody levels were found by *L. infantum* ELISA when compared with *L. braziliensis* ELISA in dogs from Venezuela. This finding is not surprising due to the fact that it is well known that humans produce low antibody levels against *L. braziliensis* (Fagundes-Silva et al., 2012) and the same appears to occur in canines (Figueiredo et al., 2009). Finally, as it is already well known and as found in this study, ITS-1 RT-PCR was less sensitive than kinetoplast RT-PCR in amplify ing *Leishmania* DNA (Talmi-Frank et al., 2010).

The limitations of this study include the small number of dogs and provinces studied from Venezuela, the lack of testing for other pathogens such as *Trypanosoma cruzi* or *Trypanosoma caninum*, the type of tissue sampled used (blood), and the lack of use of other more sensitive serological techniques such as western-blot (Persichetti et al., 2017; Solano-Gallego et al., 2014).

Other limitations are the absence of CBC, biochemical profile and urinalysis as well as a more detailed questionnaire to further define the demographic details of the dogs from Venezuela.

5. **CONCLUSION**

We conclude that the serological and PCR rates of *L. infantum* and *L. braziliensis* infection observed indicates that dogs might be frequently infected by these *Leishmania* species or closey related species in endemic areas in Venezuela.

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Ethics approval and consent to participate

Residual samples were used for all the testing described in the present study. Ethical approval was not required.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LSG designed the research study. LSG supervised technical work. LSG, AKR, and PMO contributed with data analysis and interpretation. LSG and AKR wrote the manuscript. AKR examined and collected samples from dogs living in Venezuela. PMO, MA, CR and AKR performed serological testing. RF, AKR, MA, SM, GB and YNB performed the molecular diagnosis in this study. All authors read and approved the final version of the manuscript.

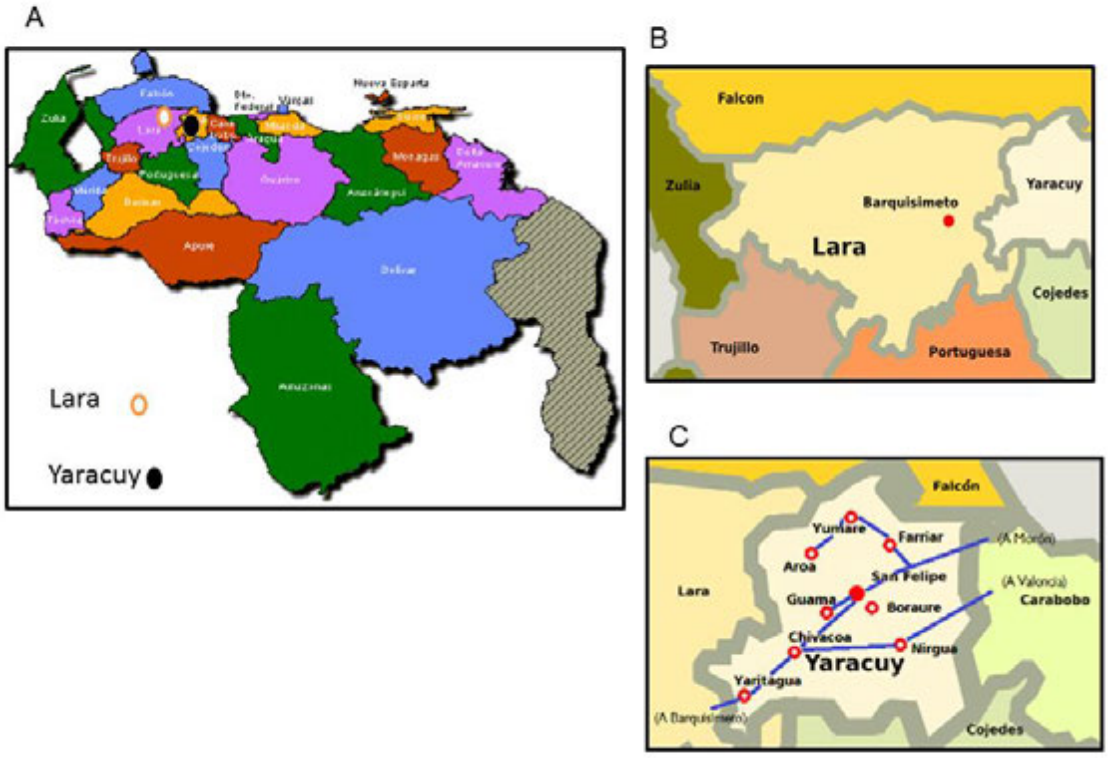

Figure 5.1 A map of Venezuela highlighting Yaracuy and Lara states (a) and the cities (b and c) where canine sampling was performed.

Table 5.1 Summary of serological and molecular results from dogs living in Yaracuy and Lara states (Venezuela).

Risk factors / Diagnostic tests		Number (%, 95% CI) of positive dogs													
		Age		P-value	Sex		P-value[a]	Breed		P-value	Location[b]		P-value	Clinical status		P-value
		Young (n=77)	Adult (n=75)		Male (n=106)	Female (n=46)		Pure Breed (n=56)	Mixed Breed (n=96)		Lara	Yaracuy		Healthy Dogs (n=134)	Sick Dogs (n=18)	
ELISA (n=144)	*L. infantum*	0	3 (3.8%,0.9-11.3)	0.2448	0	3 (6.1%,1.5-17.2)	**0.0272**	0	3 (3%,0.7-8.8)	0.2973	3 (5.2%, 1.2-14.7)	0	**0.0633**	3 (2.1%, 0.5-64.5)	0	1.0000
	L. braziliensis	2 (2.5%, 0.2-9.2)	6 (7.8%,3.3-16.3)	0.2750	8 (7.4%,3.6-14.1)	4 (8.2%,2.7-19.7)	1.0000	6 (10.5%, 4.5-21.5)	6 (6%,2.5-12.7)	0.3585	8 (13.8%,6.9-25.2)	4 (6.6%, 2.1-16.1)	**0.0670**	10 (7.2%,3.8-12.9)	2 (10.5%, 1.7-32.6)	0.6600
Kinetoplast RT-PCR[c] (n=152)		4 (5.1%, 1.6-13.0.)	4 (5.3%, 1.6-13.3)	1.0000	5 (4.7.%, 1.8-10.8)	3 6.5%, 1.6-18.1)	0.6987	4 (7.0%, 2.3-17.4)	4 (4.0%, 1.3-10.5)	0.4672	4 (6.5%, 2.1-16.1)	4 (4.4%, 1.3-11.1)	0.7144	8 (6.5%, 2.8-11.5)	0	0.5968
L. infantum or *L. braziliensis* ELISA or RT-PCR[d]		6 (7.7%, 3.3-16.2)	13 (17.3%, 10.2-27.5)	**0.0893**	13 (12.2.%, 7.2-19.9)	10 (21.7%,12.0-35.7)	0.1457	10 (17.8%, 9.8-30.0)	13 (13.5%, 7.9-21.9)	0.4894	15 (17.4%, 10.7-26.9)	8 (8.7%, 4.3-16.6)	0.1172	21 (15.6%, 10.4-22.8)	2 (11.1%, 1.8-34.0)	1.0000

Abbreviations: CI, confidence interval; RT-PCR, real-time polymerase chain reaction [a]p value < 0.05 o close to 0.05 is highlighted in bold. [b]Sera and blood samples studied from dogs living in Yaracuy state were 58 and 61 and from Lara state were 86 and 91, respectively. [c]one adult male dog from Yaracuy state was confirmed as infected with *L. infantum* by partially sequence ITS1 with 100% of identity. [d]Calculated based on number of positive dogs based on *L. infantum* ELISA and/or *L. braziliensis* ELISA and/or RT-PCR without repetition of dogs if positive to more than one technique.

Table 5.2 Summary of dogs diagnosed with *Leishmania* infection based on serological or molecular tests.

Geographical location	ID dog: signalment	ELISA (EU)		Blood RT-PCR *Leishmania* Kinetoplast *spp*
		L. infantum	*L. braziliensis*	
Lara state	Tve 5:4 years old female Mixed breed	-4.5	-0.0	+
	Tve 4: 2 years old female Mixed breed	+163.3	+52.6	-
	Tve 12 : 5 years old femaleMixed breed	+73.1	+19.3	-
	Tve 22 : 3 years old maleDaschund	-10.5	-1.4	+
	Sve 29: 2 years old male Mixed breed	-7.6	+11.1	-
	Sve 20: 5 years old male Pitbull	-15.3	+10.3	-
	Sve 19: 2 years old male Mastiff	-17.2	+10.3	-
	Sve 18: 1 years old female Mixed breed	-34.6	+14.2	-
	Sve 11: 6 years old female Mixed breed	+68.5	+18.1	-
	Sve 9: 2 months old male Mixed breed	NP	NP	+
	Sve 8: 7 years old female Poodle	-3.2	-2.3	+

	Sve 6: 12 years old male Mixed breed	-20.0	+13.0	-
Yaracuy state	Yve7:3 years old male Husky	-30.1	+8.9	-
	Yve 35:1 year old male Husky	-10.2	+8.5	-
	Yve 43 :5 years old Female German shepherd	-21.2	+10.5	-
	Cve 20:2 years old male Mixed breed	-24.0	+16.7	-
	*Cve 50:2 years old male Mixed breed	-7.4	-4.4	+
	Cve 32:4 months old male Mixed breed	-8.6	-5.0	+
	Cve 28:1 years old female Pitbull	-6.6	-3.5	+
	Yve 36:4 months old male Poodle	-2.1	-3.2	+

+: positive, -: negative, * dog confirmed infected with *L. infantum* based on sequencing, NP: not performed

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DISCUSIÓN

CAPÍTULO 6

DISCUSIÓN

La leishmaniosis es una enfermedad parasitaria que en Venezuela es de importancia en la salud pública (Oletta et al., 2011). Sin embargo, en el perro y en el gato, los estudios son limitados, existen fallas en el registro de casos en estas especies, debido a dificultades en la certeza del diagnóstico, que podría atribuirse a la falta de las diferentes herramientas diagnósticas y desconocimiento de los aspectos clínicos y epidemiológicos de esta enfermedad.

Generalmente, en el ejercicio de la clínica diaria, se realizan solo pruebas directas tales como citología y biopsia en los animales que presentan clínica compatible con leishmaniosis, pero no en todos los casos se evidencian las formas parasitarias para confirmar el diagnóstico. Un resultado positivo mediante la detección directa del parásito en un animal sospechoso confirma el diagnóstico de leishmaniosis; pero, un resultado negativo no permite descartar la enfermedad (Solano-Gallego et al., 2011). Es necesario hacer otras pruebas que forman parte del abanico de diagnóstico de la enfermedad, para evitar que pasen desapercibidos animales infectados.

Por otro lado, las medidas de control vectorial no se realizan de forma rutinaria, y aún se recomienda la eutanasia a los animales afectados en algunos países como Brasil y Venezuela, siendo esta completamente descartada en otros países como en Europa. Se han realizado estudios donde se confirma que la eutanasia no controla ni disminuye el número de casos clínicos en personas y animales (Miró et al., 2017).

En la presente tesis doctoral el primer estudio que se describe en el capítulo 3 fue basado en los aspectos clínicos de la leishmaniosis cutánea felina. Los signos

clínicos fueron lesiones nodulares y ulcerativas múltiples y/o solitarias. Estos casos fueron diagnosticados en Baquisimeto, Quíbor y Cabudare del estado Lara. En el año 1991 fue publicado un artículo que reportaba casos de leishmaniosis felina en Barquisimeto estado Lara (Bonfante-Garrido et al., 1991) desde entonces no se habían realizado publicaciones al respecto. Por este motivo, en Venezuela, en el diagnóstico diferencial de lesiones nodulares y ulcerativas, principalmente las ubicadas en la nariz y orejas, en el gato debe ser incluida la infección por *Leishmania*.

En este estudio se utilizaron diferentes técnicas en gatos con lesiones tales como citología, biopsia e inmunohistoquímica para el diagnóstico de *Leishmania spp.*. Las técnicas serológicas y moleculares fueron realizadas en los gatos enfermos y también en los clínicamente sanos. Dos de los cinco gatos enfermos resultaron positivos a ELISA para ambos antígenos, observándose un mayor nivel de anticuerpos de *L. braziliensis* al comparar con *L. infantum*, el resultado de PCR de sangre de estos dos gatos fue negativo.

Sin embargo, el resto de gatos enfermos presentaron un resultado positivo a PCR de sangre, todos los gatos clínicamente sanos resultaron negativos a la ELISA y PCR de sangre.

En relación a los resultados de WB, se observó un número mayor de bandas compatibles con *L. braziliensis* que con *L. infantum* en gatos enfermos y sanos. Basado en los resultados de nuestro estudio y en una investigación previa realizada en gatos (Persichetti et al., 2017), es posible determinar infección temprana a través de WB tanto en animales enfermos y como clínicamente sanos que presenten resultados negativos a ELISA y PCR. Por lo tanto, es recomendable realizar western blot para

detectar infección subclínica en gatos. En la figura 1 se describe un algoritmo para el uso de pruebas diagnósticas de leishmaniosis en perros y gatos-

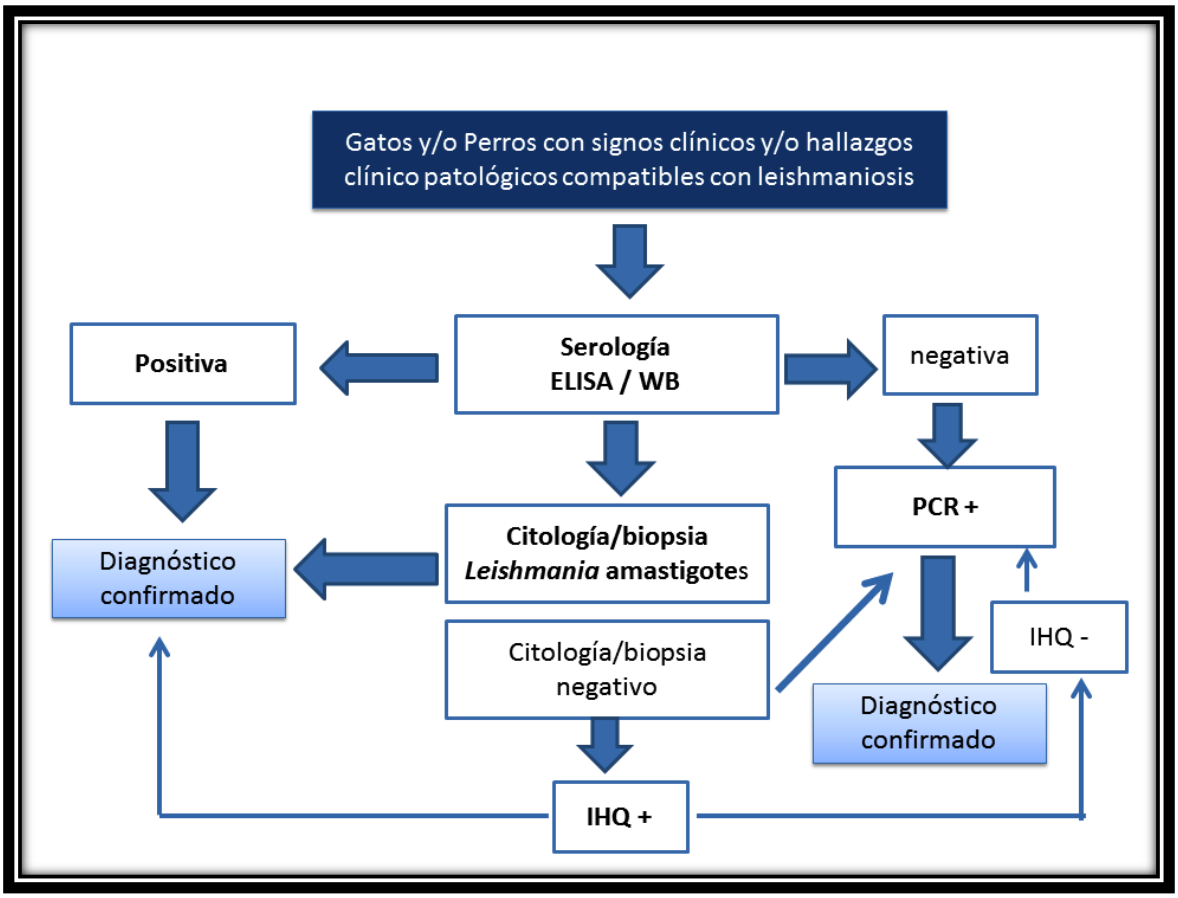

Figura 6.1. Algoritmo para el diagnóstico de leishmaniosis en perros y gatos modificado de (Solano-Gallego et al., 2011)

La identificación de especies de *Leishmania* fue posible realizar en solo dos gatos en los cuales previamente se realizó PCR para el diagnóstico de *Leishmania spp*. de citologías y biopsias de las lesiones cutáneas. De la identificación de la especie se obtuvo como resultado *L. mexicana* en dos de 3 casos enfermos. Estos serían los primeros casos de leishmaniosis cutánea felina causada por *L. mexicana* en Venezuela estado Lara.

Es importante destacar que la infección por *L. mexicana* ha sido previamente reportada en humanos con leishmaniosis cutánea en el estado Lara, estos autores sugieren que *L. venezuelensis* es una variante de *L. mexicana* (Kato et al., 2011). Por lo tanto, la investigación realizada anteriormente con gatos con lesiones similares compatibles con *L. venezuelensis* en Barquisimeto-Lara (Bonfante-Garrido et al., 1991), es probable de que se trate de una variante de *L. mexicana* como en el presente estudio.

En la tabla 6.1 se resume la distribución geográfica de la leishmaniosis felina en Sudamérica y los principales hallazgos clínicos.

Tabla 6.1. Especies de *Leishmania* identificadas en gatos en Sudamérica así como los signos clínicos y las alteraciones de laboratorio asociadas, modificado y actualizado (Pennisi et al., 2015).

Especie	País	Signos clínicos	Alteraciones de laboratorio	Referencias
Leishmania amazonensis	Brasil (Mato Grosso do Sul state)	Lesiones nodulares y ulcerativas múltiples y/o solitarias.	Sin hallazgos relevantes	(De Souza et al., 2005)
Leishmania braziliensis	Brasil (Belo Horizonte)	Lesiones nodulares y ulcerativas múltiples y/o solitarias.	Sin hallazgos relevantes	(Passos et al., 1996) (Schubach et al., 2004)
Leishmania infantum	Brasil (Cotia, São Paulo state) Brasil (Rio de Janeiro) Brasil (Andradina, São Paulo state) Brasil (Araçatuba, São Paulo state)	Lesiones nodulares y ulcerativas múltiples y/o solitarias, dermatitis exfoliativa, pápulas hemorrágicas.. Signos sistémicos: ictericia, hepatomegalia, esplenomegalia, poliuria,polidipsia, caquexia, fiebre, vómito, diarrea, anorexia, signos oculares, lesiones orales, letargia	Anemia no regenerativa, pancitopenia, linfocitosis relativa, hiperproteinemia con hipergammaglobulinemia, proteinuria, aumento de creatinina, aumento de ALT	(Savani et al., 2004) (da Silva et al., 2008) (Coelho et al., 2011) (Coelho et al., 2010) (Metzdorf et al., 2017)
Leishmania venezuelensis	Venezuela Barquisimeto , estado Lara	Lesiones nodulares y ulcerativas múltiples y/o solitarias	Sin hallazgos relevantes	(Bonfante-Garrido et al., 1991)
Leishmania mexicana	Venezuela Barquisimeto , Quíbor, estado Lara	Lesiones nodulares y ulcerativas múltiples y/o solitarias	Sin hallazgos relevantes	(Rivas et al., 2018)

El segundo estudio descrito en el capítulo 4 se realizó una comparación de técnicas serológicas para la detección de anticuerpos frente a *L. infantum* y *L. braziliensis* en perros de diferentes regiones. Las técnicas de ELISA y WB fueron realizadas en perros que vivían en áreas endémicas de Venezuela y Cataluña, con el fin de evaluar la respuesta de anticuerpos específicos a cada antígeno y discriminar entre las especies de *Leishmania* estudiadas. Los estudios realizados con pruebas serológicas que permitan discriminar la respuesta de anticuerpos específicos en caninos infectados por diferentes especies de *Leishmania* son limitados (Trevisan et al., 2015).

En el presente estudio un mayor porcentaje de perros clínicamente sanos provenientes del estado Lara y Yaracuy en Venezuela fueron seroreactivos a ELISA para antígeno de *L. braziliensis* en comparación con *L. infantum.* Lo contrario se observó en los perros enfermos provenientes de Cataluña, los cuales presentaron mayores niveles de anticuerpos frente a ELISA para *L. infantum* que para *L. braziliensis.*

De acuerdo a los resultados de esta investigación la técnica de ELISA cuantitativa con diferentes diluciones puede ser útil para evitar resultados con alta saturación (Solano-Gallego et al., 2016) y contribuye a sugerir cual especie de *Leishmania* es más probable que este ocasionando la infección en perros de las respectivas áreas endémicas estudiadas. Este hallazgo es muy importante en las áreas donde varias especies del parásito coexisten como es el caso de Venezuela.

La técnica de WB reveló resultados similares en los perros de Venezuela, se evidenció un alto número de polipéptidos para *L. braziliensis* en comparación con *L. infantum* Estos hallazgos son similares a los observados en los resultados de ELISA y WB obtenidos en el estudio número 1 realizado en gatos clínicamente sanos y con

leishmaniosis cutánea. Los antígenos de bajo peso molecular (12-14 y 14-18 kDa) parecen ser los más específicos para reconocer la infección subclínica en el perro y en el gato en concordancia con estudios previos realizados en Europa (Aisa et al., 1998; Persichetti et al., 2017). Estos fueron identificados en las WB realizadas en perros y gatos del estudio 1 y 2 de esta tesis doctoral.

En el caso de los perros de Cataluña fue observado un alto número de polipéptidos de *L. infantum* en comparación con *L. braziliensis*. Los resultados de WB para *L. infantum* en perros es similar a la demostrada en estudios anteriores (Aisa et al., 1998; Trevisan et al., 2015).

Es importante destacar, que el rol del perro como reservorio de *L. braziliensis* es incidental, y se han realizado eutanasias en perros serológicamente positivos sin discriminar la especie de *Leishmania* involucrada (Morgado et al., 2016). Este estudio brinda una alternativa para discriminar entre especies y evitar eutanasias innecesarias, además de promover el uso las técnicas serológicas con otros antígenos, entre los cuales podría utilizarse el de *L.mexicana* ya que ha sido esta especie reportada en diversas zonas endémicas en Venezuela(Kato et al., 2011) .

El tercer estudio descrito en el capítulo 5 determino la tasa de infección mediante técnicas serológicas y moleculares en perros clínicamente sanos del estado Lara y Yaracuy, áreas endémicas de leishmaniosis cutánea y visceral en Venezuela, en las cuales las especies de *L. infantum* y *L. braziliensis* coexisten. Por lo tanto, la ELISA para determinar ambos antígenos fue realizada de manera similar al estudio número 1 y 2.

Los hallazgos obtenidos en este estudio en perros sanos diagnosticados serológicamente positivos a *L. braziliensis,* no habían sido anteriormente reportados en el país. También se realizaron estudios moleculares a través del análisis de PCR de sangre, en los cuales se obtuvo un porcentaje de positividad del 5.3%. Sin embargo, los perros PCR positivos no presentaron resultados positivos a ELISA para ambos antígenos.

En este caso no fue evidenciada una correlación entre los resultados de las pruebas serológicas y moleculares, hallazgo similar fue observado en un estudio anterior en Brasil que presento una alta tasa de infección para *L. braziliensis* en perros sin lesiones compatibles (Carvalho et al., 2015).

Diversos factores de riesgo fueron investigados en el presente estudio como edad, sexo, raza, estadio clínico y localización geográfica, pero no se hallaron resultados estadísticamente significativos a excepción de la asociación encontrada entre los resultados de ELISA por *L. infantum* y el sexo hembra. En varios estudios realizados anteriormente, no se encontró una predisposición evidente para alguno de los factores de riesgo mencionados (Cardoso and Solano-Gallego, 2013; Miranda et al., 2008).

Por otro lado, a pesar de que no hubo un resultado estadísticamente significativo en relación a la ubicación geográfica, se observó una mayor tendencia a la seropositividad a ambos antígenos en el estado Lara que en Yaracuy. Estudios previos ya han identificado al estado Lara como área endémica de leishmaniosis canina y humana (Feliciangeli et al., 2005; Kato et al., 2011).

En relación a los resultados de las pruebas moleculares para la identificación de especies de *Leishmania,* un solo perro del estado Yaracuy resultó infectado por *L. infantum.* El presente estudio documenta, por primera vez, la infección por *L. infantum* en un perro clínicamente sano del estado Yaracuy.

En Venezuela hasta 1962 no existía un programa de control y tratamiento de esta enfermedad, por ello se creó la División de Dermatología Sanitaria y con esto se comenzaron a llevar los primeros registros de casos en humanos. Este registro se inició con muchas deficiencias dado que se contaba solo con unos pocos servicios con personal capacitado, y por lo tanto la mayoría de los pacientes no eran diagnosticados ni reportados. Adicionalmente, no se tenía la infraestructura adecuada para llevar un registro de los casos diagnosticados (De Lima et al., 2010).

Con el transcurso del tiempo ha mejorado de forma notable el registro de casos, sin embargo, se mantiene un subregistro importante por diversas razones tales como: persistencia de áreas endémicas sin acceso o con dificultades de acceso a los servicios de salud; fallas en el sistema de registro de casos, y un número significativo de casos que son diagnosticados y tratados por servicios médicos o centros de investigación ajenos al programa de control y que no son reportados (De Lima et al., 2010).

En Venezuela, se han reportado casos de leishmaniosis cutánea en humanos en los siguientes estados: Trujillo, Mérida, Lara, Sucre, Táchira, Cojedes, Miranda, Barinas, Bolívar, Portuguesa, Guárico, Apure, Vargas, Amazonas, Anzoategui, Yaracuy, Aragua, Monagas, Carabobo, Falcón, Zulia, Distrito Capital, Delta Amacuro, Nueva Esparta (De Lima et al., 2010).

A su vez, estos estados han sido clasificados por grupo. Un primer grupo está conformado por los estados Trujillo y Mérida con tasas superiores a 30 por 100.000 habitantes, un segundo grupo de estados (Lara, Sucre, Táchira, Cojedes) con tasas de 20 o más pero menores de 30, en tercer lugar los estados Yaracuy, Miranda y Anzoátegui con tasas que son de 10 o más y menores de 20 y finalmente el resto de los estados (quince en total, incluido el Distrito Capital) con tasas inferiores a 10 por 100.000 habitantes (Oletta et al., 2011).

En relación a la leishmaniosis visceral (LV), esta también constituye un problema de salud pública en Venezuela tanto por su morbilidad como por los cambios de comportamiento en su patrón epidemiológico, siendo de carácter endémico y focal, reportando casos desde 1941 hasta el presente. Se han descrito tres principales focos de LV: un foco Central que comprende los estados Aragua, Carabobo, Guárico, Cojedes y Yaracuy; un foco Occidental conformado por los estados Falcón, Lara, Portuguesa, Trujillo y Zulia y un foco Oriental constituido por los estados Nueva Esparta, Sucre, Anzoátegui y Monagas (Romero et al., 2012).

Casos de leishmaniosis visceral en humanos y perros han sido reportados principalmente en las áreas endémicas anteriormente mencionadas (Breve et al., 2007), así como también se han realizado estudios que reportan casos de leishmaniosis cutánea en personas y otros mamíferos (Aguilar et al., 1984), además del reporte de casos clínicos de gatos con leishmaniosis cutánea por *L. venezuelensis* previamente descrito en el estado Lara (Bonfante-Garrido et al., 1991).

La presente tesis doctoral se enfocó en el estado Lara y Yaracuy. Los hallazgos obtenidos coinciden con estudios previos en el cual el estado Lara presenta un mayor número de casos de infección por *Leishmania* tanto en animales como en humanos (Feliciangeli et al., 2005). Sin embargo, es posible que existan más casos de animales infectados por *Leishmania* en el estado Yaracuy y que no se esté realizando un registro correcto para reportarlos, a través de esta investigación se pudo demostrar el caso de un perro clínicamente sano portador de *L. infantum*, y otros animales seropositivos a *L. braziliensis*, es decir que existen perros con infección subclínica en esta región.

Es probable que durante esta investigación se hubiesen podido obtener más hallazgos, si se hubiese tenido la oportunidad de realizar hemograma y bioquímica sanguínea tanto en los perros como en los gatos, para de esta manera interpretar y relacionar los cambios clínico-patológicos con la infección clínica y subclínica. Lamentablemente no fue posible, debido a que no se contaba con suficientes recursos para hacerlo, con lo cual, esta sería una limitación de la presente tesis doctoral. Igualmente, el número de casos incluidos tanto gatos enfermos como sanos fue reducido y por tanto también limitada la información sobre la epidemiologia y clínica de la leishmaniosis felina en Venezuela.

A su vez, también fue reducido el número de perros estudiados en Venezuela Otras limitaciones de la presente tesis doctoral son: 1) el uso solo de sangre para la detección de ADN del parasito tanto en perros como en gatos, 2) haber estudiado más variables demográficas para evaluar factores de riesgo asociados en animales y personas entre otros.

Sería interesante realizar investigaciones futuras en áreas endémicas, en perros y gatos clínicamente sanos, con lesiones cutáneas compatibles con leishmaniosis y también que presenten únicamente signos clínicos inespecíficos como anorexia, hepatomegalia, esplenomegalia, anorexia, linfadenomegalia, vómito, diarrea, letargia. En estos animales se podría elaborar protocolo de diagnóstico que abarcara las diferentes pruebas desarrolladas en esta tesis e incluir en las pruebas serológicas el antígeno de *L. mexicana,* ya con el conocimiento que ha aportado esta tesis al identificar mediante técnicas moleculares esta especie en Venezuela en gatos.

A pesar de las limitaciones, el desarrollo de estos estudios de la presente tesis doctoral proporcionan un avance en el conocimiento de la clínica, epidemiología e inmunopatogénesis de la leishmaniosis canina y felina producida por *L. braziliensis*, *L. infantum,* y *L. mexicana.*

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CONCLUSIONES

CAPÍTULO 7

CONCLUSIONES

Basado en los resultados de cada estudio, las conclusiones de esta tesis doctoral son las siguientes:

1. Lesiones nodulares y ulcerativas en los gatos, principalmente las ubicadas en la nariz y orejas, pueden ser causadas por *L. mexicana* o especies altamente relacionadas en Venezuela.
2. Las técnicas de diagnóstico más adecuadas en gatos enfermos son el examen citológico o histopatológico junto con la inmunohistoquímica, ya que la PCR sanguínea y la serología mediante ELISA frecuentemente resultan negativas.
3. Las técnicas serológicas de ELISA y WB con diferentes antígenos como *L. infantum* y *L. braziliensis* pueden ayudar a discriminar entre la infección entre diferentes especies de *Leishmania.*
4. La técnica de WB permite detectar anticuerpos para *L. infantum* y para *L. braziliensis* en perros y gatos sanos en mayor proporción que al comparar con ELISA, lo cual ayuda a identificar infecciones subclínicas.
5. Los hallazgos del estudio serológico y molecular en perros clínicamente sanos en áreas endémicas de Venezuela demuestran que los perros pueden ser infectados por *L. infantum* y/o *L. braziliensis* de forma subclínica.
6. Los únicos factores de riesgo asociados a la infección por *Leishmania* en Venezuela en perros parecen ser el sexo y la distribución geográfica.