Universidad de Granada

Departamento de Parasitología

Programa Oficial de Doctorado en Farmacia

Diseño y evaluación de nuevas técnicas moleculares para la detección y diagnóstico de *Leishmania* spp. y su aplicación en estudios epidemiológicos.

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Memoria de Tesis Doctoral presentada por la licenciada en Biología

Gemma Merino Espinosa para aspirar al grado de

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Granada, 3 de abril de 2017

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Directores de la Tesis

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Fdo.:

A mi familia

A Víctor

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"I am among those who think that science has a great beauty. A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena which impress him as though they were fairy tales"

Marie Curie

Índice

I. Abstract

I. Abstract

Molecular-based diagnostic methods, such as PCR, are highly sensitive and specific assays for the detection of *Leishmania* DNA and the differentiation of species, thus considered the cornerstone in the diagnosis of leishmaniasis, but they are variable depending on the sample used. However, lack of detailed standardization and global homogenization and validation together with the reduction of specificity in endemic areas are the main disadvantages of these useful diagnostic tools.

Molecular biological techniques are now becoming powerful tools for sandfly research. Therefore, more detailed information on the risk factors for leishmaniasis, such as the prevalent sand fly species or lineage as well as the seasonal variation in the infection rate and transmission risk, can be accumulated using such techniques in various endemic areas in different seasons.

Molecular techniques undoubtedly contribute to reservoir research: a variety of samples, from hair to bone marrow can be used to establish the infection rate in a given host. In addition, the mammal blood meal can be identified from blood-fed female sandflies.

The relationships between the parasite, its vector and reservoirs build a complex and dynamic network that can be disentangled by the use of molecular tools in order to contribute to the molecular diagnosis and epidemiology of the disease. Therefore, the objectives of the present work are: To evaluate the influence of the epidemiological scenario from two endemic areas, Spain and Morocco, on the reliability of the PCR techniques and contribute to the selection of the most efficient one for cutaneous leishmaniosis and typing *Leishmania* species. To contribute to the improvement in the diagnosis of cutaneous and mucosal leishmaniasis through the establishment of a diagnosis protocol from histological samples. To analyse the composition and distribution of *Phlebotomus sergenti* mitochondrial lineages in Southwestern Europe using a novel PCR-RFLP for the Cytochrome B population analysis. To investigate *Leishmania infantum* infection in wild rabbits (*Oryctolagus cuniculus*) and discuss their potential role as reservoirs in a recognized endemic area with ample epidemiological knowledge, as is the case of Southeastern Spain. The sympatric and syntrophic presence of other kinetoplastid, *Trypanosoma nabiasi*, was taken into consideration to avoid confusion. To detect and characterize *L. infantum* infection in wild rodents in an epidemiological study in a well-known focus of canine leishmaniasis due to *L. infantum* in Southern Spain. To provide an insight into the genetic variability of *Trypanosoma nabiasi*, its *in vitro* cultivation and infectivity, and finally investigate the course of infection in its host (*O. cuniculus*).

The sensitivities and specificities of parasitological 35 methods and four PCRs were compared in cutaneous samples from 77 patients from Spanish (SPH) and Moroccan hospitals (PMH). Exudates and fresh or paraffin-embedded tissue biopsies were used. Sandflies were captured in the Iberian Peninsula and on the Canary and Balearic Islands. Mitochondrial lineage identification of 137 P. sergenti was performed using a novel PCR-RFLP that avoids the necessity of gene sequencing. In an endemic area for canine leishmaniasis in the southeast of Spain, 150 rabbits were captured over a period of three years. Samples of blood, bone marrow, liver, spleen, heart and skin were taken and analysed through parasitological, serological and molecular techniques in order to detect *Leishmania* and *Trypanosoma*. Blood, liver, spleen, bonemarrow, and skin from 37 rodents (24 *Apodemus sylvaticus*, 9 *Rattus rattus*, and 4 *Mus musculus*) were analysed by optical microscopy, culture, and two different polymerase chain reactions

None of the PCRs used in this study allows the diagnosis of all CL cases, showing also some drawbacks. Lmj4/Uni21-PCR displayed the best sensitivity with PMH but it did not provide positive results in PSH, although they were positive with other PCRs. Conversely, JW13/JW14-PCR and *L. infantum*-PCR-ELISA displayed good sensitivities with PSH that were not achieved with PMH. Nested-ITS-1-PCR showed higher sensitivity with PSH than PMH. False negative results were obtained in 19% of PSH due to unspecific hybridizations of ITS-1 primers with human chromosome. Two lineages were evidenced, the typical Iberian one (lineage I) and another, held in common with North Africa (lineage III), that show a distinctive distribution. *P. sergenti* lineage I shows a better correlation to the bioclimatic diversity in southwestern Europe. Conversely, *P. sergenti* lineage III prefers warmer temperatures and less precipitation, which are typical of the Mediterranean. 20.7% of the rabbits were infected with *L. infantum* and 82.4% with *Trypanosoma nabiasi*, and 14.8%of mixed infections were detected. Both parasites were found in all the animal organs analysed, a factor which, along with the presence of serological cross-reactions, must be taken into account in epidemiological studies on leishmaniasis. *L. infantum* DNA was found in 27 % (10 out of 37) of the trapped rodents, in a variety of tissues: bone marrow, spleen, or healthy skin (ear lobe). High prevalences of *L. infantum* infection were found in the three investigated rodent species. The presence of other trypanosomatids was also evidenced. The parasite was detected in all the type of samples of 121 wild rabbits. Epimastigotes were visualized and isolated from all the organ cultures types except from skin, and twenty-six strains were isolated and grown in mass. Epimastigote infectivity was assessed in vitro and in vivo. Amastigotes were obtained in infected

macrophages from cultured epimastigotes. Furthermore, trypomastigotes were found in the peripheral bloodstream of an experimentally infected naïve domestic rabbit with cultured epimastigotes at the fourth day after infection. The rising titre of antibodies led to the disappearance of the parasite from blood. In addition, this study reports the existence of two *T. nabiasi* genetic lineages in southern Spain. Phylogenetic analysis places *T. nabiasi* in the same clade as *T. lewisi* and other rodent trypanosomes of the subgenus *Herpetosoma*.

The combination of parasitological and molecular methods, using at least two different PCRs, is a reliable and accurate procedure for the diagnosis of CL, allowing the simultaneous identification of the parasite. The selection of these PCR techniques will be influenced by the epidemiological scenario: in areas where *L. infantum* is endemic, the use of the PCR-ELISA joint with JW13/JW14 PCR or ITS-1 seems an appropriate choice whereas in areas like Morocco, where *L. tropica* and *L. major* are the main CL causative agents with sporadic CL cases due to *L. infantum*, PCR-ELISA is the least useful while Lmj4/Uni21 and ITS-1 provide satisfactory results. The possibility of using FFPE samples for DNA extraction and PCR allows discarding CL in patients with skin lesions of unknown etiology for which leishmaniasis was not suspected. A high percentage of GLUO are due to *L. infantum* infection. Given that the success of direct microscopy analysis is associated to the experience of the analyst, we suggest the generalized implementation of a PCR technique, such as those indicated: these show high sensitivity and specificity and could help overcome the drawbacks of direct diagnosis. Additionally, we recommend that all granulomatous cutaneous lesions from *L. infantum* endemic areas should be systematically investigated in by these techniques. *Phlebotomus sergenti* lineage I shows a better correlation to the bioclimatic diversity in southwestern Europe. Conversely, *P. sergenti* lineage III prefers warmer temperatures and less precipitation, which are typical of the Mediterranean. Therefore, lineage I would seem more suitable to lead a potential geographical expansion towards the rest of Europe. Despite the fact that in southwestern Europe *L. infantum* antibodies and DNA have been found in a wide array of domestic and wild animals, and that in some cases it has been possible to isolate the parasite and submit it to isoenzymatic characterization, no clear evidence has ever been found of their involvement as reservoirs, with the possible exception of the recent study on hares. Without a doubt, the impossibility of finding an area where there are no dogs further hinders the process of evaluating the possible contribution of these other hosts to the epidemiology of leishmaniasis. In the systems that have been adequately described, the reservoir

host is abundant, forming a large proportion of the mammalian biomass and it is often a gregarious species, all characteristics which are true of the wild rabbit to an even greater extent than the dog. An effective reservoir host can be expected to be long-lived, at least surviving through any non-transmission season. Although the average lifespan of rabbits is shorter than that of dogs, it is long enough to ensure the transmission of *L. infantum.* If a rabbit reaches adulthood it may live for 4–5 years, clearly a much lower figure than the estimated average 14-year lifespan of dogs. The presence of the parasite in high proportions in the skin and peripheral blood of these rabbits with no apparent signs of acute dis-ease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit. The rabbit appears to fulfil the most of conditions which would justify it being considered a reservoir host of *L. infantum* and it would be interesting to conduct xenodiagnostic experiments using the local phlebotomine vectors. The sympatric and syntrophic presence of *T. nabiasi* must be taken into account in order to avoid any confusion. The results of this research, analysed in the context of the contributions from other authors and eco-biological data of rodents, support its role as wild reservoirs in zoonotic leishmaniasis foci. Nevertheless, more research is necessary to definitely confirm this fact. The successful isolation and mass culture of *T. nabiasi* from all the rabbit organs was accomplished using modified EMTM medium, allowing for conducting in vitro and in vivo infection experiments. These epimastigotes are infective to both primary macrophages and naïve domestic rabbits, transforming into amastigotes and trypomastigotes respectively. The formation and multiplication of intracellular amastigotes seems to occur in a greater variety of rabbit organs than previously reported. Two lineages and four haplotypes of *T. nabiasi* were identified in southern Spain. In the phylogenetic trees, *T. nabiasi* fell in the same clade of *T. lewisi* and other rodent trypanosomes, and it is included in a subclade with those trypanosomes whose division stage is the amastigote.

II. Introducción

1. Leishmaniosis: Concepto y generalidades

Leishmaniosis es la denominación que reciben aquellas enfermedades parasitarias producidas por protozoos flagelados del género *Leishmania* Ross, 1903 (Gállego, 2004; WHO, 2004). Este género incluye cerca de 30 especies patógenas, siendo más de veinte las especies capaces de infectar al ser humano. La transmisión de esta enfermedad se produce a través de la picadura de dípteros nematóceros de la familia Psychodidae, perteneciendo habitualmente al género *Phlebotomus* en el Viejo Mundo y al género *Lutzomyia* en el Nuevo Mundo. Existen tres formas clínicas principales: la leishmaniosis visceral, la más peligrosa y mortal si no se trata; la leishmaniosis cutánea y la leishmaniosis mucosa o cutáneo-mucosa (WHO 2010). Tanto el estado inmune del hospedador como la especie responsable de la infección determinarán el cuadro clínico.

La leishmaniosis se encuentra ampliamente extendida en zonas de clima cálido y templado del planeta, representando un importante problema de salud pública. A pesar de que la infección y enfermedad se puede presentar en cualquier individuo, afecta principalmente a grupos en situación de pobreza y se asocia con la malnutrición, precariedad de la vivienda, debilidad en el sistema inmune y falta de recursos. La mayoría de los casos se presentan en países con escasez económica o en vías de desarrollo de África, Asia y Latinoamérica.

Son más de 98 los países en los que la leishmaniosis es endémica, con una población en riesgo de 350 millones de personas. Se estima que su incidencia anual es de 0,2-0,4 millones de casos de leishmaniosis visceral y 0,7-1,2 millones de casos de leishmaniosis cutánea y mucosa. Además, la leishmaniosis visceral ocasiona más de 50.000 muertes al año, cifra que sólo es superada por la malaria entre otras parasitosis (WHO 2010). En cuanto a la leishmaniosis visceral debida a *L. infantum*, se estiman 4500-6800 casos anuales en la región americana, localizándose el 93% de éstos en Brasil. Además, esta especie es el agente causal de los 1200-2000 casos de leishmaniosis visceral en la Cuenca Mediterránea y de los 5.000-10.000 casos descritos en Oriente Medio (Alvar et al., 2012). En los países europeos mediterráneos se registra una incidencia de 630-850 casos de leishmaniosis visceral anuales (Alvar et al., 2012). Contrariamente a lo que se podría pensar debido a la elevada incidencia mundial, se considera una enfermedad olvidada debido a la escasa prioridad que representa tanto para organismos internacionales como para la industria farmacéutica (WHO, 2004).

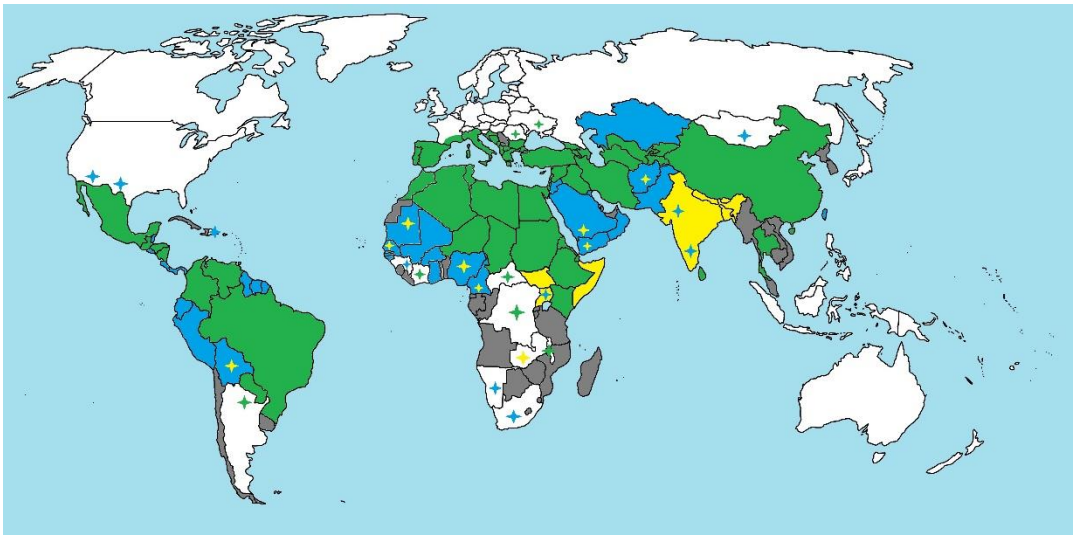

Figura 1. Distribución mundial de las leishmaniosis según Alvar et al. (2012). El color amarillo denota la presencia de leishmaniosis visceral, el color azul indica presencia de leishmaniosis cutánea o mucosa y el color verde supone la presencia de ambas. Las estrellas de color señalan presencia demostrada del parásito o de casos aislados de la enfermedad. El color blanco muestra ausencia de enfermedad y el gris ausencia de datos.

Además, se han citado casos autóctonos en áreas no endémicas como EEUU o Canadá, donde han encontrado perros con anticuerpos frente a *Leishmania* (Enserink, 2000), así como en Australia, donde se han descrito casos de leishmaniosis cutánea en canguros (Rose et al., 2004). La leishmaniosis se considera emergente en el norte de Europa, como demuestran los once casos autóctonos de leishmaniosis visceral confirmados en Alemania (Naucke et al., 2008), además de casos caninos y equinos (Bogdan et al., 2001; Koehler et al., 2002); y la aparición de diversos brotes de leishmaniosis en el norte de Italia (Varani et al., 2013). Gramiccia y Gradoni, (2005) sugieren que la leishmaniosis es una enfermedad dinámica en la que los factores ambientales, demográficos y propios del comportamiento humano juegan un papel fundamental.

1.1. Clasificación taxonómica

Los protozoos parásitos del Orden Kinetoplastida se caracterizan por ser uninucleados y poseer una mitocondria única que ocupa gran parte del citoplasma y en cuyo interior se observa el ADN mitocondrial enrollado, conocido como kinetoplasto (ADNk) (Gállego-Berenguer, 2006). Presentan división por fisión binaria longitudinal aunque se admite la existencia en el género de reproducción sexual y la existencia de híbridos (Chargui et al., 2009; Ravel et al., 2006; Rogers et al., 2014; Rougeron et al., 2016, 2010).

La clasificación propuesta por Adl et al. en 2005 actualiza la clasificación de Levine et al. 1980. En la nueva clasificación, se divide a los organismos eucariotas (Dominio Eukarya) en 6 supergrupos:

- Amoebozoa
- Opisthokonta,
- Rhizaria,
- Archaeplastida
- Chromalveolata
- Excavata

Dentro del supergrupo Excavata, se encontraría el Phylum Euglenozoa (Cavalier-Smith, 1998) y que, a su vez, incluiría a la clase Kinetoplastea, de la que forman parte los clados Prokinetoplastina y Metakinetoplastina. En este último se incluyen los órdenes:

- Neobodonida
- Parabodonida
- Eubodonida
- Trypanosomatida

El orden Trypanosomatida (donde se encontraría la familia Trypanosomatidae) abarca los siguientes géneros:

- *Blastocrithidia*
- *Crithidia*
- *Endotrypanum*
- *Herpetomonas*
- *Leishmania*
- *Leptomonas*
- *Phytomonas*
- *Rhynchoidomonas*
- *Sauroleishmania*
- *Trypanosoma*
- *Wallaceina*

Por otro lado, Kraeva et al. en 2015 consideran una división entre géneros con especies monoxénicas, donde incluyen a *Anfomonas, Blastocrithidia, Blechomonas, Crithidia, Herpetomonas, Kerotomonas, Leptomonas, Paratrypanosoma, Sergeia, Strigomonas* y *Wallacemonas* (obviando *Wallaceina* y

Rhynchoidomonas); y géneros con especies dixénicas, que incluye a *Trypanosoma, Leishmania* y *Phytomonas* (no considerándose *Sauroleishmania*). También cabe decir que Jirků et al. en 2012 propusieron la subfamilia Leishmaniinae en la que incluyen los géneros *Leishmania, Crithidia* y *Wallaceina*, así como algunas especies de *Leptomonas*. Como ejemplo de la complejidad del tema cabe destacar que estos autores consideran *Wallaceina* mientras que Kraeva et al. en 2015 consideran el género *Wallacemonas*, firmando algunos de los autores ambos artículos. Por último Votýpka et al., en 2015 incluyen en la subfamilia Leishmaniinae los géneros *Leishmania, Leptomonas, Lotmaria* y *Crithidia*, basándose en análisis de secuencias de la subunidad pequeña del ARN ribosómico (SSU rRNA).

Es importante señalar que se han aislado flagelados de los géneros *Blechomonas, Crithidia, Herpetomonas* y *Leptomonas* en casos clínicos en humanos (Kraeva et al., 2015; Singh et al., 2013), algunos en coinfección con *L. donovani*.

Dominio	**Eukarya**
	Excavata
	Euglenozoa
Clase	**Kinetoplastea**
	Metakinetoplastina
Orden	**Trypanosomatida**
Género	***Leishmania***

Figura 2. Encuadramiento taxonómico del género *Leishmania* de acuerdo con la clasificación de Adl et al (2005).

Hasta el momento se han descrito alrededor de una veintena de especies de *Leishmania* divididas en los subgéneros *Leishmania* y *Viannia*, como se puede observar en la figura 3 y en la tabla 1. Esta división tiene en cuenta el lugar de multiplicación del parásito en el tracto digestivo del vector, siendo el intestino medio en el caso de *Leishmania* y el intestino posterior en el de *Viannia.* Gracias a la implementación de la bioquímica y la biología molecular, la taxonomía se ha apoyado en los análisis de isoenzimas (Haouas et al., 2012; Pratlong et al., 2009) o en técnicas que estudian el ADNk entre otros (Bañuls et al., 2007). De este modo se ha respaldado la división en estos subgéneros propuesta por Lainson & Shaw en 1987 (Bañuls et al., 2007; Schönian et al., 2010). Gracias al análisis de isoenzimas, Rioux et al. (1990) introdujo un nuevo sistema de clasificación que permitía identificar y asociar zimodemos con regiones geográficas, tipos de reservorios y

hospedadores y con las manifestaciones clínicas que presenta cada especie; lo que supuso una mejor comprensión en la epidemiología de la leishmaniosis.

Los parásitos del subgénero *Leishmania* se encuentran presentes tanto en el Viejo como en el Nuevo Mundo mientras que los del subgénero *Viannia* se encuentran solo en el Nuevo Mundo (Tabla 1). Recientemente se han descrito las especies *L. martiniquensis, L. siamensis* y *L. waltoni* (Leelayoova et al. 2013; Pothirat et al. 2014; Shaw et al. 2015).

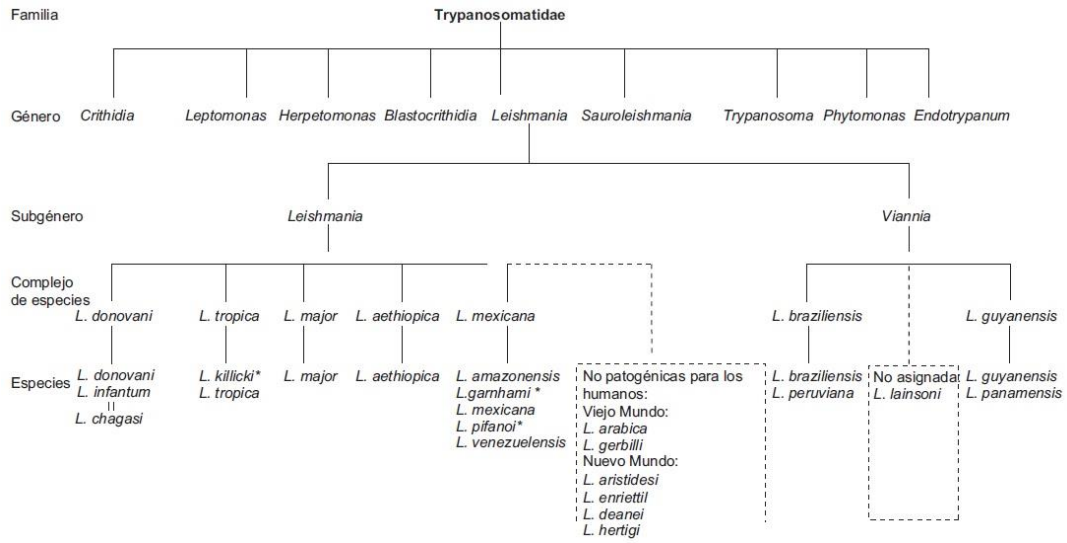

Figura 3. Familia Trypanosomatidae. División de géneros de la familia y subgéneros de *Leishmania* con sus correspondientes especies (WHO, 2010).

Subgénero	*L. (Leishmania)*	*L. (Leishmania)*	*L. (Viannia)*	*L. (Viannia)*
Viejo Mundo	*L. donovani* *L. infantum*	*L. major* *L. tropica* *L. killicki* *L. aethiopica* *L. infantum*		
Nuevo Mundo	*L. infantum*	*L. infantum* *L. mexicana* *L. pifanoi* *L. venezuelensis* *L. garnhami* *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naïffi* *L. lainsoni* *L. lindenbergi* *L. peruviana* *L. colombiensis*	*L. braziliensis* *L. panamensis*
Tropismo principal	Viscerotrópica	Dermotrópica	Dermotrópica	Mucotrópica

Tabla 1. Especies de *Leishmania* encontradas en humanos (WHO, 2010).

Leishmania infantum es la especie con mayor distribución mundial y se encuentra presente en el Viejo Mundo y el Nuevo Mundo, donde algunos autores prefieren denominarla *L. chagasi*.

Como se puede observar en la figura 3, dentro del complejo de *L. donovani*, *L. chagasi* se considera sinónima a *L. infantum.* Gracias a la técnica "Multilocus Microsatellite Typing (MLMT)", basada en el análisis de marcadores genéticos altamente polimórficos capaz de diferenciar a nivel de intra-especie, Kuhls et al. (2011) aseguran que no hay una justificación para la separación taxonómica de *L. chagasi* y *L. infantum* a nivel de especie o subespecie. Señalan la existencia de dos poblaciones principales de *L. infantum* en el Nuevo Mundo, que se corresponden con la división de zimodemos MON-1 y no MON-1 y que es MON-1 el más extendido en el continente. Encuentran un bajo polimorfismo entre las cepas del Nuevo Mundo en comparación con las del Viejo Mundo, lo que lleva a pensar que se trataría de una importación reciente desde el Viejo Mundo. Sus resultados indican que *L. infantum* fue introducida desde el Suroeste de Europa hacia el Nuevo Mundo en varias ocasiones y en distintos puntos del continente, donde se adaptó y expandió rápidamente por el continente gracias a las poblaciones locales de *Lutzomyia longipalpis*. (Fig. 4). Los genotipos encontrados en el centro y sur de América también se hallaron en Europa, concretamente en España, Portugal, Francia e Italia; de modo que se trataría de un "Efecto fundador" debido a una reciente introducción de una parte restringida de la población original de *L. infantum*, sufriendo una posible deriva genética o una expansión clonal de sólo algunos genotipos.

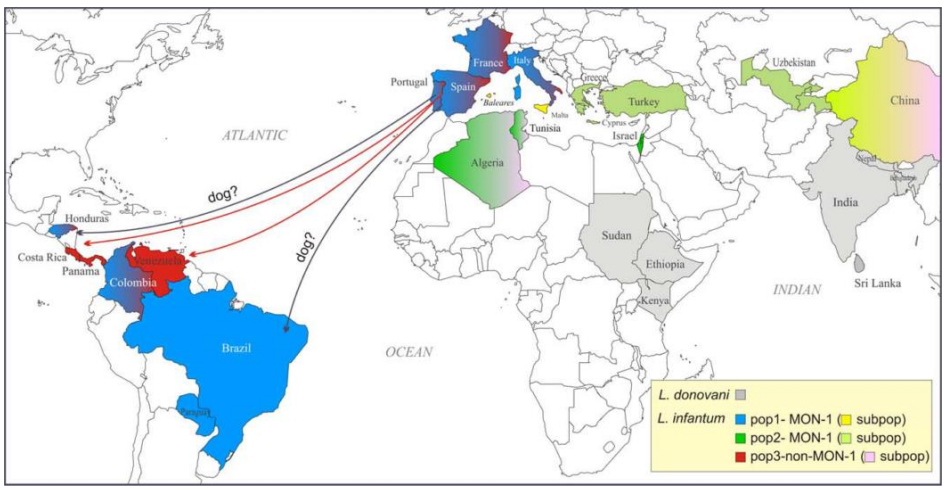

Figura 4. Mapa que muestra el posible origen de *L. infantum* en el Nuevo Mundo. Tomado de Kuhls et al. (2011).

Algo parecido sucede con las especies *L. tropica* y *L. killicki*, incluidas ambas en el complejo *L. tropica.* El análisis taxonómico mediante electroforesis de isoenzimas (MLEE) incluyó primero a *L. killicki* en el complejo *L. tropica* (Pratlong et al., 1986; Rioux et al., 1990). Sin embargo, tras la revisión de la clasificación del género *Leishmania*, se consideró a *L. killicki* como un complejo filogenético independiente (Rioux and Lanotte, 1993). Recientemente, un estudio actualizado llevado a cabo por Pratlong et al. (2009) confirmó la inclusión de *L. killicki* dentro del complejo *L. tropica*.

1.2. Morfología

Son parásitos pleomórficos que muestran dos formas claramente definidas en su ciclo biológico conocidas como amastigotes y promastigotes.

Formas promastigotes (Fig. 5a): fase que se encuentra en el tubo digestivo del hospedador invertebrado (flebotomo hembra). Presentan un cuerpo fusiforme de 15-20 µm de longitud y 3-5 µm de ancho del que parte un flagelo que puede alcanzar una longitud de 20 µm. A su vez, se distinguen dos formas de promastigotes: el procíclico, con escasa capacidad infectiva, y el metacíclico, forma infectiva por excelencia. Entre ellos existen diferencias estructurales, especialmente en la cantidad y tamaño de las glicoproteínas de superficie y otras de carácter metabólico. El promastigote procíclico se transforma en metacíclico mediante división durante aproximadamente diez días tras la ingesta de sangre por parte del vector. Sin embargo, las formas metacíclicas no son capaces de dividirse. En la transformación de procíclico a metacíclico algunos autores también consideran las formas intermedias haptomonadas y nectomonadas (Dostálová and Volf, 2012).

Formas amastigotes (Fig. 5b): fase intracelular obligada que parasita a las células del sistema fagocítico mononuclear, con frecuencia macrófagos, en el hospedador vertebrado. Se tratan de formas redondeadas con tendencia elipsoide y carentes de flagelo (solo raíz flagelar). Son más pequeñas que los promastigotes presentando en torno a 3-5 µm de diámetro, y en su interior se observa el núcleo excéntrico y el kinetoplasto.

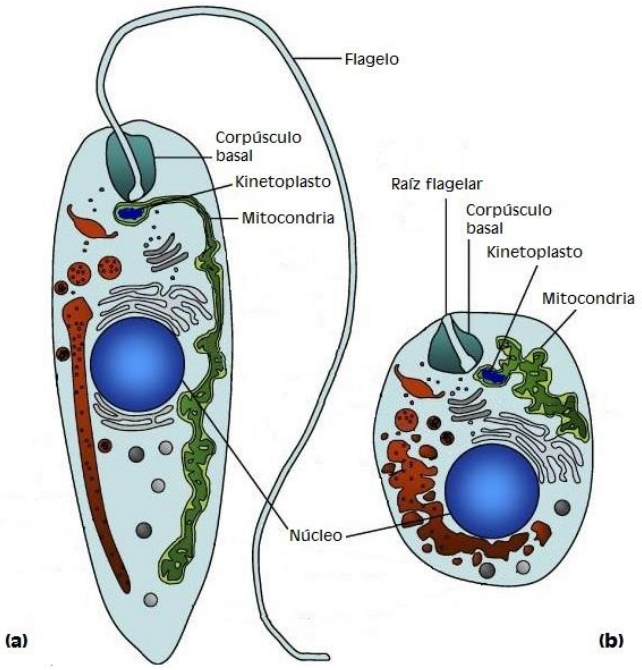

Figura 5. Estructura celular de las fases: (a) promastigote, (b) amastigote. Imagen obtenida de Besteiro et al. (2007). Modificadas por la autora.

1.3. Ciclo biológico

Las especies pertenecientes al género *Leishmania* presentan un ciclo de vida heteroxeno (Fig. 6), del que forman parte un hospedador vertebrado y otro invertebrado que actúa como vector. El artrópodo vector es un díptero nematócero del género *Phlebotomus* en el Viejo Mundo y del género *Lutzomyia* en el Nuevo Mundo, mientras que el hospedador vertebrado incluye varios grupos de mamíferos entre los que se encuentra el hombre.

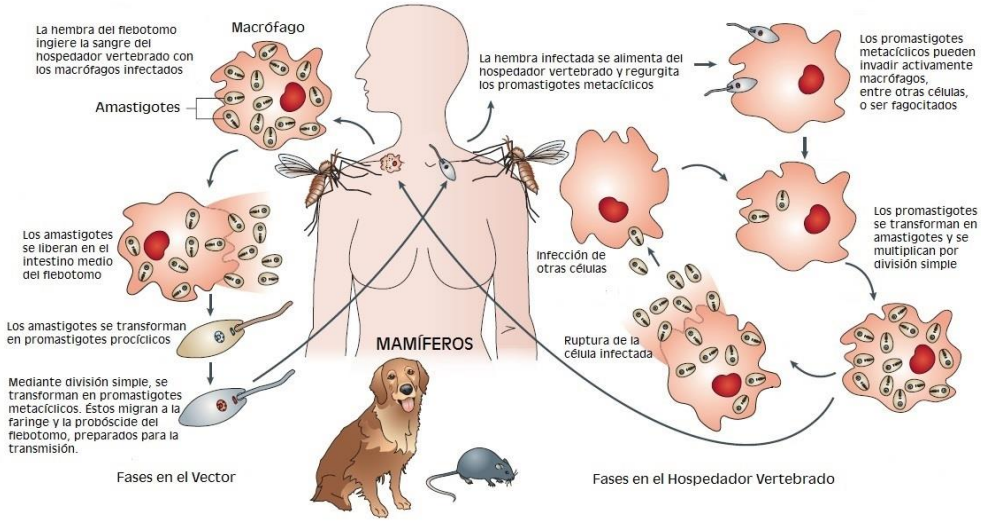

Figura 6. Ciclo biológico de *Leishmania.* Imagen tomada de Lipoldová & Demant (2006). Modificada por la autora.

La hembra del flebotomo ingurgita la sangre del hospedador vertebrado que contiene los macrófagos en cuyo interior se encuentra la vacuola parasitófora donde reside el parásito en su forma amastigote; una vez en el tubo digestivo del flebotomo el protozoo sale del macrófago para transformarse en promastigote, adherirse al intestino medio del vector y multiplicarse activamente. Posteriormente, y después de pasar por una serie de estadios intermedios dan lugar a la forma infectiva conocida como promastigote metacíclico, que migran a la región anterior del intestino (válvula estomodeal), faringe y probóscide del vector. El parásito pasa al hospedador vertebrado a través de la picadura del flebótomo.

En el hospedador vertebrado, el parásito es capturado por los macrófagos de la dermis. Una vez dentro lo engloban en una vacuola parasitófora, donde se transforma en amastigote y se multiplica activamente por división binaria hasta que el macrófago estalla y salen al exterior para ser nuevamente fagocitados. A su vez, el trauma mecánico que produce la picadura induce la llegada de neutrófilos a la zona, englobando a los promastigotes metacíclicos, que se dividen en su interior hasta que los neutrófilos son lisados. Para garantizar su supervivencia, estos amastigotes necesitan volver a ser fagocitados por otras células mieloides. Algunos autores hacen alusión a un proceso exocítico que implica la formación de pequeñas vacuolas que se fusionan a la superficie del macrófago y permiten que los amastigotes sean liberados al torrente sanguíneo (Rittig and Bogdan, 2000). Los amastigotes libres van a infectar otros macrófagos, o bien se diseminan a

través del torrente circulatorio o vía linfática para infectar órganos ricos en estas células inmunes como son hígado, bazo o médula ósea (Gállego-Berenguer, 2006; Kaye and Scott, 2011).

1.4. Leishmaniosis debida a *Leishmania infantum* en España

La leishmaniosis cursa de forma hipoendémica en España (0,41 casos por 100.000 habitantes) y es causada por *Leishmania infantum*, única especie que se presenta de forma autóctona en España y agente causal de leishmaniosis visceral, cutánea y cutáneo-mucosa (Aliaga et al., 2003; Cobo et al., 2007; Faucher et al., 2011) para la que se ha descrito una elevada variabilidad isoenzimática en nuestro país (Martín-Sánchez et al., 2004). El ciclo de vida de *L. infantum* constaría de un ciclo selvático, en el que sus reservorios serían animales salvajes como el lobo, zorro, roedores y lagomorfos; y un ciclo doméstico, en el que su principal reservorio sería el perro doméstico.

En España, ha sido una enfermedad de declaración obligatoria (EDO) desde 1982, pero a partir de 1996 se estableció la Red Nacional de Vigilancia Epidemiológica, siendo la notificación de los casos competencia a nivel regional para proceder al registro total de los casos en el último mes de cada año. Gracias al análisis de estos datos se sabe que se trata de una enfermedad con tendencia creciente (Herrador et al., 2015) que afecta a casi la totalidad de las Comunidades Autónomas, pero especialmente a toda la zona mediterránea y a la Meseta Central. Sin embargo, hay que resaltar el hecho de que existe una subestimación de los casos reales frente a los declarados anualmente, ya que se estima que sólo se registran entre el 25-40% de leishmaniosis viscerales y no hay registros de prácticamente la totalidad de los casos de leishmaniosis cutáneas (Alvar et al. 2012).

La infección producida por *L. infantum* afecta a personas de todas las edades y condiciones, aunque las manifestaciones clínicas se dan con frecuencia en niños y personas inmunodeprimidas. La susceptibilidad de los individuos adultos sanos al parásito sería baja, siendo la mayoría de las infecciones asintomáticas. Sin embargo, los estados de inmunosupresión comprometen esta resistencia natural al parásito. Este grupo fue determinante para el aumento que experimentó la enfermedad en las décadas de los 80 y 90 no solo en España sino también en otros países de Europa y en Sudamérica, como consecuencia de la coinfección entre el parásito y el virus de la inmunodeficiencia humana (VIH). A partir de la introducción de la terapia antirretroviral de gran actividad a mediados de los 90, en el ámbito nacional se ha venido observando un notorio cambio en la situación

epidemiológica de la enfermedad con reducción notable de los casos de coinfección (de La Rosa et al., 2002; Pintado and López-Vélez, 2001). En concreto, en España y sur de Europa hubo un pico de incidencia de los casos de coinfección entre 1996 y 1998, mientras que entre 1998 y 2001 la incidencia de coinfección descendió a niveles que se han mantenido hasta 2006 (Alvar et al., 2008). Curiosamente la situación no mejoró en Portugal, donde en el periodo comprendido entre los años 2000 y 2009, aparecieron 107 casos de coinfección del total de 173 casos de leishmaniosis visceral diagnosticados (Campino and Maia, 2010).

La leishmaniosis por *L. infantum* es una zoonosis en la que el perro (*Canis lupus familiaris*) es el principal hospedador reservorio, que sufre la enfermedad en forma de un síndrome con afectación visceral, cutánea y mucosa (Alvar et al., 2004; Ballart et al., 2012a; Gálvez et al., 2010; Martín-Sánchez et al., 2009), conocido como leishmaniosis canina (LCa).

El reservorio es el principal responsable del mantenimiento del parásito durante aquellos periodos en los que la persistencia de éste está comprometida de forma natural además de por el desarrollo de programas de control u otras medidas con fines sanitarios o agrícolas, como la introducción del DDT u otros insecticidas implicados en la lucha contra la malaria (Ashford, 2000).

En los estudios realizados en nuestro país acerca de la sero-prevalencia de LCa, se deducen valores medios de entre el 10 y el 20%, variando de forma notable de unas regiones a otras (Fisa et al., 1999). Así, en la provincia de Granada, las sero-prevalencias alcanzan valores de 20,1%, 13,3% y 1,1% en los pisos bioclimáticos Meso, Termo y Supra-Mediterráneo, respectivamente (Martín-Sánchez et al., 2009). En la Axarquía malagueña (Morillas et al. 1996) la sero-prevalencia canina es del 34,7%.

También se han descrito otros hospedadores domésticos como son los mulos, caballos (Gállego, 2004; Solano-Gallego et al., 2003), y especialmente en gatos en los que al emplear métodos moleculares la prevalencia alcanza valores del 26,1% (Martín-Sánchez et al., 2007). Estos autores sugieren un posible papel protector de la respuesta inmune humoral en los gatos infectados por *Leishmania.* Entre los animales salvajes citados como hospedadores secundarios de *L. infantum* podemos destacar los lobos (*Canis lupus*), zorros (*Vulpes vulpes*), ginetas (*Genetta genetta*), linces (*Lynx pardinus*) chacales (*Canis aureus*) entre otros (Hervás et al. 1996; Portús et al. 2003; Sobrino et al. 2008).

En el período comprendido entre 2009-2012 se declaró un brote epidémico de leishmaniosis en la Comunidad de Madrid, pasando de 12-25 casos anuales entre los años 2000-2009 a registrar 542 casos (36% de ellos de leishmaniosis visceral) entre julio de 2009 y diciembre de 2012 (Arce et al., 2013); produciéndose la mayoría en una zona urbana del sur de la región. Al realizar estudios entomológicos, se encontró una elevada densidad media de 144 flebotomos/m^2 (contando en algunas estaciones más de 1000 flebotomos/m^2); mientras que en muestreos aleatorios de los perros del entorno se encontró una sero-prevalencia del 1,6%. Tras encontrar una prevalencia tan baja de la enfermedad en el perro, llevaron a cabo estudios sobre otros posibles reservorios, encontrando elevados porcentajes de infección en las liebres (*Lepus granatensis*) y conejos del entorno (Carrillo et al., 2013), señalándolos como responsables del foco de leishmaniosis en la zona.

Aunque como se ha mencionado, el ciclo natural de *Leishmania infantum* es una zoonosis, se señaló la posibilidad de un ciclo antroponótico asociado a casos de coinfección con el virus de la inmunodeficiencia humana "VIH" (Alvar et al., 1989; Alvar and Jiménez, 1994; Molina et al., 2003). En personas drogodependientes coinfectadas por VIH, el papel vectorial del flebotomo parece haberse sustituido por las jeringuillas y la transmisión paralela entre pacientes actuaría de forma sinérgica entre ambas (Alvar and Jiménez, 1994; Pineda et al., 2002, 2001). Morillas-Márquez et al. (2002) encontraron que la pequeña cantidad de sangre que queda en el émbolo y aguja de las jeringas que utilizan los drogodependientes infectados con VIH-*L. infantum* podría contener suficiente cantidad de parásitos como para producir la infección en animales de experimentación. Para ello, la inyectaron junto con solución salina por vía intraperitoneal en ratones y hámsteres, que sacrificaron al cabo de 2 meses. Al analizar las muestras de sangre completa y suspensión de bazo, todas fueron positivas con la PCR-ELISA específica de *L. infantum* (Martin-Sanchez et al., 2001); lo que demostró que se consiguió la transmisión y establecimiento de la infección parasitaria.

El perro doméstico, además de ser el principal reservorio de la leishmaniosis debida a *L. infantum* (Morales-Yuste et al., 2012), sufre una compleja enfermedad denominada leishmaniosis canina. Se trata de una enfermedad multisistémica en la que el parásito infecta virtualmente todos los órganos y tejidos del animal conduciéndole a la muerte (Baneth and Aroch, 2008).

Aunque pretendemos centrarnos en la situación en España, hemos de indicar que es endémica en los países de la Cuenca Mediterránea, Oriente Medio y Sudamérica

(Baneth, 2013). También se ha detectado la importación de casos de leishmaniosis canina hacia regiones no endémicas de Europa, como Reino Unido (Shaw et al., 2009) y Alemania (Mencke, 2011); así como en el Sur de Estados Unidos (Petersen and Barr, 2009). En todos ellos, los perros habían viajado a zonas endémicas del sureste de Europa, como España, Francia o Italia.

En España se han realizado distintos muestreos aleatorios para determinar la prevalencia de la leishmaniosis canina (Martín-Sánchez et al., 2009), encontrando valores de entre el 0 y el 100% dependiendo de la región y de la técnica diagnóstica empleada, lo que confirma la naturaleza endémica y la distribución parcheada de la enfermedad (Morales-Yuste et al., 2012).

En las regiones endémicas, aunque la enfermedad se encuentra ampliamente distribuida, no todos los perros están infectados y entre los infectados, no todos los animales muestran signos clínicos de la enfermedad. Estos últimos pueden permanecer asintomáticos a largo plazo o evolucionar a estados más severos de la enfermedad con el tiempo, seguramente debido a cambios en el estado inmunológico del animal (Baneth and Aroch, 2008). Precisamente por ello tienen una gran importancia en la epidemiología de la enfermedad, y es que éstos son tan competentes para transmitir el parásito al flebotomo como lo son los perros clínicamente enfermos(Alvar et al., 1994; Laurenti and Marcondes, 2014; Molina et al., 1994). Tanto los perros asintomáticos como los enfermos, son capaces de transmitir el parásito al vector incluso después del tratamiento (Alvar et al., 1994; Miró et al., 2011).

Podría considerarse entonces que los perros con enfermedad aparente suponen solamente la punta de un "iceberg", en la que en la base estarían los perros sanos no infectados y entre esos se encontraría un segmento de perros infectados clínicamente sanos (Baneth and Aroch, 2008).

1.5. Casos importados y posibilidad de establecimiento de otras especies en España

El aumento de los viajes internacionales es uno de los principales factores para el desarrollo y la propagación de patógenos emergentes una amenaza, lo que supone una amenaza a la salud pública mundial y requiere atención a nivel nacional e internacional. El riesgo de adquirir una enfermedad infecciosa durante un viaje se ve influenciado, entre otros factores, por el lugar de destino, el tipo y

la duración de los viajes, las actividades de exposición y el uso de medidas preventivas como vacunas o quimioprofilaxis.

En la última década, la leishmaniosis, especialmente la LC, se ha convertido en una amenaza para el turismo internacional (Spinello Antinori et al., 2005; Zamarrón-Fuertes et al., 2010). Además de éstos, el personal militar y trabajadores del sector del turismo ecológico/de aventura están expuestos a un alto riesgo de adquirir la enfermedad. Esta tendencia no sólo se asocia con el aumento de los viajes a destinos endémicos, sino también con la inmigración (Monge-Maillo et al. 2014) o las poblaciones de refugiados que provienen de zonas endémicas (Al-Salem et al., 2016). En los países y regiones no endémicos de Europa, el aumento de los casos importados de leishmaniosis se ha documentado en Austria, Francia, Alemania, Países Bajos y Reino Unido (El Hajj et al., 2004; Harms et al., 2003; Herremans et al., 2010; Lawn et al., 2004; Malik et al., 2006; Pavli and Maltezou, 2010). Estos autores destacan que la LV se adquirió principalmente en el sur de Europa; mientras que la LC, que fue la más numerosa en cuanto a casos, se adquirió en África subsahariana y septentrional, Oriente Medio, Asia central y América del Sur. En las zonas no endémicas, los casos importados pueden no ser reconocidos como leishmaniosis debido a la falta de familiaridad de los médicos con esta enfermedad y los que se detectan se registran como importados (Bart et al., 2013). Sin embargo, los médicos y laboratorios de países endémicos pueden ofrecer una mayor experiencia clínica y diagnóstica, aunque deben estar alerta ante aquellos casos que puedan ser importados, ya sea por inmigrantes o turistas que han viajado a zonas endémicas (Pérez-Ayala et al., 2009).

También es importante la monitorización de la posible introducción de especies de *Leishmania* en áreas endémicas de otras especies autóctonas, de modo que puedan ser transmitidas potencialmente a través de los flebotomos locales, específicos o permisivos, con consecuencias imprevisibles (Antoniou et al., 2013). Aparte de la introducción de *L. infantum* en el Nuevo Mundo desde el sur de Europa (Kuhls et al., 2011; Rotureau et al., 2006) que pone de manifiesto la plasticidad parasitaria en la adaptación a nuevos ecosistemas, surgen en Europa nuevos focos con especies importadas de *Leishmania*, como *L. donovani* en Chipre (Antoniou et al., 2008) y los híbridos *L. infantum/L. major* descritos en Portugal (Ravel et al., 2006). Debido a los cambios ambientales, no sólo los vectores, sino también los huéspedes mamíferos pueden actuar en nuevos ciclos de transmisión (Antoniou et al., 2013), de modo que los turistas/inmigrantes infectados en el

extranjero también podrían convertirse en potenciales reservorios de parásitos antroponóticos de *Leishmania*.

Aunque en el suroeste europeo no se han detectado casos autóctonos de LC producida por *L. tropica* o *L. major*, el sur de la Península Ibérica es una zona susceptible de sufrir la introducción de este parásito debido a la proximidad con Marruecos, al flujo migratorio entre ambos territorios y a la presencia de sus principales vectores (*P. sergenti y P. papatasi*, respectivamente).

Para que se origine un foco autóctono de *L. tropica* o *L. major* se necesita la introducción del parásito a través de personas infectadas procedentes de zonas endémicas o que las poblaciones de flebotomos que transmiten estas especies posean capacidad vectorial. Se tiene constancia del flujo humano migratorio procedente de África y Oriente Medio, donde se encuentran presentes estas especies (Aghaei et al., 2014; Ajaoud et al., 2013; Beldi et al., 2017; El-Beshbishy et al., 2013; Kavarizadeh et al., 2017; Moncaz et al., 2012; Rhajaoui, 2011) y en las que están en contacto con su vector. La inmigración a España, según Instituto Nacional de Estadística, ha aumentado dramáticamente: del 2,9% en 1998 al 10,9% en 2012, con aproximadamente el 75% de los inmigrantes procedentes de países en vías de desarrollo o con pocos recursos (INE, 2013). A estas cifras habría que sumarle la inmigración ilegal sobre la que no es posible establecer un control sanitario. En España se ha demostrado que la gran mayoría de los casos de LC no se declaran (Alvar et al., 2012), y la identificación de las especie que produce la enfermedad no se suele realizar con rutina, por lo que no hay un control sobre un posible establecimiento de especies foráneas en el territorio español. La importación de casos de LC por *L. tropica* se ha comprobado en otros países europeos (S. Antinori et al., 2005; Gramiccia and Gradoni, 2005; Morizot et al., 2007) y en España ya se han descrito casos importados de LC por *L. tropica* (Giavedoni et al., 2015) y por *L. major* (Fernandez-Flores et al., 2016; González-Llavona et al., 2007).

Tanto *P. sergenti* como *P. papatasi* están ampliamente distribuidos en la Península Ibérica (Barón et al., 2011; Morales Yuste, 2012), de igual forma que en Marruecos (Ajaoud et al., 2013; Boussaa et al., 2016; Kahime et al., 2015). Además, algunos estudios muestran la afinidad bioclimática entre Marruecos y el sudeste de España, así como la concordancia de especies con respecto a cada nivel bioclimático (Martinez-Ortega, 1988). En cuanto a *P. sergenti*, cabe destacar la existencia de una línea mitocondrial en común con Marruecos, en donde se encuentra *L. tropica,* de modo que las poblaciones españolas que pertenezcan a esta línea mitocondrial podrían ser capaces de actuar como vectores.

La LC producida por *L. tropica* se considera una antroponosis en el que el ser humano es el principal reservorio y los casos que se producen se dan en ciudades muy pobladas y sus alrededores con bajo predominio en zonas semi-rurales (WHO 2010). Sin embargo, la LC producida por *L. major* es una zoonosis en la que el principal reservorio es un roedor, como los pertenecientes al género *Meriones* spp, la rata de arena (*Psammomys obesus*) o el gran gerbo (*Rhombomys opimus*) (Motazedian et al., 2006). En Marruecos, *P. obesus* se encuentra en el oeste (Postigo, 2010), mientras que *M. shawi* es el principal reservorio en el sur (Rhajaoui, 2011).

De modo que la introducción y establecimiento de *L. major* en nuestro país sería improbable debido a la carencia de sus reservorios naturales. Por el contrario, el riesgo de importación de *L. tropica* sería más que posible al tratarse de una antroponosis y estar presente la línea mitocondrial de *P. sergenti* en común con Marruecos.

Así, la Península Ibérica podría convertirse en un crisol donde estos factores podrían fusionarse y contribuir a la importación de nuevas especies de *Leishmania*, representando una carga para el sistema médico y un riesgo potencial para la salud pública de las personas en nuestro país.

2. Diagnóstico de la leishmaniosis

Los signos y síntomas de leishmaniosis son insuficientes para diagnosticar la enfermedad y diferenciarla de otras infecciones sistémicas, además de verse complicado por la variedad de formas clínicas existentes. Por ello, es necesario recurrir a pruebas de laboratorio específicas (WHO 2010). Un test diagnóstico ideal debe ser sensible, específico, reproducible y capaz de diferenciar entre individuos asintomáticos y enfermos con el fin de discriminar cuándo el tratamiento es necesario (Chappuis et al., 2007).

2.1. Formas clínicas y signos clínicos asociados

Las diferentes especies de *Leishmania,* dentro de su tropismo y su espectro de enfermedades, producen manifestaciones clínicas diversas, con síntomas y signos en su mayoría no patognomónicos. Esta diversidad no está completamente explicada por la diversidad genética del parásito y la patogenicidad también depende de las poblaciones humanas, cuya respuesta inmunológica, que es crucial, da lugar a las distintas formas de leishmaniosis. Un claro ejemplo de esta diversidad es la que produce la especie *L. infantum*, agente causal de leishmaniosis visceral, cutánea y cutáneo-mucosa.

A continuación se describen las principales características de las diversas formas clínicas de la leishmaniosis (WHO 2010).

Leishmaniosis visceral (LV): Esta forma de leishmaniosis, causada por *L. infantum* (zoonótica) y *L. donovani* (antroponótica), resulta de la infección por el parásito de las células del sistema fagocítico mononuclear del hígado, bazo y médula ósea, provocando hepatomegalia, esplenomegalia y alteración de la médula ósea que conduce a pancitopenia. Además cursa con otros síntomas como episodios intermitentes de fiebre alta, irregular y persistente, alteración de la fracción albúmina/globulinas, anorexia, astenia, delgadez progresiva que puede evolucionar en caquexia y linfoadenopatías; y la muerte (Ashford 2000; WHO 2010). Esta forma de leishmaniosis se presenta en individuos de todas las edades, pero circunstancias como la malnutrición o la inmunosupresión predisponen a la enfermedad.

En algunos estudios se ha descrito de forma ocasional una patología visceral debida a *L. tropica* (Alborzi et al., 2008; Lemrani et al., 2002; Magill et al., 1993; Weiss et al., 2009). Este hecho podría tener relación con zimodemo de las cepas de *L. tropica* implicadas o con la propia respuesta inmune específica del

hospedador (Weiss et al., 2009). Los síntomas clínicos de esta patología visceral son comunes a los causados por otras especies de *Leishmania*: pérdida de peso, anorexia, esplenomegalia, anemia y transaminitis leve (Weiss et al., 2009).

La leishmaniosis visceral se asocia a respuestas inmunitarias mixtas con predominio Th2 y el progreso de la enfermedad viene determinado por bajos niveles de interferón-γ y otras interleuquinas como IL-12, IL-2 o IL-17 (Kip et al., 2015). Una característica de la leishmaniosis visceral es la hiperglobulinemia debida a IgG policlonal.

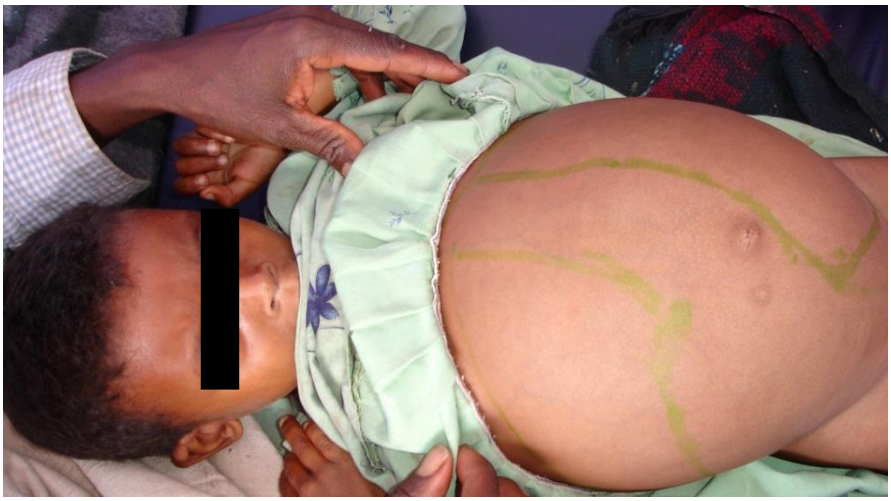

Figura 7. Leishmaniosis visceral producida por *L. infantum* en la que se aprecia una marcada hepatoesplenomegalia. Imagen tomada de www. who.int.

Leishmaniosis cutánea (LC): Sus manifestaciones clínicas presentan una gran variabilidad entre las regiones donde es endémica y dentro de la misma región. Esta variabilidad depende de la especie del parásito y del sistema inmunológico del hospedador.

- **Leishmaniosis cutánea producida por *L. infantum*:** se ha descrito una gran variabilidad clínica que abarca lesiones ulcerosas, papulares, nodulares, etc. La lesión típica empieza en el lugar de inoculación del parásito en forma de pápula o nódulo que aumenta hasta alcanzar un tamaño máximo. En la zona central aparece una costra que finalmente se desprende dando lugar a las lesiones ulcerosas de varios centímetros de diámetro, con un borde elevado e induración periulcerosa; aunque también se presentan lesiones papulosas sin costra (Urrutia et al., 2000). En la mayoría de los casos es benigna y cura sola espontáneamente. Este

tipo de leishmaniosis puede producir lesiones crónicas que presentan un nódulo crónico indoloro que presenta, a menudo, una placa eritematosa rodeada de pápulas (Urrutia, 2001). En los casos de inmunosupresión, el parásito evade al sistema inmune y se extiende produciendo una leishmaniosis cutáneo difusa o, en los casos en los que se dé una infección de las mucosas, leishmaniosis mucosa (WHO 2010). Aliaga et al. (2003) han descrito en España casos de leishmaniosis mucosas estrictas causadas por *L. infantum*.

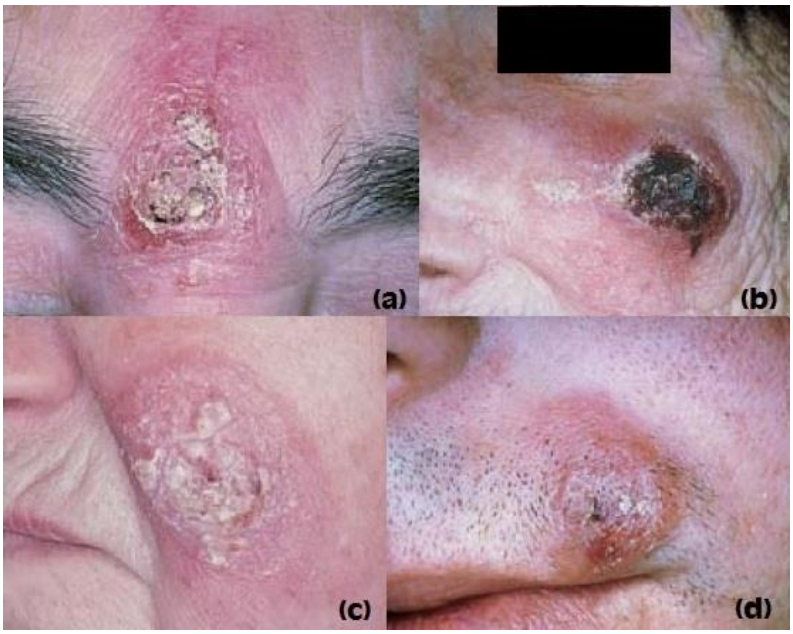

Figura 8. Lesiones características de la leishmaniosis cutánea producida por *L. infantum*. (a) y (b) nódulos eritematocostrosos, (c) placa eritematocostrosa, (d) nódulo eritematoso. Imagen tomada de Urrutia et al. (2000).

- **Leishmaniosis cutánea producida por *L. tropica*:** se caracteriza por ser indolora, con más de una lesión a la vez y se manifiesta con úlceras secas (Fig. 9) (WHO 2010). El período de incubación suele estar comprendido entre semanas hasta 8 meses, aunque se han descrito casos años después de la infección (Weiss et al., 2009). En la mayoría de los casos sanan espontáneamente en el plazo de un año después de su aparición. En otras ocasiones la sanación tarda más tiempo y pueden quedar cicatrices desfigurantes. También se puede presentar una forma crónica recidivante que puede durar varios años. Uno o dos años tras la curación de la lesión aguda, aparecen nuevos nódulos y pápulas de aspecto granulomatoso y

escamoso en el margen de la cicatriz que se genera tras esta curación. Las lesiones progresan lentamente y, si no se trata, es destructiva y desfigurante (Fig 9c). Este tipo de leishmaniosis cutánea crónica presenta un gran parecido clínico e histológico con el 'lupus vulgaris'. Su dificultad diagnóstica radica en la escasez de amastigotes en las lesiones crónicas. *Leishmania tropica* es el agente causal más frecuente de la forma cutánea recidivante, entre otras especies de *Leishmania* (Khaled et al. 2011; Nilforoushzadeh et al. 2008; WHO 2010).

Figura 9. Lesiones características de la leishmaniosis cutánea producida por *L. tropica*. (a) forma clásica de la lesión, (b) lesión en nariz, (c) lesiones nodulares hiperqueratósicas propias de leishmaniosis recidivante (Imágenes cedidas por el equipo de la Dra. M. Riyad, Facultad de Medicina y Farmacia, Universidad Hassan II de Casablanca, Marruecos).

- **Leishmaniosis cutánea producida por *L. major*:** la enfermedad suele presentarse como lesiones ulceradas que pueden alcanzar hasta 8 cm de diámetro por su crecimiento hacia el exterior, de aspecto "húmedo" por el exudado purulento que producen y agravadas por infecciones bacterianas, y que con el tiempo se recubren de una costra. Se acompañan de inflamación de los vasos linfáticos y linfadenopatía satélite (WHO 2010). Las lesiones se encuentran en la cara y, más frecuentemente, en los miembros. El período de incubación es relativamente corto (2-3 meses), y más del 90% de los casos se describen durante el primer y cuarto trimestre del año, lo que se corresponde con el periodo de transmisión de verano a otoño (Riyad et al., 2013). La curación ocurre espontáneamente en 4-6 meses.

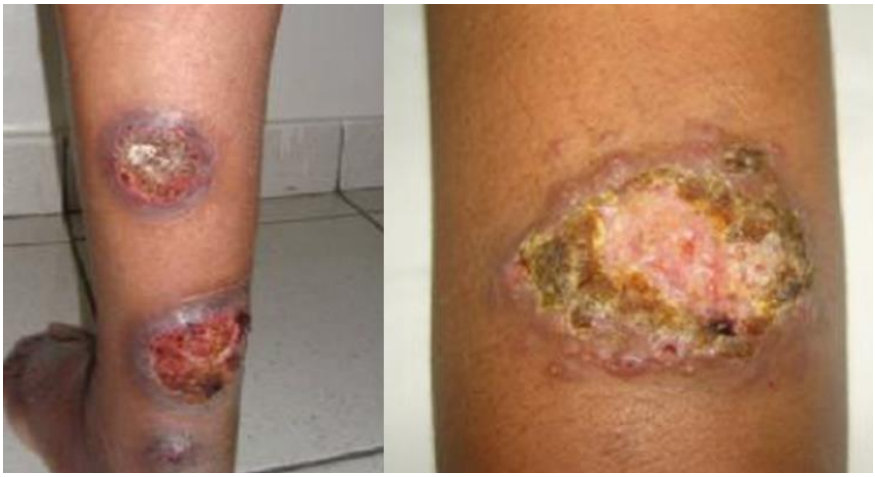

Figura 10. Lesiones características de la leishmaniosis cutánea producida por *L. major*. (Imágenes cedidas por el equipo de la Dra. M. Riyad, Facultad de Medicina y Farmacia, Universidad Hassan II de Casablanca, Marruecos).

- **Leishmaniosis cutánea producida por otras especies de *Leishmania*:** la mayoría de los casos importados pertenecen a especies procedentes del Nuevo Mundo y adquiridas por turistas (Schwartz et al., 2006). Son más complejos de diagnosticar al existir una gran diversidad de especies, de modo que, por ejemplo, las lesiones producidas por especies como *L (L.) mexicana* pueden curarse espontáneamente en un período de pocos meses mientras que las lesiones por *L (V.) braziliensis* pueden progresar hacia una leishmaniosis mucosa si no se trata (2% -10% de los casos) (Blum et al., 2004).

Leishmaniosis cutáneo-mucosa o mucosa estricta (LM): Principalmente causada por *L. braziliensis*, puede considerarse una extensión de la lesión cutánea inicial hacia las mucosas a través de los vasos linfáticos o sanguíneos (McGwire and Satoskar, 2014). Es muy desfigurante debido a la destrucción local del tejido en nariz, boca y nasofaringe, llegando a dificultar la respiración y hasta la alimentación. Puede presentarse varios meses después, incluso años, tras la cicatrización de la forma cutánea; o bien se manifiesta cuando las lesiones cutáneas aún están presentes. La malnutrición, aparición de lesiones primarias por encima de la cintura y la demora en la curación de dichas lesiones son algunos de los factores que aumentan el riesgo de padecer esta forma clínica (Salazar & Castro 2001; Sánchez-Saldaña et al. 2004; WHO 2010). Este tipo de leishmaniosis casi nunca cura de forma espontánea y puede ser mortal si no se aplica el tratamiento adecuado (Sánchez-Saldaña et al. 2004; WHO 2010). *Leishmania infantum* también

ha sido identificada como agente causal de esta forma de leishmaniosis en España, Francia e Italia (Aliaga et al., 2003; Faucher et al., 2011; Casolari et al. 2005). En España, la LM debida a *L. infantum* está infradiagnosticada y a menudo es confundida con procesos tumorales (Cobo et al., 2016).

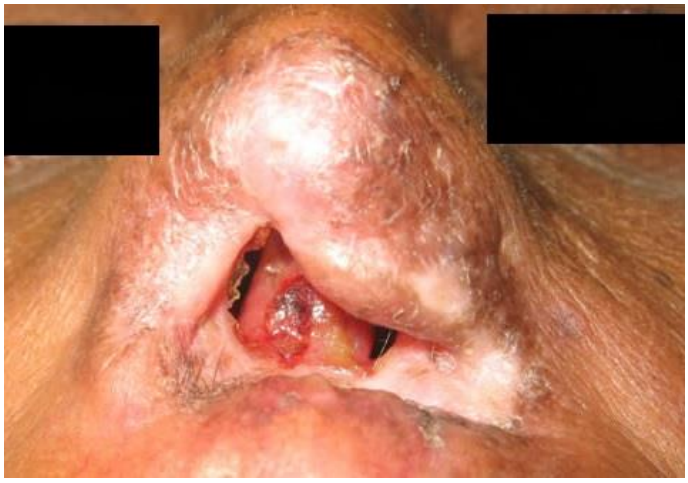

Figura 11. Lesiones características de la leishmaniosis cutáneo-mucosa. Imagen tomada de Aranzazu et al. (2007).

Leishmaniosis dérmica post-Kala-azar: Consiste en la aparición de lesiones cutáneas entre 6 meses y un año tras la aparente curación de la leishmaniosis visceral debida a *L. donovani*. Las lesiones que aparecen son máculas que pueden volverse papulares, nodulares o infiltrativas. Aparece especialmente en la cara (Fig. 12), aunque puede afectar a la mucosa bucal y genital y a la conjuntiva (WHO 2010). Hay muchas incógnitas alrededor de esta forma de leishmaniosis y se baraja la influencia del tratamiento así como de factores genéticos del parásito o el hospedador (Mukhopadhyay et al., 2014). Las lesiones producidas en los casos de África pueden curar espontáneamente pero raramente en los casos de la India (WHO 2010).

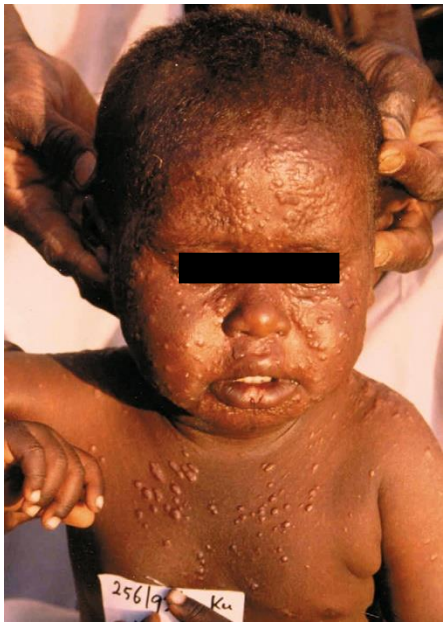

Figura 12. Lesiones características de la Leishmaniosis dérmica post-Kala-azar. Imagen tomada de Zijlstra et al. (2003).

Leishmaniosis asociada a inmunodepresión: No se trata de una forma de leishmaniosis, pero es importante señalar que en las personas inmunodeprimidas, además de mostrar una mayor susceptibilidad a la enfermedad, ésta adquiere manifestaciones clínicas más variables, apareciendo lesiones en zonas atípicas como los pulmones, por ejemplo, mientras que otros signos como la esplenomegalia suelen ser menos frecuentes (Monge-Maillo et al. 2014). También es de destacar que la leishmaniosis visceral reduce la eficacia de la terapia antirretroviral en pacientes con VIH (López-Vélez, 2003). Aunque en la leishmaniosis asociada a inmunodepresión se suele destacar el papel de la co-infección con VIH, no hay que olvidar a personas inmunodeprimidas por otras causas como el tratamiento de enfermedades autoinmunes o trasplantes de órganos (van Griensven et al., 2014) cuya relevancia epidemiológica es considerable y a la que según algunos autores debería de prestarse más atención (Herrador et al., 2015; Ramos et al., 2015).

Los fármacos anti-TNFα están indicados para el tratamiento de enfermedades inmunológicas como artritis reumatoide, espondilitis anquilosante, enfermedad de Crohn y psoriasis (moderada a grave). En la revisión realizada por Català et al. (2015) muestran que los pacientes que reciben tratamiento anti-TNFα en áreas endémicas podrían tener un riesgo elevado de sufrir leishmaniosis, ya que en condiciones de inmunosupresión la actividad leishmanicida de los macrófagos se reduciría drásticamente, lo que haría factible el desarrollo de LV además de LC o LM. Además, hay evidencias de la activación de la infección latente tras varios años

después de la exposición al parásito en este tipo de pacientes (Jewell and Giles, 1996). De modo que la detección serológica debe tenerse en cuenta para detectar la enfermedad latente y prevenir su reactivación y hacer seguimientos exhaustivos de los pacientes con el fin de evitar una nueva infección durante el tratamiento.

Elmahallawy et al. (2015), en un estudio realizado en Granada con pacientes asintomáticos trasplantados de riñón, encuentran que la prevalencia de leishmaniosis es de 4,8%, lo que concuerda con valores obtenidos en estudios realizados en las Islas Baleares y Castilla y León (Garrote et al., 2004; Riera et al., 2008). A pesar de no saber el momento en el que los pacientes asintomáticos desarrollarán la LV, parece existir una fuerte asociación entre la magnitud de la serología positiva y el riesgo de desarrollar la enfermedad (Hasker et al., 2014).

Leishmaniosis críptica o asintomática: en estudios llevados a cabo con donantes de sangre en el sur de Francia y España se demuestra que existe una alta tasa de infección por *Leishmania* en individuos asintomáticos (Le Fichoux et al., 1999; Riera et al., 2004). La presencia de *L. infantum* en sangre periférica de estos portadores puede ser sólo ocasional y de duración variable dependiendo de la virulencia del parásito y del sistema inmune del individuo (Le Fichoux et al., 1999; Riera et al., 2004). Se han descrito casos en los que el parásito puede permanecer en pacientes tras su recuperación clínica (Riera et al., 2005).

En un estudio reciente llevado a cabo con donantes de sangre en las Islas Baleares, Riera et al. (2008) describen una prevalencia de 3,1%. Seleccionaron aleatoriamente una muestra de 304 donantes de Mallorca y 18 de los 304 donantes probados (5,9%) fueron positivos a ADN de *L. infantum*. Además, dos cultivos de esta muestra aleatoria fueron positivos a Leishmania (0,6%). La falta de una técnica de referencia y métodos fiables para detectar la leishmaniosis críptica hace difícil determinar la extensión de esta forma de la enfermedad. Estudios previos indican que en áreas endémicas el ADN de *Leishmania* puede estar presente en la sangre de personas sanas sin que se detecte respuesta humoral (Mary et al., 2006; Riera et al., 2004). Esta falta de respuesta inmune humoral durante la infección asintomática también se ha observado en perros con infección críptica de *L. infantum* (Iniesta et al., 2002).

Leishmaniosis canina (LCa): La mayoría de los perros presentan estado de delgadez con tendencia hacia la anorexia. Los signos cutáneos incluyen lesiones cutáneas nodulares, ulcerativas y pustulosas, junto con dermatitis exfoliativa. También alopecia, palidez de las mucosas y reacciones eritematosas. Así mismo,

se pueden encontrar signos oculares como blefaritis, uveítis y conjuntivitis. La onicogrifosis es consecuencia de la gran cantidad de inmunoglobulinas circulantes. La adenopatía en los perros sintomáticos está caracterizada por un agrandamiento de los nódulos linfáticos debido a la hipertrofia de sus estructuras tisulares. Se puede encontrar el parásito en estos tejidos aunque la carga parasitaria no se correlaciona perfectamente con su tamaño o el estado clínico del animal (Koutinas and Koutinas, 2014). Se detecta, a su vez, glomerulonefritis asociada a la deposición de inmunocomplejos, que puede evolucionar hacia un fallo renal, siendo ésta la principal causa de muerte en los perros con leishmaniosis canina (Solano-Gallego et al., 2009). Debido a desórdenes de coagulación pueden presentarse epistaxis, hematuria y diarrea hemorrágica (Koutinas and Koutinas, 2014). En el bazo y en hígado, la presencia de amastigotes da lugar al agrandamiento de los órganos y cambios en su microestructura. El parásito invade gran parte del organismo del animal llegando hasta la médula ósea, considerado el tejido más profundo y responsable de la persistencia de la enfermedad y de sus recidivas (Momo et al., 2014). La parasitación de este tejido provoca cambios en la producción de células, dando lugar a pancitopenia en general y anemia arregenerativa en particular.

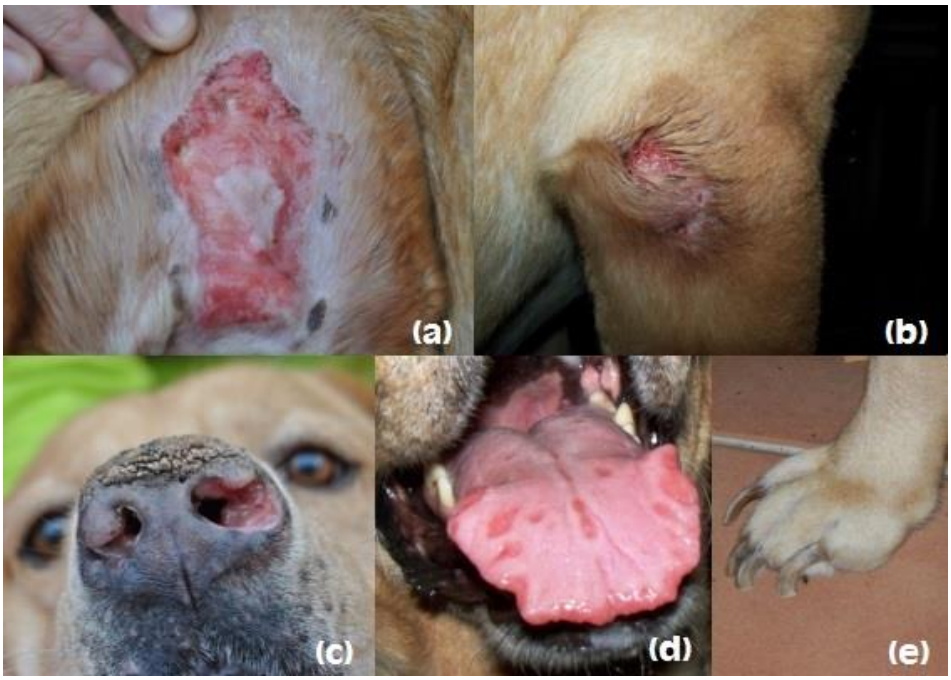

Figura 13. Lesiones características de la leishmaniosis canina. (a) lesión con infiltración en oreja, (b) lesión cutánea en codo de pata delantera, (c) lesión mucosa con pérdida de tejido, (d) lesiones nodulares en lengua, (e) onicogrifosis. Imágenes tomadas de Morales-Yuste (2012).

2.2. Diagnóstico de la leishmaniosis visceral

Diagnóstico parasitológico: La **observación microscópica** del parásito en preparaciones teñidas con Giemsa de aspirados medulares obtenidos del esternón o cresta ilíaca en niños, esplénicos, o ganglionares es la prueba clásica, de alta especificidad pero sensibilidad variable dependiendo del tejido (53-99%) y de la especialización del analista.

El **cultivo del parásito** de estas muestras aumenta la sensibilidad diagnóstica, aunque el resultado puede necesitar de un mes. Los medios bifásicos NNN (Novy, Nicolle y McNeal) y EMTM (Evans' Modified Tobie's Medium) son los más utilizados para el aislamiento del parásito. También se pueden utilizar medios líquidos, donde el suero bovino fetal se ha sustituido parcialmente por algunos autores por orina humana estéril al 1-2%, dando buenos resultados para *L. infantum* (Tasew et al., 2009). Finalmente se suelen suplementar con antimicrobianos y/o antifúngicos. La temperatura de cultivo suele situarse entre 23-26 ºC. Estos cultivos no se deben rechazar hasta pasados 2-3 meses de seguimiento.

Diagnóstico molecular: El diagnóstico mediante PCR detecta el ADN del parásito, mejorando considerablemente la sensibilidad. En los últimos años se han desarrollado un gran número de técnicas de PCR convencional, PCR anidadas y PCR en tiempo real basadas en la detección de diversos genes o regiones genómicas que ofrecen distintas sensibilidades y especificidades. Todas estas técnicas se pueden aplicar en una gran variedad de muestras, no solo las clásicas (sangre, aspirado medular, punción esplénica, o aspirado de ganglio) sino también en otras como orina (Silva et al. 2014) o raspado bucal (Vaish and Mehrotra, 2011), que resultan mucho menos invasivas.

Las técnicas basadas en PCR en tiempo real son más rápidas a la hora de dar un resultado pero requieren equipamiento complejo; sin embargo éstas aportan un valor añadido a la hora de cuantificar el ADN del parásito para el seguimiento de la enfermedad durante el tratamiento (Bastien et al., 2008; Sudarshan et al., 2014, 2011; Sudarshan and Sundar, 2014). Sin embargo, (Bastien et al., 2008), advierten que los resultados analíticos de PCR en tiempo real no son "automáticamente" mejores en comparación con los que se obtienen con la modalidad de PCR convencional, la aplicación de la PCR en tiempo real no garantiza la excelencia. El factor esencial para implantación de una técnica diagnóstica, y sobre todo de una técnica molecular, es la continua optimización de las condiciones de reacción así como el dominio de los fundamentos de la técnica, ya que requieren personal con

habilidades técnicas y conocimiento para desarrollar y validar estos ensayos, así como superar las dificultades técnicas experimentadas durante su realización rutinaria (Beld et al., 2007).

Las técnicas basadas en **LAMP** (Loop-Mediated Isothermal Amplification) se presentan como una alternativa muy prometedora a las basadas en PCR por varias razones (Khan et al., 2012; Nzelu et al., 2016; Verma et al., 2017): son adaptables al trabajo de campo ya que no necesitan ciclos de temperatura, solo una temperatura estable y los reactivos son termoestables; su detección se basa en la turbidez de la muestra, facilitando la obtención del resultado. Por tanto se presentan como pruebas fáciles, robustas, rápidas y sobretodo económicas.

Diagnóstico serológico: La detección de anticuerpos mediante pruebas serológicas como la inmunofluorescencia indirecta (IFI o IFAT), ELISA, Dot-ELISA, el test de aglutinación directa (DAT), y el inmunoblotting o Western blot muestran buenas sensibilidades y especificidades diagnósticas, siendo la IFI la técnica considerada de referencia (WHO 2010). También está disponible la prueba FAST (Fast Agglutination Screening Test) en muestras de suero (Boelaert et al., 2014). Los inconvenientes de estas técnicas es que pueden aparecer reacciones cruzadas con otras parasitosis (tripanosomosis, paludismo) o enfermedades autoinmunes o linfoproliferativas. Además los anticuerpos permanecen en niveles detectables durante mucho tiempo en el organismo, dificultando el diagnóstico de las recaídas.

Inmunocromatografía basada en el antígeno rK39: Las pruebas basadas en el antígeno rK39 son fáciles de realizar, rápidas (15-20 minutos), baratas (1$ por prueba) y reproducibles, pudiéndose usar en el diagnóstico de la leishmaniosis visceral en etapas tempranas. Es la prueba de primera línea ante la sospecha de la enfermedad en países en desarrollo endémicos, que se debe combinar con pruebas serológicas o parasitológicas adicionales en zonas donde su sensibilidad sea inferior al 90% (WHO 2010).

Pruebas de detección de antígenos: La prueba de aglutinación de látex (Sundar et al., 2005) muestra una elevada especificidad pero una sensibilidad moderada (48-87%) en el subcontinente indio y en África Oriental. Su principal ventaja es la correlación entre el resultado de la prueba y la eficacia del tratamiento en la enfermedad, aunque es difícil distinguir entre negativos y positivos débiles, afectando a la reproducibilidad de la técnica (Chappuis et al., 2007). La prueba KAtex tiene la ventaja de poder usarse en muestras de orina (Boelaert et al., 2014).

Intrademorreacción de Montenegro: Es el método clásico de detección de la inmunidad celular, aunque tiene más utilidad en la leishmaniosis cutánea. En zonas endémicas suele obtenerse resultado positivo por la elevada tasa de infecciones subclínicas y se utiliza como indicativo de infecciones asintomáticas (Acedo Sánchez et al., 1996; Morillas et al., 1996; Sanchis-Marín et al., 1997). Consiste en la inoculación de leishmanina (solución fenolada y autoclavada de promastigotes del parásito) de forma intradérmica en el brazo del paciente y la aparición de una induración debida a la respuesta celular.

2.3. Diagnóstico de la leishmaniosis cutánea y cutáneo-mucosa

Dada su similitud con otras enfermedades cutáneas, es necesaria su confirmación diagnóstica y recomendable llegar hasta la especie causante.

Los métodos parasitológicos clásicos, como la observación de improntas y cultivos, son el método de referencia en estas formas de la enfermedad (Reithinger and Dujardin, 2007; Vega-López, 2003). Las muestras se toman del exudado del borde de la lesión cutánea, pero no del centro porque puede estar infectado secundariamente y porque contiene menos parásitos. Se pueden realizar raspados o cortes para tomar la porción serosa de la lesión. Otros métodos son aspirados con jeringa de insulina (sin aguja) con solución de suero fisiológico, o mediante biopsia; en este último caso tiene la ventaja de obtener mayor cantidad de muestra, aunque la desventaja de ser un método más invasivo. En la leishmaniosis cutáneo-mucosa el examen microscópico o el cultivo son de poca utilidad debido a la poca cantidad de parásito en las lesiones por la fuerte reacción inmunitaria (Andrade-Narvaez et al., 2005; Motazedian et al., 2002).

El material obtenido por estos procedimientos se puede utilizar para examen microscópico del material teñido en Giemsa, cultivo o técnicas moleculares (Garcia et al., 2007).

El diagnóstico molecular permite no solo hacer el diagnóstico de la enfermedad sino también diferenciar la especie causante de la lesión, lo que resulta particularmente útil en regiones donde coexisten varias especies de *Leishmania* (Al-Jawabreh et al., 2006; Nicolas et al., 2002; Reithinger and Dujardin, 2007; Schönian et al., 2003). Una de las grandes ventajas de la aplicación de la PCR para el diagnóstico de la LC es la variedad de muestras que se pueden utilizar (Vega-López, 2003). En el diagnóstico de la leishmaniosis cutáneo-mucosa y mucosa la PCR ha demostrado ser el método más sensible (Martín-Sánchez et al., 2002).

El diagnóstico serológico es de uso limitado por su baja sensibilidad y especificidad variable en la leishmaniosis cutánea, obteniéndose títulos de anticuerpos nulos o bajos aunque su positividad aumenta la sospecha clínica en casos de leishmaniosis cutáneo-mucosa (WHO 2010).

2.4. Diagnóstico de Leishmaniosis dérmica post kala-azar

En estos casos el diagnóstico es clínico y puede confirmarse encontrando el parásito en muestras de las lesiones cutáneas obtenidas por raspado o biopsia. El diagnóstico molecular en estas muestras es también muy útil por su sensibilidad (WHO 2010).

2.5. Diagnóstico en caso de coinfección *Leishmania* y VIH

Dada la baja reacción inmunitaria, los diagnósticos parasitológico y molecular son los más adecuados, hecho reforzado por la mayor cantidad de parásito en sangre que los facilitan. Es de destacar el test de aglutinación de látex aplicado a orina de estos pacientes, que tiene una alta sensibilidad. Esta sensibilidad se puede aumentar utilizando una combinación de prueba rápida rK39 y aglutinación directa (WHO 2010).

2.6. Diagnóstico de la Leishmaniosis Canina

El diagnóstico de la enfermedad se basa en la observación de signos clínicos compatibles y anormalidades analíticas, la detección del parásito por técnicas parasitológicas o moleculares directas y la detección de anticuerpos IgG frente al parásito mediante IFI (Noli and Saridomichelakis, 2014). Las mismas técnicas utilizadas en la leishmaniosis visceral se pueden aplicar al diagnóstico de la leishmaniosis canina.

Un perro con signos clínicos compatibles y título elevado de anticuerpos frente al parásito puede ser diagnosticado como enfermo, dado que los perros enfermos suelen mostrar elevados títulos de anticuerpos (Miró et al., 2008).

En caso de títulos más bajos en ausencia de signos clínicos, se puede confirmar la infección asintomática mediante la detección del parásito por técnicas moleculares sensibles. Para descartar la enfermedad sería necesario repetir las pruebas pasados 3 meses (Miró et al., 2008).

3. Tratamiento de la leishmaniosis

La leishmaniosis es tratada fundamentalmente con fármacos inicialmente desarrollados para otras enfermedades y de hecho, sus mecanismos de acción en la mayoría de los casos no están claros (Singh et al., 2012). Sin embargo, el refinamiento en las dosis y las pautas en el tratamiento de la leishmaniosis junto con la combinación de fármacos ha llevado a que actualmente, el tratamiento de la leishmaniosis tenga un porcentaje de cura del 95% (Matlashewski et al., 2011).

El tratamiento de la leishmaniosis se debe administrar tras la confirmación de la enfermedad, de ahí la importancia de un diagnóstico precoz y eficaz de la misma. Es recomendable que se identifique la especie responsable de la enfermedad debido a la variabilidad en la respuesta y en las formas clínicas de la enfermedad, lo cual es especialmente importante en el caso de la leishmaniosis cutánea en el Nuevo Mundo o en zonas en las que coexisten varias especies de *Leishmania* que produzcan el mismo cuadro clínico, como la leishmaniosis cutánea producida por *L. tropica* y *L. major* en el Viejo Mundo.

Los principales problemas a resolver en la terapéutica de la leishmaniosis son el elevado coste del tratamiento, los efectos adversos del arsenal terapéutico, la aparición de resistencias y la vía de administración y duración del tratamiento (Freitas-Junior et al., 2012).

3.1. Arsenal farmacológico

Antimoniales pentavalentes: son derivados del ácido estibónico, en los que el antimonio (Sb^V) se encuentra unido a una molécula de glucosa. Dos son los fármacos de este grupo, el antimoniato de meglumina (Glucantime®) y el estibogluconato sódico (Pentostam®). Tanto su eficacia como su toxicidad se basan en su contenido en antimonio. Se llevan utilizando en el tratamiento de la leishmaniosis desde hace más de 50 años y aun así se consideran la primera línea de tratamiento en las regiones donde no existe resistencia. A pesar de su elevado porcentaje de éxito (90%), estos medicamentos han encontrado problemas de resistencias en Bihar (India) y Nepal, con fracaso terapéutico de hasta un 60% (WHO 2010). Se considera un profármaco que es reducido en el macrófago o en el propio parásito a antimonito trivalente, la forma activa del fármaco. El mecanismo de acción exacto es desconocido, aunque ambas formas de antimonio provocan la fragmentación del ADN (con posible implicación en la apoptosis), beta oxidación de ácidos grasos y fosforilación del ADP. Una interesante observación,

realizada recientemente es que la exposición crónica a arsénico en el agua de bebida podría ser la causa de la aparición de resistencia a los antimoniales (Perry et al., 2013). El tratamiento con antimonio se debe administrar por vía intramuscular o intravenosa en perfusión, o inyección lenta con aguja fina con el fin de evitar trombosis. En el caso de la leishmaniosis cutánea, se administra intralesionalmente y en perros se administra de forma subcutánea. Son el primer fármaco de elección en el tratamiento de la leishmaniosis canina en España (Miró et al., 2008). Los efectos adversos constituyen un gran problema: anorexia, náuseas y vómitos, dolor abdominal, malestar, mialgia, altralgia, cefaleas, letargia, alteraciones electrocardiográficas (inversión de la onda T, prolongación del intervalo QT), arritmias, elevación de enzimas pancreáticas y hepáticas, leucopenia, anemia y trombocitopenia. Sus efectos cardiotóxicos y hepatotóxicos pueden llevar a la retirada del tratamiento (WHO 2010). Se ha comprobado la generación de resistencia a antimoniales en casos de leishmaniosis canina (Carrió and Portús, 2002; Gramiccia et al., 1992) aunque en estos casos no se ha caracterizado el origen de esa resistencia, limitándose a describir el fracaso terapéutico y la sensibilidad *in vitro* de los parásitos aislados antes y después del tratamiento. En un estudio reciente, Gómez-Pérez et al. (2016) realizaron ensayos de susceptibilidad a antimonio, observando un incremento en la resistencia a antimonio, lo que podría explicar el fracaso terapéutico del Glucantime®. Este aumento en la resistencia se ha atribuido a niveles de expresión inferiores en la proteína acuagliceroporina-1, responsable del transporte de antimonio al interior del parásito.

Anfotericina B: este fármaco muestra una gran afinidad por el ergosterol, el esterol predominante en las membranas celulares de *Leishmania*, formando complejos con éstos y abriendo poros que desequilibran el equilibrio iónico, llevando a la muerte celular (Roberts et al., 2003). Poseen una gran eficacia frente a *Leishmania,* por lo que prácticamente han sustituido a los antimoniales en algunas regiones como España o donde existen resistencias a los antimoniales. La formulación de deoxicolato de anfotericina B (Fungizone®), aunque muy eficaz, no está exenta de reacciones adversas: son frecuentes la fiebre, los escalofríos y la tromboflebitis, siendo la nefrotoxicidad también común. Por estas razones el tratamiento debe ser en régimen de hospitalización, lo que dificulta su administración y cumplimiento. Las formulaciones lipídicas de anfotericina B se desarrollaron con el fin de mejorar su biodisponibilidad, propiedades farmacocinéticas y reducir sus efectos adversos sin perder su eficacia: son la anfotericina B liposómica (Ambisome®), el complejo lipídico de anfotericina B (Abelcet®) y la dispersión coloidal (Amphocil®). Presentan una toxicidad

significativamente menor y su administración se realiza en infusión durante 2h. Tienen una eficacia del 95%. El principal inconveniente de estas formulaciones lipídicas es el precio. También se han detectado resistencias asociadas al tratamiento, donde las cepas resistentes de *L. donovani* presentan ergosterol no alquilado, aunque no es el único mecanismo citado (Freitas-Junior et al., 2012).

Aminosidina (Paromomicina o Humatin®): antibiótico aminoglucosídico con actividad antibacteriana y frente a *Leishmania.* Su mecanismo de acción es poco claro, aunque se sugiere que penetra en *Leishmania* por endocitosis, siendo la mitocondria su principal diana. En otros estudios, Hirokawa et al. (2007) demostraron que la paromomicina interactúa con las subunidades ribosómicas 30S y 50S, inhibiendo la síntesis proteica. Es muy útil en el tratamiento de la leishmaniosis cutánea y visceral, con una elevada eficacia y tolerabilidad. En ensayos clínicos en la India y África Oriental se demostró la eficacia de su tratamiento, poniéndose de manifiesto su utilidad en casos de resistencia a antimoniales (Jha et al., 1998; Sundar and Chakravarty, 2015) y en la leishmaniosis cutánea se presenta como una alternativa de fácil aplicación tópica (Monge-Maillo and Lopez-Velez, 2014). Su principal limitación es su poca disponibilidad en zonas endémicas. Aún no se han mostrado resistencias en uso clínico debido a su poco uso, pero se conocen *in vitro*.

Miltefosina (Impavido®): Es un alquilfosfolípido desarrollado inicialmente como antineoplásico; se convirtió en el primer fármaco utilizado frente a la leishmaniosis por vía oral, suponiendo un enorme avance. De hecho se ha recomendado para el tratamiento de la leishmaniosis visceral en niños (Bhattacharya et al., 2007; Dorlo et al., 2012a). Su actividad se basa en su acumulación intracelular. Causa procesos similares a apoptosis en amastigotes de *L. donovani*. Otro efecto es la estimulación de la óxido nítrico sintasa 2, que cataliza la generación de óxido nítrico y la eliminación del parásito (Wadhone et al., 2009). Provoca efectos adversos gastrointestinales, mayoritariamente leves como anorexia, náuseas, vómitos y diarrea. Es potencialmente teratógeno y abortivo, por lo que está contraindicado en mujeres en edad fértil. Su posible mal uso (dada su administración oral), puede dar lugar a la aparición de resistencias (Cojean et al., 2012; Hendrickx et al., 2014; Mondelaers et al., 2016; Rijal et al., 2013). Además de las resistencias, se han descrito casos de adulteración o baja calidad del medicamento (Dorlo et al., 2012b).

Pentamidina: es una diamidina aromática utilizada como tratamiento de segunda línea en casos de resistencia a antimonio, particularmente en India, aunque su

utilización está en decadencia debido a su eficacia cada vez más reducida y a las resistencias (Sundar, 2001) aunque aún posee valor para la terapia combinatoria (Croft and Coombs, 2003). Como otros fármacos, su mecanismo de acción es confuso, aunque podría estar implicada alguna función de la mitocondria (Sun and Zhang, 2008). Este fármaco es bastante tóxico, provocando hipoglucemia grave, nefrotoxicidad, baja tensión arterial, miocarditis y diabetes mellitus.

Derivados azólicos: Ketoconazol, fluconazol e itraconazol son antifúngicos orales con eficacia variable en el tratamiento de la leishmaniosis.

Imiquimod: inmunofármaco que estimula la producción de citoquinas como el interferón-γ, induciendo la síntesis de óxido nítrico y la eliminación del parásito. Utilizado recientemente frente a la leishmaniosis cutánea en forma de pomada (Croft and Coombs, 2003; Hervás et al., 2012) y en combinación con antimoniales (Miranda-Verástegui et al., 2005).

Alopurinol (Zyloric®): isómero de la hipoxantina e inhibidor de la xantina oxidasa. Con eficacia probada en varios estudios frente a *Leishmania* (Balaña-Fouce et al., 1998), se emplea en la leishmaniosis canina como preventivo o terapia de mantenimiento tras el tratamiento con Glucantime.

4. El Vector

Como se ha mencionado anteriormente, las especies de protozoos pertenecientes al género *Leishmania* presentan un ciclo heteroxeno, lo que supone que parte de éste se desarrolla en el hospedador invertebrado que actúa como vector. Los flebotomos son dípteros nematóceros pertenecientes a la familia Psychodidae, al género *Lutzomyia* en el Nuevo Mundo y al género *Phlebotomus* en el Viejo Mundo; y se distribuyen principalmente en las partes tropicales y cálidas del mundo, incluidos el sur de Europa, Asia, África, Australia, América central y Sudamérica. Del total de las aproximadamente 700 especies conocidas, sólo en unas 60 se ha demostrado este papel vectorial (Gil Collado et al., 1989.; Ready, 2010).

Tanto machos como hembras se alimentan de zumos vegetales y savia, pero las hembras además son obligatoriamente (y salvo excepciones) hematófagas, y responsables, por tanto, del papel vectorial de *Leishmania* y otros agentes infecciosos.

Son insectos de hábitos nocturnos y crepusculares que tienden a salir con el crepúsculo hasta pasada la media noche; suelen encontrarse en regiones donde la temperatura diurna es superior a 15,6 ºC durante al menos tres meses al año y a altitudes que oscilan entre el nivel del mar y los 1.500 metros sobre el nivel del mar (m s. n. m) e incluso más (Lane, 1993). En los estudios realizados en la provincia de Granada se han encontrado ejemplares de *P. ariasi* hasta los 2613 m s .n. m (Franco et al., 2010), *P. perniciosus* hasta los 1534 m s. n. m (Barón et al., 2011) (Barón et al. 2011) y *P. sergenti* hasta los 1353 m s. n. m (Barón et al., 2013). En un estudio reciente en la provincia de Lleida se han capturado individuos de *P. perniciosus* y *P. ariasi* a 1630 y 1620 m s. n. m respectivamente (Ballart et al., 2014).

La ovoposición tiene lugar sobre un soporte sólido de origen vegetal o animal, en lugares oscuros, de humedad relativa elevada y temperatura constante tales como huecos de árboles, agujeros de muros o casas abandonadas, leñeras o vertederos. Esta puesta de huevos se produce aproximadamente 3-7 días después de la ingesta de sangre y el número oscila entre 30-70 unidades, siendo la media de 50.

La vía de transmisión principal de la leishmaniosis es a través de la picadura del flebotomo, aunque se han descrito casos de infección accidental por transfusión de sangre (Mestra et al., 2011) y transmisión congénita (Meinecke et al., 1999; Osorio et al., 2012). Cabe destacar la transmisión a partir de agujas infectadas con

el parásito entre drogodependientes, siendo de gran relevancia en la coinfección VIH-*Leishmania* (Alvar et al., 1997; Morillas-Marquez et al., 2002). Según la WHO (2010), los criterios generalmente aceptados para incriminar vectores de *Leishmania* son:

1. **El vector ha de ser antropofílico**. Se determina mediante la captura nocturna cuando van a picar a humanos o por el análisis de la sangre ingerida por las hembras que se han capturado en las viviendas.
2. **El vector debe picar al reservorio**. La mayoría de las leishmaniosis son zoonosis por lo que el hecho de que un supuesto vector se alimente del reservorio evidencia su posible papel vectorial. Para determinar las preferencias de alimentación se utiliza la comparación de las capturas en trampas cebadas con diferentes animales. Mediante el análisis de la sangre ingerida por las hembras, se pueden determinar los animales de los que se alimenta el vector, pero no la preferencia de alimentación.
3. **El vector se debe infectar en la naturaleza por la misma especie de *Leishmania* que afecta a los humanos**. Las técnicas que se suelen usar para determinar la especie son las isoenzimáticas o análisis de ADN. Los resultados más significativos se obtienen de hembras sin sangre, o con ésta digerida, ya que los parásitos podrían detectarse en la sangre fresca ingerida por flebotomos no vectores, a pesar de que en éstos no puedan completar su ciclo.
4. **El vector debe ser compatible con el crecimiento del parásito que transmite**. Cuando los flebotomos vectores están infectados por *Leishmania* adquirida en la naturaleza, el parásito persiste después de que la sangre haya sido digerida. Estos estudios se pueden complementar con infecciones experimentales.
5. **El vector ha de ser capaz de transmitir al parásito mediante la picadura**. Verificar este aspecto actualmente es difícil para determinadas especies, por su dificultad de cultivo y por la dificultad de persuadir a las hembras para que piquen más veces logrando así la transmisión.

4.1. Taxonomía del vector

Estos pequeños insectos pertenecen al orden Diptera, familia Psychodidae, subfamilia Phlebotominae. Según la clasificación de Lewis (1982) revisada por Young & Duran (1994), esta subfamilia está compuesta por 6 géneros, 3 en el Viejo Mundo (*Sergentomyia*, *Phlebotomus* y *Chinius*) y 3 en el Nuevo Mundo (*Lutzomyia*, *Brumptomyia* y *Warileya*); siendo los géneros *Phlebotomus* y *Lutzomyia* los vectores de la leishmaniosis humana.

Reino	**Animal**	
Phyllum	**Arthropoda**	
Clase	**Insecta**	
Orden	**Diptera**	
Suborden	**Nematocera**	
Familia	**Psychodidae**	
Subfamilia	**Phlebotominae**	
Géneros	***Sergentomyia***	Viejo Mundo
	Phlebotomus	
	Chinius	
	Brumptomyia	Nuevo Mundo
	Lutzomyia	
	Warileya	

Figura 14. Encuadramiento taxonómico de la subfamilia Phlebotominae y los géneros que la componen de acuerdo con la clasificación de Lewis (1982) y actualizada por Young & Duran (1994).

4.2. Morfología del vector

Los flebotomos son dípteros de pequeño tamaño (1,5-3,5 mm), color amarillento, con cuerpo y alas densamente cubiertos de pelo. Su cuerpo es giboso y presentan dos alas lanceoladas con nerviación longitudinal característica y que en posición de reposo se disponen en ángulo de 45º con el cuerpo. Se caracterizan por la presencia de piezas bucales largas y mandíbulas bien desarrolladas adaptadas a la succión, palpos divididos en 5 segmentos, antenas cilíndricas, y venas radiales de las alas distribuidas en 5 ramas. Su constitución es frágil y vuelan a saltos. Su cuerpo se divide en: **cabeza, tórax y abdomen** (Gállego-Berenguer, 2006).

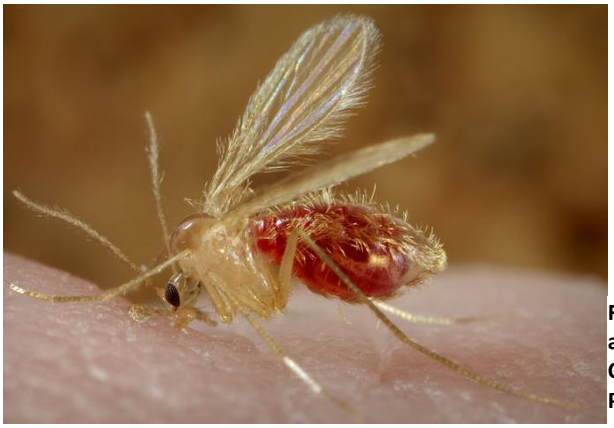

Figura 15. Hembra de flebotomo alimentándose. Imagen tomada de Centers for Disease Control and Prevention (CDC).

4.2.1. Cabeza

Se encuentra constituida por una estructura quitinosa capsular o epicráneo y dos grandes ojos multifacetados. El epicráneo se divide en: occipucio, vértice y frente. Esta última se prolonga hasta el clípeo, donde se inserta el aparato picador-chupador de la hembra de flebotomo o probóscide. Entre los ojos se localiza la sutura interocular, y a su altura unas piezas muy irregulares conocidas como escape o primera pieza de la antena. De los 16 fragmentos que constituyen cada antena el segundo es el pedicelo o torus, en el que se fijan los 14 artejos restantes. Desde la tercera pieza de la antena hasta el final se pueden ver una serie de finas sedas y otros elementos más gruesos, refringentes y acodados en su base, que Newstead denominó espinas geniculadas o ascoides con función sensorial. En la hembra, la probóscide está constituida por seis piezas que se hayan protegidas ventralmente por el labium, y cuyo conjunto se denomina fascículo:

- **Labro-epifaringe**: muy quitinizada con forma de hoja de espada y cruzada ventralmente por un canal de papilas sensoriales**.**
- **Dos mandíbulas:** largas palas afiladas una sobre la otra. En el borde interno del extremo terminal presentan pequeñas denticulaciones.
- **Dos maxilas:** más gruesas que las mandíbulas y se disponen a ambos lados del canal labial. Presentan denticulaciones en los dos bordes del extremo terminal. De la base de las maxilas parten los palpos, constituidos por cinco segmentos, de los cuales el quinto es más largo.
- **Hipofaringe**. Pieza simétrica terminada en dientes de sierra delgados y flexibles y que está cruzada por el canal salival. En su porción anterior, a la altura del clípeo, se une al labro-epifaringe prolongándose al interior de la cavidad cefálica.

El labium constituye la pieza más voluminosa de la probóscide. La parte basal está constituida por una placa pseudotriangular o mentum, en cuyo extremo descansan las labelas, que son apéndices móviles. El cibarium es una estructura con gran capacidad suctoria situada dentro de la cabeza. A veces, en él aparece un engrosamiento en posición posterior conocido como mancha pigmentaria, donde se pueden observar unos dientes más o menos desarrollados denominados dientes cibariales. A continuación se dispone la faringe, que está constituida por la unión de tres placas soldadas, dando lugar a una conformación parecida a una botella de sección triangular. En el extremo posterior de la faringe se ubica una estructura reticulada, la armadura faríngea. Tanto el cibarium como la faringe y su armadura faríngea son elementos que, dada su variabilidad morfológica, ostentan gran importancia taxonómica (Abonnenc 1972; Léger & Depaquit 1999; Morillas-Márquez 1981).

4.2.2. Tórax

De aspecto giboso y recubierto de pelos, está compuesto de 3 segmentos llamados: protórax, mesotórax y metatórax. De cada uno de éstos salen un par de patas de hasta tres veces la longitud corporal. El protórax es muy pequeño y parcialmente cubierto por el mesotórax, más desarrollado y con dos alas dorso-lateralmente. En el metatórax se distinguen los dos balancines, que son pequeños apéndices de un segundo par de alas vestigiales cuya función es estabilizar el vuelo del flebotomo (Abonnenc, 1972; Davis, 1967; Morillas-Márquez, 1981). Desde un punto de vista sistemático las estructuras más destacables de esta unidad funcional son las alas. Éstas son lanceoladas y muy pilosas. Presentan abundantes nerviaciones longitudinales y algunas transversas. Los parámetros taxonómicos empleados en su caso son las posiciones relativas de diversos puntos de confluencia de dichas nerviaciones (Rioux & Golvan 1969).

. De este modo, en las especies del género *Phlebotomus* $\alpha > \beta$, mientras que en las pertenecientes al género *Sergentomyia* $\alpha < \beta$ (Fig. 17).

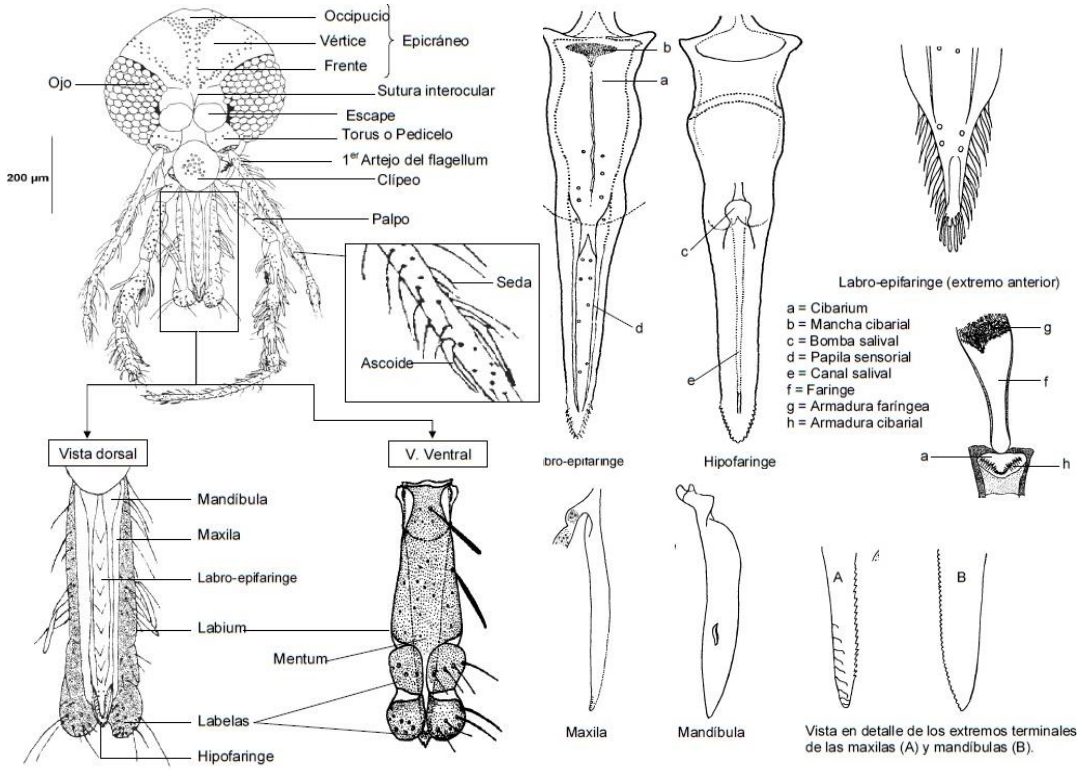

Figura 16. Estructura de la cabeza, probóscide y aparato bucal del flebotomo (Abonnenc 1972, Léger y Depaquit 1999). Imágenes tomadas de Morales-Yuste (2012).

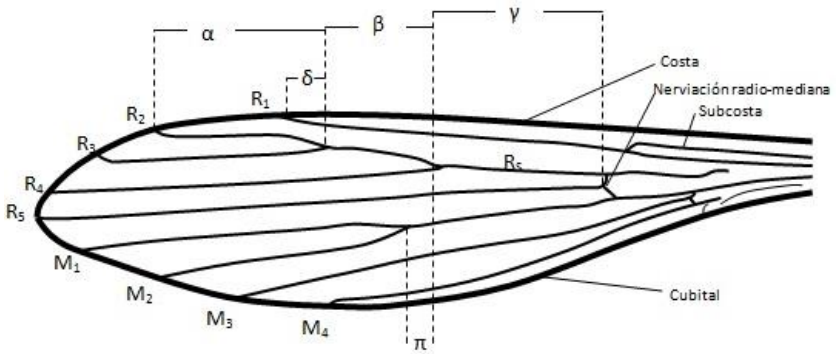

Figura 17. Esquema del ala de flebotomo en el que se señalan las nerviaciones de interés taxonómico. Imagen tomada de Morales-Yuste (2012).

Estas nerviaciones reciben la siguiente nomenclatura:

- 1ª Longitudinal simple: R1.
- 2ª Longitudinal o sector radial: Se divide en R2, R3 y R4.
- 3ª Longitudinal simple: R5.
- 4ª Longitudinal: M1 y M2.

- 5ª Longitudinal simple: M3.
- 6ª Longitudinal simple: M4.
- 7ª Longitudinal simple o cubital.

4.2.3. Abdomen

En gran parte es cilíndrico y está formado por la unión de 10 segmentos. Todos los segmentos que conforman el abdomen tienen una placa quitinosa llamada tergito en su parte dorsal, y otra llamada esternito en su parte ventral. Ambas estructuras se encuentran separadas lateralmente por la membrana pleural, que permite la dilatación del abdomen en el momento de la alimentación. El primero de estos 10 segmentos se fija al tórax, y los dos últimos segmentos, muy modificados, conforman el aparato genital externo. Los 7 segmentos no modificados restantes presentan cada uno un par de estigmas respiratorios, cuya apertura se encuentra generalmente en el borde anterior del segmento sobre la membrana pleural.

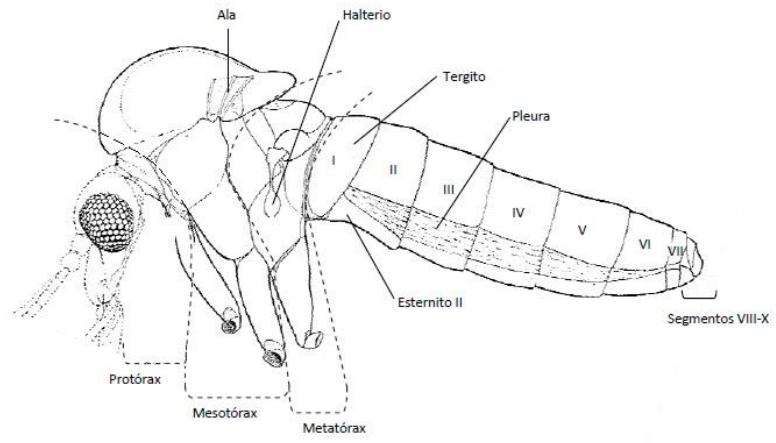

Figura 18. Esquema del abdomen del flebotomo (Davis 1967). Imagen tomada de Morales-Yuste (2012).

Principales características del abdomen de la hembra de flebotomo: En estado de reposo los segmentos 8 al 10 se encuentran involucionados dentro del séptimo tergito y esternito. Estas piezas sólo se encuentran extendidas durante la ovoposición, apareamiento y defecación (Davis, 1967). El octavo tergito, más desarrollado que los precedentes, continúa en la región ventral con un esternito lobulado denominado gonapófisis ventral. A continuación se dispone el noveno tergito, bajo el cual se observa una región membranosa o área genital donde convergen los orificios de los conductos espermáticos de las espermatecas. Al

último segmento abdominal se fijan dos placas laterales conocidas como cercas o gonapófisis dorsales, que se hallan separadas por una estructura quitinosa central que constituye el ano (Abonnenc, 1972). En la práctica, uno de los elementos más utilizados para la clasificación de especies es la espermateca con su cabeza (a), cuello (b), cuerpo o reservorio (c) y conductos espermáticos (d) (Fig. 19).

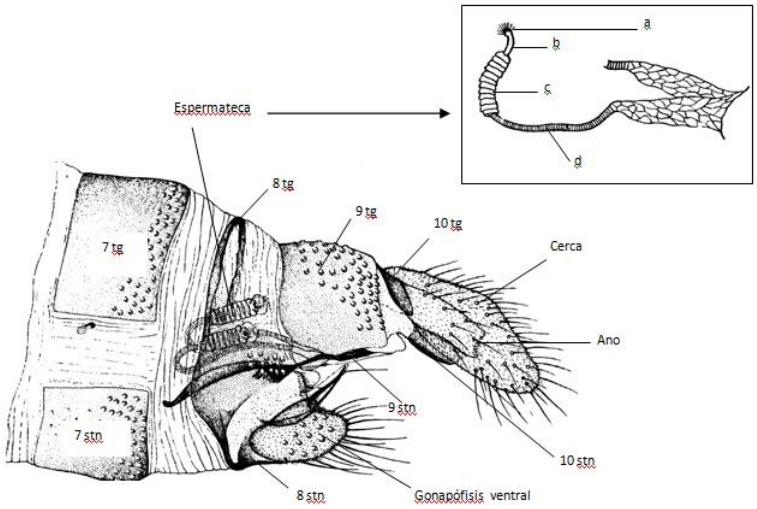

Figura 19. Abdomen terminal de hembra de *P.papatasi*. Espermateca de *P. ariasi*. tg, tergito; stn, esternito (Jobling, 1987; Theodor, 1958)**. Imagen tomada de Morales-Yuste (2012).**

Principales características del abdomen del macho de flebotomo: los segmentos 7 y 8 se encuentran poco desarrollados. Por el contrario, los segmentos terminales se encuentran muy evolucionados y a las 24-48 horas de la eclosión se da una rotación de 180° que hace que a partir del octavo fragmento los esternitos se dispongan dorsalmente y los tergitos en posición ventral (Abonnenc, 1972). De este modo, la genitalia está formada por dos apéndices laterales alargados (coxitos) que articulan con sendas prolongaciones llamadas estilos. En la porción distal de cada estilo se sitúan una serie de espinas cuya disposición y longitud son elementos empleados en la sistemática de diversas especies. De la base de los coxitos salen dos piezas ventrales denominadas parámeros, entre los cuales se observan unas estructuras fuertemente quitinizadas que reciben el nombre de aedeagus o valvas del pene. Estas valvas del pene se disponen rodeando el extremo terminal del filamento genital o canal eyaculador, el cual se prolonga al interior del abdomen para conectar con la pompa genital, que a su vez se encuentra ensamblada al pistón (Fig. 20). Finalmente, en posición ventral se disponen dos estructuras que reciben la denominación de lóbulos laterales, los

cuales se hallan soldados a las cercas por su cara interna (Abonnenc 1972; Léger & Depaquit 1999; Morillas-Márquez 1981).

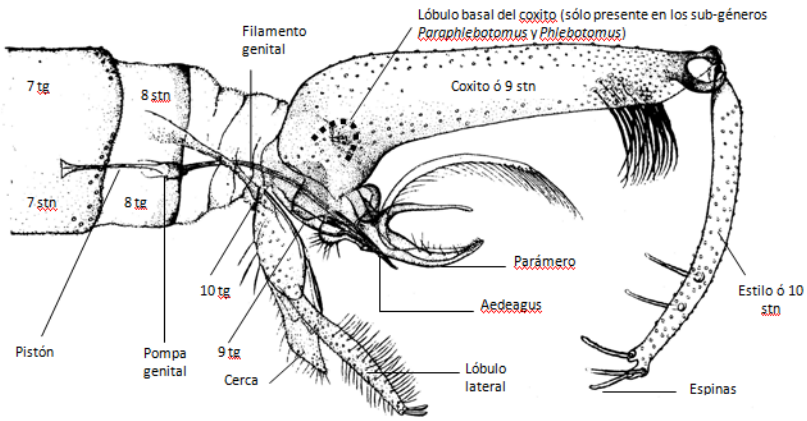

Figura 20. Abdomen terminal de macho de *P.papatasi*. tg, tergito; stn, esternito (Jobling 1987). Imagen tomada de Morales-Yuste (2012).

4.3. Distribución de las principales especies presentes en España

A lo largo de los años se ha podido comprobar que en total hay 11 especies de flebotomos presentes en nuestro país pertenecientes a dos géneros: *Sergentomyia* y *Phlebotomus*. Dentro del género *Sergentomyia* están las especies *S. minuta* y *S. fallax* pertenecientes al subgénero *Sergentomyia*. En el género *Phlebotomus* se diferencian 5 subgéneros que agrupan a las 9 especies restantes: *Phlebotomus* (*P. papatasi*), *Larroussius* (*P. perniciosus*, *P. ariasi* y *P. langeroni*), *Paraphlebotomus* (*P. sergenti*, *P. alexandri* y *P. chabaudi*), *Adlerius* (*P. mascittii*), y *Abonnencius* (*P. fortunatarum*).

En España los vectores principales son las hembras de *Phlebotomus perniciosus* en zonas áridas y semiáridas mientras que *P. ariasi* lo sería principalmente en zonas más húmedas (Gil Collado et al. 1989; Morillas et al. 1995). La especie *P. longicuspis* se ha citado como vector de *L. infantum* en el Norte de África, pero su presencia en España se ha descartado (Collantes & Ortega 1997; Martín-Sánchez et al. 2000; Morillas-Márquez et al. 1991).

Otras especies que tienen notable importancia en España son *P. papatasi* (subgénero *Phlebotomus*) y *P. sergenti* (subgénero *Paraphlebotomus*), que representan entre el 5-10% de las capturas en algunos lugares. Estas especies transmiten *L. major* y *L. tropica* respectivamente, especies que hasta ahora no se han descrito como autóctonas en nuestro país. Otro vector de *L. infantum, P.*

langeroni se encuentra también en nuestro país con una distribución que se creía muy limitada (Martinez Ortega et al., 1996). Recientemente, hemos puesto de manifiesto su presencia en el sudeste de España en asociación con madrigueras de conejos y su posible papel vectorial (Díaz-Sáez et al., 2013).

El mapa de distribución de las especies de flebotomos existentes en España se completa como sigue (Fig. 21) (Gil Collado et al. 1989; Morales Yuste 2012): *P. mascitti*, que sólo se ha descrito en Cataluña, pero en escaso número. Esta especie también existe en Francia, Bélgica y Alemania. En las Islas Canarias se encuentran *P. fortunatarum* (exclusivo de las islas) y en escaso número, *Sergentomya fallax* presente también en el Norte de África. Por último, *S. minut*a es la especie más ubicua y abundante en toda España cuando se hacen capturas con trampas adhesivas, sin embargo es herpetófila (las hembras se alimentan de la sangre de reptiles).

Figura 21. Especies de flebotomos descritas en España y mapa de distribución (Aransay et al. 2004; Conesa Gallego et al. 1997; Díaz-Sáez et al. 2013; Gállego-Berenguer et al. 1992; Gil Collado et al. 1989; Lucientes et al. 1994; Lucientes et al. 2001; Martinez Ortega et al. 1996; Merino-Espinosa et al. 2016; Morillas-Márquez et al. 2017). Imagen tomada de Morales-Yuste (2012) y completada por la autora.

4.4. Análisis moleculares aplicados al estudio de poblaciones de flebotomos

En las últimas décadas ha habido un notorio aumento en los marcadores genéticos disponibles el estudio de diversidad genética de los flebotomos, enfocados a

determinar la variabilidad inter e intraespecífica. Conocer la similitud entre los individuos y las poblaciones es de gran utilidad a la hora de comprender la epidemiología de la leishmaniosis y poder elaborar programas de control del vector más eficaces y específicos. Además la existencia de razas o especies hermanas, sólo identificables a nivel molecular, podría influir en la capacidad vectorial de las diferentes especies de flebotomos.

Los marcadores genéticos más utilizados para el estudio de las diferentes especies de flebotomos son el gen ribosómico ITS-2 y los mitocondriales ND4 (subunidad 4 de la NADH deshidrogenasa), Cyt b (citocromo b) y Cox I (subunidad I de la citocromo oxidasa) (Belen et al., 2011; Depaquit et al., 2015, 2013, 2008; Moin-Vaziri et al., 2007).

En los estudios realizados con marcadores mitocondriales en *P. perniciosus,* vector de *L. infantum* en la Península Ibérica, se describen dos líneas mitocondriales: una "línea típica" encontrada en Marruecos, Túnez, Malta e Italia, y una "línea ibérica" (Aransay et al., 2003; Esseghir et al., 2000, 1997; Perrotey et al., 2005; Pesson et al., 2004). Además, la "línea ibérica" estaría formada por dos poblaciones: una que englobaría la zona norte de la península y otra que abarcaría el centro-sur de España (Aransay et al., 2003).

Al estudiar las poblaciones de *P. ariasi*, vector de *L. infantum* en la Península Ibérica, en el sur de España se identificaron dos poblaciones distintas que se asociaban con áreas que presentaban diferentes características bioclimáticas (Martín-Sánchez et al., 2000). De modo que se llevó a cabo un estudio intraespecífico con el uso del marcador Cyt b, en el que se comprobó la amplia diversidad genética (Franco et al., 2010) distribuida en dos líneas mitocondriales: una que limita geográficamente con Argelia y la otra situada en el resto de países del Mediterráneo. Cabe destacar que en Andalucía se encontraron una gran cantidad de haplotipos.

En cuanto a *P. sergenti,* vector de *L. tropica* ampliamente distribuido en la Península Ibérica donde no se han detectado casos autóctonos de *L. tropica,* se analizaron varias poblaciones en España y Marruecos (Barón et al., 2008). El estudio permitió identificar una única línea ribosómica y 4 líneas mitocondriales: dos exclusivas de Marruecos, una exclusiva de España y una de ellas (línea III) común entre estos dos países. Esta línea coincide con la llamada "línea Taza" de Yahia et al. (2004), que fue encontrada en 3 focos de LC antroponótica investigados por

estos autores. La presencia en España de una línea mitocondrial exclusiva junto con una en común con el norte de Marruecos donde la LC por *L. tropica* se considera epidémico-emergente podría tener importantes repercusiones epidemiológicas.

III. Objectives

Molecular-based diagnostic methods, such as PCR, are highly sensitive and specific assays for the detection of *Leishmania* DNA and the differentiation of species, thus considered the cornerstone in the diagnosis of leishmaniases, but they are variable depending on the sample used. However, lack of detailed standardization and global homogenization and validation together with the reduction of specificity in endemic areas are the main disadvantages of these useful diagnostic tools.

Molecular biological techniques are now becoming powerful tools for sandfly research. Therefore, more detailed information on the risk factors for leishmaniasis, such as the prevalent sand fly species or lineage as well as the seasonal variation in the infection rate and transmission risk, can be accumulated using such techniques in various endemic areas in different seasons.

Molecular techniques undoubtedly contribute to reservoir research: a variety of samples, from hair to bone marrow can be used to establish the infection rate in a given host. In addition, the mammal blood meal can be identified from blood-fed female sandflies.

In conclusion, the relationships between the parasite, its vector and reservoirs build a complex and dynamic network that can be disentangled by the use of molecular tools in order to contribute to the molecular diagnosis and epidemiology of the disease. Therefore, the objectives of the present work are:

1. To evaluate the influence of the epidemiological scenario from two endemic areas, Spain and Morocco, on the reliability of the PCR techniques and contribute to the selection of the most efficient one for cutaneous leishmaniosis and typing *Leishmania* species.
2. To contribute to the improvement in the diagnosis of cutaneous and mucosal leishmaniasis through the establishment of a diagnosis protocol from histological samples.
3. To analyse the composition and distribution of *Phlebotomus sergenti* mitochondrial lineages in Southwestern Europe using a novel PCR-RFLP for the Cytochrome B population analysis.
4. To investigate *Leishmania infantum* infection in wild rabbits (*Oryctolagus cuniculus*) and discuss their potential role as reservoirs in a recognized endemic area with ample epidemiological knowledge, as is the case of Southeastern Spain. The sympatric and syntrophic presence of other kinetoplastid, *Trypanosoma nabiasi*, was taken into consideration to avoid confusion.

5. To detect and characterize *L. infantum* infection in wild rodents in an epidemiological study in a well-known focus of canine leishmaniasis due to *L. infantum* in Southern Spain.
6. To provide an insight into the genetic variability of Trypanosoma nabiasi, its *in vitro* cultivation and infectivity, and finally investigate the course of infection in its host (*O. cuniculus*).

IV. Resultados

PUBLICATION	Journal (Impact Factor)	Category Name (Journal Rank in Category)	Quartile in Category
Comparison of PCR-based methods for the diagnosis of cutaneous leishmaniasis in two different epidemiological scenarios: Spain and Morocco. **Merino-Espinosa G**, Rodríguez-Granger J, Morillas-Márquez F, Tercedor J, Corpas-López V, Chiheb S, Alcalde-Alonso M, Azaña-Defez JM, Riyad M, Díaz-Sáez V, Martín-Sánchez J. **ENVIADO A TROPICAL MEDICINE AND INTERNATIONAL HEALTH.**	Tropical Medicine and International Health (2,519)	TROPICAL MEDICINE (3/19)	Q1
Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and their epidemiological implications. **Merino-Espinosa G**, Corpas-López V, Callejón-Fernández R, Porcel-Rodríguez L, Díaz-Sáez V, Gállego M, Ballart C, Molina R, Jiménez M, Morillas-Márquez F, Martín-Sánchez J. Trop Med Int Health. 2016 May;21(5):630-41. doi: 10.1111/tmi.12686.	Tropical Medicine and International Health (2,519)	TROPICAL MEDICINE (3/19)	Q1
High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: epidemiological consequences. Díaz-Sáez V, **Merino-Espinosa G**, Morales-Yuste M, Corpas-López V, Pratlong F, Morillas-Márquez F, Martín-Sánchez J. Vet Parasitol.	Veterinary Parasitology (2,242)	VETERINARY SCIENCES (10/138)	Q1

2014 May 28;202(3-4):119-27. doi: 10.1016/j.vetpar.2014.03.029.			
Leishmania infantum in wild rodents: reservoirs or just irrelevant incidental hosts? Navea-Pérez HM, Díaz-Sáez V, Corpas-López V, **Merino-Espinosa G**, Morillas-Márquez F, Martín-Sánchez J. Parasitol Res. 2015 Jun;114(6):2363-70. doi: 10.1007/s00436-015-4434-y.	Parasitology Research (2,027)	PARASITOLOGY (15/36)	Q2
Genetic variability and infective ability of the rabbit trypanosome, *Trypanosoma nabiasi* Railliet 1895, in southern Spain. **Merino-Espinosa G**, Corpas-López V, Morillas-Márquez F, Díaz-Sáez V, Martín-Sánchez J. Infect Genet Evol. 2016 Aug 24;45:98-104. doi: 10.1016/j.meegid.2016.08.028.	Infection, Genetics and Evolution (2,591)	INFECTIOUS DISEASES (37/83)	Q2

Comparison of PCR-based methods for the diagnosis of cutaneous leishmaniasis in two different epidemiological scenarios: Spain and Morocco.

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Abstract

OBJECTIVES: Cutaneous leishmaniasis (CL) is a disfiguring and stigmatising disease occurring in more than 70 countries across de world including Spain and Morocco. The use of sensitive tests that can differentiate *Leishmania s*pecies is advised. Although many PCRs are described in the literature, more comparative studies are required to optimise the molecular diagnosis taking into account the diversity of epidemiological scenarios.

METHODS: The sensitivities and specificities of parasitological methods and four PCRs were compared in cutaneous samples from 77 patients from Spanish (SPH) and Moroccan hospitals (PMH). Exudates and fresh or paraffin-embedded tissue biopsies were used.

RESULTS: None of the PCRs used in this study allows the diagnosis of all CL cases, showing also some drawbacks. Lmj4/Uni21-PCR displayed the best sensitivity with PMH but it did not provide positive results in PSH, although they were positive with other PCRs. Conversely, JW13/JW14-PCR and *L. infantum*-PCR-ELISA displayed good sensitivities with PSH that were not achieved with PMH. Nested-ITS-1-PCR showed higher sensitivity with PSH than PMH. False negative results were obtained in 19% of PSH due to unspecific hybridizations of ITS-1 primers with human chromosome1.

CONCLUSIONS: PCR should be routinely used in patients with cutaneous lesions compatible with CL being advisable to combine the use of two PCR techniques. The selection of these PCRs will be influenced by the epidemiological scenario: in areas where *L. infantum* is endemic, the use of the PCR-ELISA joint with JW13/JW14-PCR or ITS-1 seems an appropriate choice whereas in areas like Morocco, Lmj4/Uni21 and ITS-1 provide satisfactory results.

Keywords

Cutaneous leishmaniasis, Spain, Morocco, conventional parasitological analysis, paraffin-embedded tissue biopsies, dermal exudates.

Introduction

Cutaneous leishmaniasis (CL) is a disfiguring and stigmatising disease occurring either as zoonotic or anthroponotic infections in more than 70 countries across de world. In the Old World, CL is commonly caused by parasites of the *L. tropica* complex, *L. major* and *L. infantum* (1,2).

Leishmaniasis is endemic in Spain where visceral (VL), cutaneous (CL), mucosal (ML) and canine leishmaniasis are due to *Leishmania infantum* (3–7). Additionally, imported cases caused by other *Leishmania* species are sporadically diagnosed (8,9).

In Morocco, CL is a major public health problem caused by three *Leishmania* species that often overlap: *L. major* is the causative agent of zoonotic CL, *L. tropica* is the causative agent of anthroponotic CL and the viscerotropic species *L. infantum* is also involved in sporadic CL and VL (10).

Diagnosis of CL can be difficult, especially if it is mainly based on clinical symptoms and microscopic examination of smears. Its clinical presentation is very diverse and can be similar in appearance to other skin diseases such as carcinoma, lupus, rosacea, sarcoidosis or Winkler's disease (11–14). Additionally, the chronic form of CL is easily misdiagnosed because the clinical signs are atypical. Moreover, due to the low density of *Leishmania* parasites in chronic lesions, conventional laboratory methods are much less sensitive in detecting amastigotes in the samples (15,16). All *Leishmania* species are morphologically similar and their identification using Giemsa-stained smears or cultures is not possible. Although these methods remain the gold standard in countries where leishmaniasis is an endemic disease due to its high specificity, they are generally time-consuming and do not provide enough sensitivity (14,17). For this reason, the development of sensitive laboratory tests that can differentiate *Leishmania s*pecies are required in order to evaluate the prognosis of the lesion and to contribute to a better understanding of CL epidemiology.

Polymerase chain reaction (PCR) has proved to be a sensitive tool for the diagnosis and identification of *Leishmania* species in clinical samples, avoiding the need of culture of parasites and isoenzyme identification (2,17–20). There are a variety of PCR targets in the literature, including the kinetoplast minicircle (kDNA), ribosomal DNA or the miniexon sequences (20–24). Restriction fragment length polymorphism (RFLP) is widely used for parasite characterization with the Internal Transcribed Spacer 1 (ITS-1) (20,21,24,25). Real-time PCR, however, improved molecular diagnostics by adding simplicity and rapidity (19,26–28). Other modalities, such as nested-PCR or PCR-ELISA have resulted in a gain in sensitivity accompanied by an increased risk of contamination or complexity, respectively (20,29). A valid method should be sensitive enough to detect the parasite, effective in the identification of the *Leishmania* species and as simple as possible in its performance. Although many PCR assays for *Leishmania* detection are described in the literature, more comparative studies are required to improve and optimise the CL molecular diagnosis taking into account the diversity of epidemiological scenarios.

Therefore, the sensitivities and specificities of conventional parasitological methods and four PCR assays were compared in cutaneous samples from two endemic areas with different epidemiological characteristics, Spain and Morocco. The main objective was to evaluate the influence of the epidemiological scenario on the reliability of the PCR techniques and contribute to the selection of the most efficient one for CL diagnosis and typing of *Leishmania* species.

Material and Methods

Characteristics of Patients and clinical samples

Patients from Spanish Hospitals

Forty-two patients with cutaneous lesions were attended in Spanish hospitals (PSH) between 2012 and 2014. 61.9% (26/42) were men and 38.1% (16/42) were women. The patients were in the age range from 12 to 104 years old with an average age of 50 ± 7 years. Of these, 50% were Spanish, 23.8% Moroccan immigrants and 26.2% immigrants from other countries. More than 75% (32/42) of the patients were consulting in hospitals of Granada, 21.4% (9/42) in Almería and 2.4% (1/42) in Albacete, corresponding with their residence province. The samples collected from these patients were tissue biopsies that were used as both fresh and formalin-fixed paraffin-embedded (FFPE) tissue blocks.

The study of the presence of *Leishmania* was only required in 21 (50.0%) of the 42 patients. Mycobacterial infection was suspected in 35.7% (15/42) among other non-parasitic infectious agents

Patients from Moroccan Hospitals

Thirty-five Moroccan patients (PMH) were attended in the Ibn Rochd University Hospital of Casablanca (Morocco), between 2010 and 2011. Of these, 48.6% (17/35) were men and 51.4% (18/35) were women. The patients were in the age range from 2 to 80 years old with an average age of 43.2 ± 25.7 years. Syringe-sucked dermal fluid was obtained and smeared onto glass slides. No tissue biopsies were collected.

Studies of the presence of *Leishmania* were requested in all PMH.

This study was conducted according to the ethical principles specified in the Declaration of Helsinki and the procedure was approved by both Ethics Committees of the University of Granada and the hospitals involved.

Parasitological analysis

Parasitological analyses were only performed when CL was suspected. For this purpose, smeared syringe-sucked dermal fluids and fresh skin biopsy imprints were fixed with absolute methanol and stained with Giemsa for a 100x immersion objective analysis in order to detect amastigotes. Cultures were performed in EMTM medium at 24 ºC and examined for parasite growth by optical microscopy every 3-7 days. Subcultures were made once a week for 6 weeks before they were reported as negative. Positive cultures were transferred to RPMI-1640 supplemented with 20% Foetal Bovine Serum and 5% human urine. The *Leishmania* spp. reference strains were also cultured as described above after thawing.

Molecular diagnosis

DNA extraction

Exudates were scratched from the slide with a sterile blade per sample. FFPE tissue samples were cut out from the block and washed twice with 1 mL of xylene and 1 mL of pure ethanol. DNA was obtained from the different types of samples using the MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA). The DNA was rehydrated in a final volume of 20 µL of sterile water. In order to discard DNA contamination at this stage, extraction controls were carried out. These consisted of tubes of sterile water to which the whole extraction process was applied simultaneously with the biological samples. The extracted DNA was kept at −20 ºC until its amplification by PCR.

As a totally independent procedure to the DNA extraction from biological samples, DNA was also extracted from *Leishmania* spp. reference strains: *L. infantum* promastigotes (MCAN/ES/91/DP204, MHOM/ES/08/DP532 and MHOM/ES/14/DP581), *L. tropica* promastigotes (MHOM/MA/88/LEM1314 and

MHOM/MA/88/LEM1452) and *L. major* promastigotes (MHOM/MA/81/LEM265, MRHO/SU/59/LEM129 and MHOM/IL/81/Friedlin). A commercially available kit was used (REALPURE Genomic DNA Extraction kit: REAL Durviz S. L., Valencia, Spain), according to the manufacturer's instructions. The parasites were previously washed and counted with a hemocytometer and the DNA was adjusted to a final concentration of 1000 parasites/µL.

PCR based methods

Four PCR assays were tested, all of them in a final PCR volume of 25 µl:

- Minicircle kDNA PCR with Lmj4/Uni21 primers, following the described conditions (30), allowing species differentiation.
- Internal Transcribed Spacer 1 (ITS-1) rDNA nested-PCR, following the described conditions (20), allowing species differentiation by RFLP with HaeIII (20) and MnlI (31) restriction enzymes (Thermo Scientific, Germany).
- Minicircle kDNA PCR with 9/83 primers followed by a probe hybridization and an ELISA (29). This assay is specific for *L. infantum*.
- Minicircle kDNA real-time with JW13/JW14 primers following the described conditions (19,27), allowing species differentiation. The melting temperatures (T_m) given by these authors were adjusted to 86.25-88.80 for *L. major,* 88.75-90.25 for *L. tropica* and 90.25-91.75 for *L. infantum*.

Sequencing and comparative sequence analysis

Amplified PCR products were eluted from agarose gel using a commercial kit (Zymoclean Gel DNA Recovery kit, Zymo Research, Irvine, CA, USA) and they were directly sequenced in both directions using the primers for DNA amplification. Sequences were aligned using the multiple alignment program CLUSTALX 1.81 (Conway Institute UCD Dublin, Ireland) and manually adjusted, and they were

subjected to BLAST analysis to find the most similar sequences in the GenBank database.

Comparison of diagnostic assays

To determine the efficiency in the CL diagnosis of every PCR technique used, 3 different criteria were followed: 1) the patient was considered positive when the conventional parasitological analysis (direct examination and/or culture) was positive, 2) the patient was considered positive when at least two PCR assays were positive, and 3) the patient was considered positive when a PCR assay was positive. Sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV) and Cohen's kappa coefficient, which is a measure of the agreement between two tests, were determined. The strength of agreement is defined as follows: poor (<0.20), fair (0.21- 0.40), moderate (0.41-0.60), good (0.61-0.80), and very good (0.81-1.00). Data were analysed with IBM SPSS Statistics 20.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

Cutaneous specimens from 77 patients (42 PSH and 35 MSH) were examined by parasitological and molecular (4 PCR assays) diagnostic techniques. CL was confirmed in 47.6% (20/42) of PSH by one or more techniques while 22 patients were negative for all of them. *Leishmania infantum* was the causative agent in 14 patients and *L. major* was identified in 6 cases. *Leishmania tropica* was not detected in any case. In a similar way, CL was confirmed in 57.1% (20/35) of PMH. In 11 of them the causative agent was *L. major*, 4 due to *L. tropica*, 2 cases due to *L. infantum* and 3 parasitological positive patients that were negative with all PCRs. Concordance between the diagnostic techniques evaluated is displayed in Table 1. Parasitological analyses were only performed when CL was suspected: in 50%

(21/42) of PSH and 100% (35/35) of PMH. Of them, positive results were obtained for 47.6% (10/21) and 31.4% (11/35), respectively. Ten PSH in whom CL was not suspected were positive to *Leishmania* DNA by three (1 patient), two (3 patients) or one (6 patients) PCR assays.

Comparison of PCR assays using conventional parasitological methods as the gold standard is shown in Table 2. The Lmj4/Uni21 PCR displayed the best values of all the PCRs performed on PMH samples with a good measure of agreement (0.694 ± 0.120). Conversely, this technique provided negative results in all PSH samples analysed and their use was ruled out. For PSH samples, the JW13/JW14 PCR presented the best PCR diagnostic values with a good level of agreement (0.800 ± 0.131). However, the measure of agreement provided by this assay for PMH was moderate (0.474 ± 0.123).

PCR-ELISA results are conditioned by the species of *Leishmania* involved. In this way, when imported cases were excluded in the PSH group, PCR-ELISA displayed 100% of sensitivity, 66.7% of specificity, 83.3% of PPV, 100% of NPV and 0.714 ± 0.035 of kappa value. In PMH group, only 2 CL cases were due to *L. infantum,* both of them with a negative parasitological result.

Comparison of PCR assays using two positive PCRs as the gold standard was determined in Table 3. The Lmj4/Uni21 PCR showed again the best values of all the PCR performed with PMH samples with a good measure of agreement (0.756 ± 0.113). For PSH samples, ITS-1 PCR was the assay with better global results (0.797± 0.094) although PCR-ELISA provided the highest sensitivity (90.9%).

Comparison between the four PCR assays evaluated is presented in Table 4. In this case, a patient was considered CL positive when at least one PCR technique detected *Leishmania* DNA. For Lmj4/Uni21, JW13/JW14, ITS-1 and PCR-ELISA assays, the number of positive PSH samples was 0, 14, 8 and 14; and 17, 10, 11 and 2 for PMH samples, respectively.

Leishmania species identification by these PCR assays is displayed in Table 5. The Lmj4/Uni21 PCR identified *L. major* but did not allow the differentiation between

L. infantum and *L. tropica.* A similar problem was detected with the ITS-1 PCR for 11 PSH when HaeIII was used for RFLP which was resolved using MnlI restriction enzyme (10 patients) or sequence analysis (1 patient). *L. infantum* was identified in 14 PSH by the PCR-ELISA; four of them were negative with ITS1-PCR, JW13/JW14 PCR and even with conventional parasitological detection methods.

The JW13/JW14 PCR was able to identify *Leishmania* species in 13/14 PSH and 10/10 PMH. In the remaining PSH case, the melting temperature (T_m) value was intermediate between those for *L. infantum* and *L. major*. However, with the other PCR techniques *L. infantum* was diagnosed as the causative agent of the lesion (Table 5).

An unspecific fragment with a similar band size as the expected for *Leishmania* genus was detected in 8 of the 42 PSH (19%) with ITS1-PCR, being the restriction enzymes HaeIII and MnlI unable to digest it. Sequence analysis of this fragment identified it as the result of a nonspecific hybridization of ITS primers with human chromosome 1, namely RABGAP1L gene which encodes for the RAB GTPase activating protein 1-like, located within the q-arm of chromosome 1. The homology of the sequenced fragment with the published sequence in GenBank (AL591108.18) was 99%.

Discussion

The use of more than one diagnostic technique is recommended both in leishmaniasis epidemiological studies and in clinical practice (32,33). In CL, the clinical appearance of the lesions induces a suspicion that must then be properly confirmed, usually by classical parasitological procedures. These methods have a medium-low diagnostic sensitivity for CL (33–35) with values ranging from 17-83% for microscopic observation of tissue imprints (36–38) and 17-85% for culture (23,37,38) showing the influence of the *Leishmania* species, the parasite load and the technical personnel expertise (39). In this study, 37.5% of PMH with negative

parasitological result was positive with at least one PCR assay. In a similar way, 10 of the 21 (47.6%) PSH in which CL was not suspected and therefore classical analysis was not performed, were positive with 1 to 3 PCR techniques (Table 1). These results indicate that PCR should be routinely used in patients with cutaneous lesions compatible with CL. However, none of the PCR assays used in this study allows the diagnosis of all CL cases, showing also some drawbacks, being thus advisable to combine the use of two PCR techniques.

Diagnosis of clinically suspected leishmaniasis by PCR is expected to become the gold standard for diagnosis, given the advancements in DNA extraction protocols, the celerity of the technique and the variety of samples types that can be used (14). A number of PCR techniques have been described in the literature but interlaboratory standardization is mostly absent, complicating comparisons among them and complicating the selection of the most relevant technique, even though they represent a considerable improvement in sensitivity and specificity over conventional methods (18,20,22,24). In this study we have been able to verify the different diagnostic efficiency of 4 PCR assays using cutaneous samples from two CL endemic areas with different epidemiological characteristics.

The Lmj4/Uni21 PCR displayed the best sensitivity with PMH (77.3% vs conventional parasitological methods and 84.2% vs 2 PCRs) but it did not provide positive results in PSH, although they were positives with other PCR assays. Conversely, although the JW13/JW14 PCR displayed good sensitivity with PSH (100 and 81.8%), this value was not achieved with PMH (52.6 and 58.8%).

Regarding the *L. infantum* PCR-ELISA, this assay showed sensitivity of 83.3 and 90.9% with PSH but its usefulness in PMH is limited given that it is specific for *L. infantum.* In Morocco, CL is mainly due to *L. tropica* and *L. major* while *L. infantum* is only sporadically detected (40).

The nested ITS-1 PCR showed higher sensitivity with PSH (50 and 72.7%) than PMH (55 and 61.1%). This is the most widely used technique for the diagnosis of leishmaniasis, particularly in the Mediterranean region, as well as for the

identification of imported cases (18,41,42). However, false negative results were obtained in 19% of PSH due to unspecific hybridizations of ITS-1 primers with human chromosome 1, namely RABGAP1L gene which encodes for the RAB GTPase activating protein 1-like.

Diagnostic tools are necessary both to detect the parasite and to identify the *Leishmania* species responsible of the disease either in endemic areas where different species coexist such as Morocco, or in non-endemic and endemic areas where imported CL cases occur due to travelling flow and population migration (20,41,43). In the latter, southwestern European countries are included, such as Spain, France, Italy and Portugal, where *L. infantum* is the only endemic species but other species are responsible for imported cases (8,9,44). In this study 6 CL cases due to *L. major* were detected among immigrants of the PSH group; lesions appeared after a long stay in *L. major* endemic areas of their home countries.

Three of the PCR assays used in this study allow the identification of the most important aetiological agent of CL in the Old World, *L. tropica, L. major* and *L. infantum,* depending on their T_m values (JW13/JW14 PCR) or band pattern generated directly with PCR (Lmj4/Uni21 PCR) or after RFLP (ITS-1 PCR). The fourth PCR technique is specific for *L. infantum*, the species with a broader geographical distribution, and the only one responsible for visceral, cutaneous and mucosal leishmaniasis.

Although the ITS-1 target allows the identification of almost all Old and New World medically relevant *Leishmania* parasites with the use of only one restriction enzyme (HaeIII) for the amplicon digestion (20,45,46), the species identification is hampered when the parasite load is low and the restriction pattern has low definition (47,48). In this study some difficulties were encountered when distinguishing *L. infantum* and *L. tropica* with HaeIII that were solved with the restriction enzyme MnlI (31) or sequence analysis. MnlI enzyme generates two bands in *L. tropica* ITS-1 fragment but it has not restriction site at *L. infantum* amplicon and the amplification of the human chromosome 1 should be ruled out.

Among the advantages offered by PCR as a diagnostic method of CL is the variety of types of cutaneous samples that can be used (14). In this study, tissue biopsies in PSH and dermal fluid in PMH have been used. CL lesions due to *L. infantum* contain a lower parasitic load than those due to *L. tropica* or *L. major* and a fluid aspiration / scraping of the lesion could not be sufficient for diagnosis (49). Optimizing a DNA extracting procedure from paraffin-embedded tissue biopsies has allowed the use of this type of samples for CL diagnosis (50,51). In this study, ITS-1 PCR did not show enough sensitivity with these types of samples from PSH as reported elsewhere (20,52), who observed inhibition of PCR in FFPE samples. Out of 8 confirmed CL cases from FFPE samples, only one was positive with ITS-1 PCR for *L. major*. The JW13/JW14 assay, was positive to *L major* for 6 FFPE samples and to *L. infantum* for a seventh sample. Performing *L. infantum* PCR-ELISA assay with FFPE tissue from Spanish patients proved to be a highly sensitive test for CL diagnosis, even in chronic lesions, when other methods failed to detect the parasite.

It is noteworthy that *Leishmania* was detected in 10 PSH for whom leishmaniasis was not suspected despite the endemic character of *L. infantum* in Spain. Underestimation of CL cases has been reported worldwide (1) The clinical spectrum of CL is broad and may mimic with other skin conditions, such as fungal infections, leprosy, cancer or granulomas. The clinical staff should be familiar with CL clinical features in order to be able to suspect CL both in endemic areas and after stays there.

At least two PCRs yielded a positive result for 4 of these unsuspected CL patients and 7 PMH for which parasitological techniques did not detected the parasite (Table 1). A positive result obtained simultaneously by two different PCRs strengthen the CL diagnosis in the patient, particularly when the parasitological analysis was negative or was not performed. However, a positive result obtained from a single PCR should not be considered a false positive if this technique is sufficiently validated and the DNA extraction has been performed carefully to

avoid contamination. Many factors might have influenced the PCR negative results in the 3 PMH where amastigotes were detected: a false positive, a lack of amplification due to polymorphism in the target DNA or the low parasitic load in the sample subjected to the molecular diagnosis (53).

Conclusion

The combination of parasitological and molecular methods, using at least two different PCRs, is a reliable and accurate procedure for the diagnosis of CL, allowing the simultaneous identification of the parasite. The selection of these PCR techniques will be influenced by the epidemiological scenario: in areas where *L. infantum* is endemic, the use of the PCR-ELISA joint with JW13/JW14 PCR or ITS-1 seems an appropriate choice whereas in areas like Morocco, where *L. tropica* and *L. major* are the main CL causative agents with sporadic CL cases due to *L. infantum*, PCR-ELISA is the least useful while Lmj4/Uni21 and ITS-1 provide satisfactory results. The possibility of using FFPE samples for DNA extraction and PCR allows discarding CL in patients with skin lesions of unknown etiology for which leishmaniasis was not suspected.

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	Conventional parasitological analysis						
	PSH				PMH		
N° of positive PCR assays	POSITIVE	NEGATIVE	NS	**TOTAL**	POSITIVE	NEGATIVE	**TOTAL**
0	0	11	11	**22**	3	15	**18**
1	4	0	6	**10**	2	2	**4**
2	2	0	3	**5**	3	3	**6**
3	4	0	1	**5**	3	4	**7**
TOTAL	**10**	**11**	**21**	**42**	**11**	**24**	**35**

Table 1.Concordance between the different diagnostic techniques evaluated with cutaneous samples from patients of Spanish and Moroccan hospitals. PSH: patients from Spanish Hospitals; PMH: patients from Moroccan Hospital; NS: parasitological analysis was not performed because CL was not suspected

	PSH (N=21)					PMH (N=35)				
Molecular PCR method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE
Lmj4/Uni21	ND	ND	ND	ND	ND	77.3	100	100	68.8	0.694 ± 0.120
JW13/JW14	100	80	83.3	100	0.800 ± 0.131	52.6	100	100	59.1	0.474 ± 0.123
ITS-1	50	90	83.3	64.3	0.400 ± 0.188	55	100	100	52.6	0.449 ± 0.124
L. infantum ELISA	83.3	60	71.4	75	0.441 ± 0.269	ND	ND	ND	ND	ND

Table 2. Results of the four diagnostic PCR in comparison with conventional parasitological methods as Gold Standard. PSH: patients from Spanish Hospitals; PMH: patients from Moroccan Hospital; PPV: positive predictive values; NPV: negative predictive values; ND: no data; N: number of patients

	PSH (N=42)					PMH (N=35)				
Diagnostic method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE
Direct Diagnosis	85.7	69.2	60	90	0.500 ± 0.185	55.6	71.4	71.4	55.6	0.262 ± 0.164
Lmj4/Uni21 PCR	ND	ND	ND	ND	ND	84.2	92.9	94.1	81.3	0.756 ± 0.113
JW13/JW14 PCR	81.8	83.9	64.3	92.9	0.684 ± 0.130	58.8	100	100	68.2	0.573 ± 0.129
ITS-1 PCR	72.7	100	100	91.2	0.797 ± 0.094	61.1	100	100	63.2	0.557 ± 0.131
L. infantum ELISA-PCR	90.9	81.8	71.4	94.7	0.681 ± 0.122	100*	50*	50*	100*	0.400 ± 0.392

Table 3. Results of the four diagnostic PCR and Direct Diagnosis in comparison with Two PCR Positives as Gold Standard. PSH: patients from Spanish Hospitals; PMH: patients from Moroccan Hospital; PPV: positive predictive values; NPV: negative predictive values; ND: no data: *: analysis performed in 3 PMH.

	PSH (N=42)					PMH (N=35)				
Diagnostic methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ ± SE
L. infantum ELISA-PCR vs. JW13/JW14 PCR	57.2	94.8	88.9	75	0.544 ± 0.144	ND	ND	ND	ND	ND
L. infantum ELISA-PCR vs. ITS-1 PCR	50	94.8	87.5	72	0.474 ± 0.148	ND	ND	ND	ND	ND
JW13/JW14 PCR vs. ITS-1 PCR	75	76.5	42.9	92.9	0.400 ± 0.149	77.8	84.2	70	88.9	0.602 ± 0.159
Lmj4/Uni21 PCR vs. ITS-1 PCR	ND	ND	ND	ND	ND	53.3	76.9	72.7	58.8	0.296 ± 0.172
Lmj4/Uni21 PCR vs. JW13/JW14 PCR	ND	ND	ND	ND	ND	64.3	93.8	90	75	0.591 ± 0.144

Table 4. Comparison between the four diagnostic PCR. PSH: patients from Spanish Hospitals; PMH: patients from Moroccan Hospital; PPV: positive predictive values; NPV: negative predictive values; ND: no data.

	PSH					PMH				
PCR assays	*L. infantum*	*L. infantum*/ *L. major*	*L. tropica*	*L. major*	**TOTAL CL cases**	*L. infantum*	*L. infantum*/ *L. tropica*	*L. tropica*	*L. major*	**TOTAL CL cases**
Lmj4/Uni21 PCR	ND	ND	ND	ND	**ND**	0	6	0	11	**17**
JW13/JW14 PCR	7	1	0	6	**14**	2	0	2	6	**10**
ITS-1 PCR	7*	0	0	1	**8**	2	0	4	5	**11**
L. infantum ELISA-PCR	14	0	0	0	**14**	2	0	0	0	**2**
Consensus identification	**14**		**0**	**6**	**20**	**2**		**4**	**11**	**17**

Table 5. *Leishmania* species identification. PSH: patients from Spanish Hospitals; PMH: patients from Moroccan Hospital; *: One *L. infantum* result by sequencing.

Differential ecological traits of two *Phlebotomus sergenti* mitochondrial lineages in southwestern Europe and its epidemiological implications.

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Abstract

OBJECTIVES: The introduction of leishmaniasis in a new area requires a well-established population of the sandfly vector species of the parasite. No autochthonous cases of anthroponotic cutaneous leishmaniasis have been detected in southwestern Europe and *Leishmania infantum* is the only species responsible for leishmaniasis in this area. *Phlebotomus sergenti,* the main vector

of *Leishmania tropica,* is commonly found in the Iberian Peninsula at sufficient densities to be able to act as vectors. *Phlebotomus sergenti* is characterized by high genetic diversity and it is classified in four mitochondrial lineages. Our aim was to analyse the composition and distribution of *P. sergenti* mitochondrial lineages in southwestern Europe given the possibility of phenotypic differences of biomedical importance between them.

METHODS: Sandflies were captured in the Iberian Peninsula and on the Canary and Balearic Islands. Mitochondrial lineage identification of 137 *P. sergenti* was performed using a novel PCR-RFLP that avoids the necessity of gene sequencing.

RESULTS: Two lineages were evidenced, the typical Iberian one (lineage I) and another, held in common with North Africa (lineage III), that show a distinctive distribution. *P. sergenti* lineage I shows a better correlation to the bioclimatic diversity in southwestern Europe. Conversely, *P. sergenti* lineage III prefers warmer temperatures and less precipitation, which are typical of the Mediterranean.

CONCLUSION: Lineage I seems to have adaptive advantages given its wider tolerance to temperatura and altitude than lineage III, and it would seem more suitable to lead a potential geographical expansion towards the rest of Europe.

Keywords

Phlebotomus sergenti, *Leishmania tropica*, mitochondrial lineages, Cytochrome b, PCR-RFLP, Southwestern Europe, ecological traits.

Introduction

Ten proven or potential vector species of genus *Phlebotomus* (Diptera, Psychodidae) are indigenous in Europe: *Phlebotomus ariasi* Tonnoir*,* 1921, *P. perniciosus* Newstead, 1911, *P. perfiliewi* Parrot, 1930, *P. kandelakii* Shchurenkova, 1929, and *P. balcanicus* Theodor, 1958, vectors of *Leishmania infantum; P. neglectus* Tonnoir, 1921, and *P. tobbi* Adler, Theodor & Lourie, 1940, vectors of *Leishmania donovani; P. sergenti* Parrot, 1917, and *P. similis* Perfiliew, 1963, vectors of *Leishmania tropica,* (it is important to point out that the taxonomic state of *P. similis* is under debate and some authors considered it a subspecies of *P. sergenti*) and *P. papatasi* Scopoli, 1786, vector of *Leishmania major.* Only *L. infantum* is widespread in Europe whereas *L. major* is not an endemic species and the distribution of the other two species is very limited – *L. tropica* is only found in Greece and Turkey and *L. donovani* is present in Cyprus and Turkey (1–4).

For a long time, *P. sergenti* was considered the sole vector of *Leishmania tropica*, the causative agent of anthroponotic cutaneous leishmaniasis (ACL) (5,6). However, the vectorial capacity of *P. arabicus* has been demonstrated in a focus in northern Israel (7), and *P. similis* is considered a probable vector on the island of Crete (8).

Experimental infections under laboratory conditions revealed that *P. sergenti* is a "specific vector", i.e. a vector species in which only a single *Leishmania* species, *L. tropica*, is capable of maturing and it does not support the development of any other *Leishmania* species (9,10). Nevertheless, *P. sergenti* is implicated as the main vector of ACL, based on its distribution and on the fact that *L. tropica* has been identified in infected sandflies recovered from endemic foci (5,6).

Phlebotomus sergenti has a wider geographical distribution than *L. tropica* (11). This vector is a well represented species in North Africa where it coexists with *L. tropica* (11–13). In Morocco, ACL is endemic in semi-arid areas in the centre and south of the country and is emerging with epidemic foci in the north. Currently *L.*

tropica is thought to be the species with the widest geographical distribution in Morocco (6,14–17).

In southwestern Europe, *P. sergenti* is found in the Iberian Peninsula, the south of France, on the Canary Islands, on the Balearic Islands and on the islands of Corsica and Sicily (18–24). However, no autochthonous cases of ACL have been detected and *L. infantum* is the only species responsible for leishmaniasis in this area (25,26). According to predictive risk models which establish the risk of presence of *P. sergenti*, several areas in the centre and south of Spain are considered potential hot spots for a hypothetical autochthonous focus of *L. tropica* (20). In some of these areas, *P. sergenti* density is sufficient for these sandflies species to be able to act as vector, reaching values of 35.3 sandflies/m^2 (20). Moreover, some studies have highlighted the bioclimatic affinity between northern Morocco and the southeast of the Iberian Peninsula (27), along with the high concordance of sandfly species found and its phenology (16,28–30). This similarity between northern Morocco (an emerging ACL zone) and the southeast of the Iberian Peninsula, indicate that southwestern Europe may be susceptible to the introduction of *L. tropica,* in a similar way to the situation which has occurred in northern Morocco (16). The flow of immigrants from endemic regions of North Africa and, to a lesser extent, the Middle East is known to exist, and the importation of ACL cases due to *L. tropica* has been verified in other European countries (31–34).

Three of the four mitochondrial lineages within *P. sergenti* are present in Morocco and two in Spain, interestingly one of the lineages is present in both countries (35,36). Phenotypic differences of biomedical importance may exist between these mitochondrial lineages. Population genetics can help to assess the threat of the geographical spread of vectors in relation to climate and other environmental changes. Therefore, our aim was to analyse the composition and distribution of *P. sergenti* mitochondrial lineages in southwestern Europe. In order to facilitate the Cytochrome b (mtDNA cyt b) population analysis, we designed a PCR-RFLP which avoids the necessity of gene sequencing.

Materials and methods

Study area

Phlebotomine sandflies were sampled in the Iberian Peninsula, on the Canary Islands and on the Balearic Islands, southwestern Europe (Fig. 1, Table 1).

Collection and morphological identification of specimens

Sandflies were collected with sticky traps (21x29.7-cm pieces of paper coated with castor oil) placed on uneven ground, fissures, crevices or drainage holes on roadsides for 4 days, or overnight with CDC miniature light traps (Hausherr's Machine Works, Toms River NJ, USA) and then preserved in 70% ethanol at room temperature or frozen stored dry at -20 °C until morphological identification. After transparentation with Marc-André fluid, they were slide-mounted in Berlese fluid (37) following dissection and morphologically identified based on external and internal characters of the head and the genitalia as previously described (35). The rest of the body of the specimens identified as *P. sergenti* were kept individually at -20 °C for molecular identification.

Sandfly DNA extraction

Each sandfly was placed in a sterile 1.5 ml Eppendorf tube and kept in liquid nitrogen for a few seconds to facilitate the mechanic rupture of the tissues using a pestle. The genomic DNA was extracted from the thorax and the attached anterior abdomen of individual sandflies (38). A commercially available kit was used (RealPure Genomic DNA Extraction kit: REAL Durviz S. L., Valencia, Spain), according to the manufacturer instructions. The DNA was resuspended in 20 µl of bidistilled water and kept at -20 °C until use.

PCR amplification

Polymerase chain reaction was used to amplify a 550 bp fragment containing the 3′ end of the mtDNA cyt b following the methodology described elsewhere (39): an initial denaturation at 94 °C for 3 min; 94 °C for 30 s, 40 °C for 30 s and 72 °C for 1 min 30 s for the first five cycles; and 94 °C for 30 s, 44 °C for 30 s and 72 °C for 1 min 30 for the remaining thirty cycles. The final extension time was 10 min at 72 °C. For specimens where the above PCR failed to amplify the target 550 bp fragment, we designed two primers amplifying a 250 bp internal cyt b fragment from the sequences of *P. sergenti* obtained by our group (35). The primers sequences are: SFG-F (5' TTC TCC TAT TgA CTT g 3') and SFG-R (5' AgT AAA gTT AAT AgA AAG 3'). The reaction was carried out in a final reaction volume of 25 ·l, containing 2 ·l of genomic DNA, 100 ·M of each dNTP, 1 ·M of each primer, 2.5 ·l of 10× reaction buffer, 2 mM $MgCl_2$ and 1.25 U Taq DNA polymerase. PCR conditions were optimized as initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 30.5 °C, extension at 72 °C for 1 min, and a final extension at 72 °C for 3 min. Although this is a very low annealing temperature, a single band of 250 bp was amplified.

Sequencing and comparative sequence analysis

Amplified PCR products were eluted from agarose gel using Real Clean Spin kit (REAL Durviz S. L., Valencia, Spain). Direct sequencing of both DNA strands was performed by Experimental Station of Zaidin (Granada, Spain). PCR products were bi-directional sequenced using the primers for DNA amplification. Sequences were edited and aligned to identify the mitochondrial lineage using Clustal X 1.81 software and manually adjusted, if necessary.

Restriction fragment length polymorphism (RFLP)

From the sequences obtained by our group (35), the diagnostic endonuclease restriction sites on mtDNA cyt b sequences for each haplotype and mitochondrial lineage were predicted using GenScript Restriction Enzyme Map Analysis Tools (http://www.genscript.com/cgi-bin/tools/enzyme_cuttingtool) and ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape); accordingly, Hae III (Thermo Scientific, Germany) and Msp I (BIORON, Germany) were selected among the potential diagnostic enzymes. The digestion of the amplified fragment was performed in a 20 µl total volume, containing 16 µl of PCR product, 2 µl of enzyme (10 U/µl) and 2 µl of standard buffer (10X). PCR products were digested 10 min at 37 °C for Hae III and 3 h at 55 °C for Msp I. The digested samples were separated by electrophoresis in a 3% agarose gel and their sizes determined by comparison with HyperLadder V (Bioline, UK).

Relationship between the mitochondrial lineage and bioclimatic data

In order to investigate the possible association between bioclimatic data and the presence of each mitochondrial lineage, a logistic regression analysis was carried out for each of the lineages, including as a dependent variable the absence or presence of the lineage and each bioclimatic variable as an independent variable. The variables analysed were:

- mean monthly average temperature
- maximum monthly average temperature
- minimum monthly average temperature
- mean annual average temperature
- maximum annual average temperature
- minimum annual average temperature
- monthly precipitation
- annual total precipitation
- temperature annual range

- precipitation seasonality
- altitude

This information were taken from the WorldClim global climate data (www.worldclim.org/). Data were analysed with Statgraphics Plus 5.1 (Statpoint Technologies Inc., Warrenton, Virginia, USA) and IBM SPSS Statistics 20.0 for Windows (IBM Corp., Armonk, NY, USA).

Results

Morphological identification of P. sergenti specimens

One hundred and thirty-seven *P. sergenti* (119 males and 18 females) from 45 locations were selected for genetic characterisation (Table 1).

Significant morphological variation was observed between *P. sergenti* females from the Canary Islands in comparison to those from the Iberian Peninsula and the Balearic Islands: the spermathecal complex from the peninsular and Balearic females showed 4-6 rings and a rectangular distal ring whereas the females from the Canary Islands had a spermatheca composed of 6-8 rings and its distal ring which were to be flatter and patently separated from the other rings, as shown in figure 2.

No morphological traits distinguished both lineages.

PCR-RFLP optimisation

Digestion of the 550 bp mtDNA cyt b fragment with Msp I failed to produce a banding pattern enabling the differentiation of the *P. sergenti* mitochondrial lineages as predicted via sequence analysis. Conversely, digestion with Hae III lead to a characteristic banding pattern for each of the four mitochondrial lineages, simplifying their identification (Fig. 3). This enzyme are useful to analyse a 250 bp mtDNA cyt b fragment as well (Table 2).

Lineage identification by optimized PCR-RFLP

Only two of the four mitochondrial lineages characterized by our group (35) were detected within the 137 specimens characterized in this study. 52.6% were identified as mitochondrial lineage III whereas 47.4% belonged to lineage I. The only specimen from Portugal was lineage I as well (Fig. 1). Of the 96 *P. sergenti* from the south of the Iberian Peninsula (Andalusia and Murcia), 60.4% were lineage III and 39.6% belonged to lineage I. Conversely, of the 12 specimens analysed in the northeast of the Iberian Peninsula (Catalonia), the proportion belonging to lineage III decreases to 25% while 75% of the sandflies were lineage I. All specimens from the Balearic Islands and Madrid were lineage I. Opposite to this, *P. sergenti* from the Canary Islands belonged to lineage III (Fig. 1) and were identified as two new haplotypes: S-H and S-I (GenBank accession number LN881129 and LN881130, respectively) (Table 3).

Analysis of bioclimatic factors

The analysis of climatic factors which might represent differential ecological traits for mitochondrial lineages revealed that the presence of lineage I is not related to mean annual average temperature ($P > 0.05$) maximum annual average temperature ($P > 0.05$) or minimum annual average temperature ($P > 0.05$). However, considering the monthly variation of these variables, a negative association is found in the coldest months whereas this correlation turns positive from June to September (Table 4). Conversely, the line III is positively associated with mean annual average temperature ($P = 0.001$, Odds Ratio (OR) 1.64), maximum annual average temperature ($P < 0.001$, OR 1.92) and minimum annual average temperature ($P = 0.01$, OR 1.36). When analysing the monthly values, an association is also observed except for the period from June to August. The association is positive for the maximum temperature except for December (Table 4).

Regarding precipitation, lineage I is positively associated with the total annual precipitation (306-983 mm, $P < 0.0001$, OR 1.01), while the line III is negatively associated (150-609 mm, $P < 0.0001$, OR 0.98). Both lineages are influenced differently by monthly precipitation (data not shown).

The annual temperature range (20.3-31.4 °C, $P < 0.01$, OR 1.23) is positively associated with lineage I but it is not related to lineage III ($P > 0.05$).

The precipitation seasonality is negatively associated with the presence of lineage I (18-68%, $P < 0.0001$, OR 0.91), and positively associated with lineage III (33-92%, $P = 0.0001$, OR 1.09).

In the areas where both mitochondrial lineages coexist, the presence of specimens belonging to lineage III is negatively associated to altitude [$P < 0.001$, OR 0.996 (0.994-0.996)] whereas lineage I showed no relationship with this variable ($P > 0.05$), as shown in figure 4.

Discussion

The introduction of leishmaniasis in a new area requires a well-established population of the sandfly vector species of the parasite. *P. sergenti*, the main vector of *L. tropica*, is widely distributed in the Iberian Peninsula (southwestern Europe), in some places at densities of up to 35.3 sandflies/m^2, enough to serve as a vector (20), and belongs to two different mitochondrial lineages according to previous results (35) and the present study. *Phlebotomus sergenti* have previously been reported on the Balearic Islands (Mallorca) (19) and on the island of Tenerife (Canary Islands) (22). These authors have pointed out the morphological differences between the spermatheca of females from the Canary Islands and from continental Europe.

In Moroccan foci where ACL is emerging, densities of 4 to 16 sandflies/m^2 have been found (40) whereas in a typical ACL focus in arid areas in Tunisia, a density of 1.8 sandflies/m^2 has been reported (41).

Phlebotomus sergenti is characterized by high genetic diversity and it is classified in at least twenty haplotypes in four mitochondrial lineages (35,36). The polymorphism described in North Africa is larger than in southwestern Europe, where only two of the described four mitochondrial lineages are present, and some of the haplotypes are present in both continents. This is consistent with the accepted hypothesis of dispersion of *P. sergenti* (11,12). *Phlebotomus sergenti* lineage I is unique in southwestern Europe. *Phlebotomus sergenti* lineage III is widely represented in all studied Moroccan foci of leishmaniasis due to *L. tropica,* both stable in the southcentral region and emerging in the northern region (35,36). In Morocco, where the parasite coexists with the vector, no evidence supports that mtDNA might be a marker of susceptibility to *L. tropica* (36). The molecules that serve as midgut receptors for parasite attachment can vary between different phlebotomine species (42) and no differences have been found among the same species to date.

In order to facilitate the population study through cyt b analysis and therefore analyse the distribution of both mitochondrial lineages in southwestern Europe, a novel PCR-RFLP which avoids the necessity of sequencing was designed. Moreover, new primers for the amplification of an internal cyt b fragment were found useful for those specimens for which cyt b amplification is unfavourable using the conditions described by Esseghir *et al.* (39), for example, when analysing sandflies which are poorly preserved, as noticed by this research group. The restriction enzyme Hae III is of particular use for the digestion of both the 550 bp cyt b mitochondrial fragment and the internal 250 bp internal fragment.

The obtained results show an irregular distribution of both mitochondrial lineages in southwestern Europe probably derived from its different preferential climatic characteristics. Mitochondrial lineage III is limited to Mediterranean peninsular areas and to the Canary Islands whereas lineage I is more widespread in the Iberian Peninsula and the only one present on the Balearic Islands (Fig. 1). Furthermore, due to its presence in Portugal, it is advisable to denominate it the Iberian Lineage.

According to Depaquit *et al.* (11,12), the history of subgenus *Paraphlebotomus* seems to have begun in the Eocene, at the time where the African continent was completely isolated from other continents by the Tethys Sea at its north, by the Atlantic Ocean at its west and by the Indian Ocean at its East (43). The African ancestor of *P. sergenti* might well have appeared in the middle-eastern area of the continent. Due to the progressive drift of Africa towards north, it would have progressively closed the Tethys Sea, delimitating the current boundaries of the Mediterranean Sea by the end of the Oligocene (44). These authors also suggested that the western distribution of *P. sergenti* is due to a migration along the southern limits of the Tethys Sea through Lebanon, Egypt and North Africa. From Morocco it would have colonized Madeira Island and some of the Canary Islands. Before the opening of the Strait of Gibraltar, some 5 million years ago (43,45), the joint between Africa and Europe would have allowed it to reach Spain, where it is currently found, followed by the Balearic Islands, Corsica and southern France.

The molecular clock calibration suggested by Esseghir *et al.* (39) revealed that the mitochondrial lineage III might have been the origin of the other lineages. Therefore, the first mitochondrial lineages which were divided were lineages III and IV some 3.8 – 2.2 million years ago, while lineage II originated from lineage III later (2.9 – 1.7 million years ago). The division between mitochondrial lineages I and III is predicted to have occurred 2.4 – 1.4 million years ago in the Iberian Peninsula. Thus, mitochondrial lineage I (Iberian Lineage) should have derived from the first settlers which belonged to lineage III whereas on the Canary Islands this mutation did not happen.

Temperature determines the biology and ecology of sandflies (i.e. sandfly survival, speed of development of the different stages of its life cycle) and also the development of the parasite inside the vector (46–48). Precipitation determines sandfly survival, it promotes adult emergence and appropriate oviposition sites and it has been associated with their activity period in some South American foci (49). Lineage I seems to have adaptative advantages given its wide tolerance to

extreme minimum and maximum temperatures (-1.8-32.4 °C), its preference for higher precipitations (306-983 mm) (Fig. 5), its wider altitudinal range (from 19 to 1819 metres above the sea level or m.a.s.l.) and geographical distribution. Conversely, presence of lineage III only occur within a range of warm temperatures (12.2-20.4 °C) (Fig. 6), lower precipitations (150-609mm) and lower altitude (19-1251 m.a.s.l.), which are typical on areas of Mediterranean influence.

Conclusions

Phlebotomus sergenti lineage I shows a better correlation to the bioclimatic diversity in southwestern Europe. Conversely, *P. sergenti* lineage III prefers warmer temperatures and lower precipitations which are typical on areas with Mediterranean influence. Therefore, lineage I would seem more suitable to lead a potential geographical expansion towards the rest of Europe.

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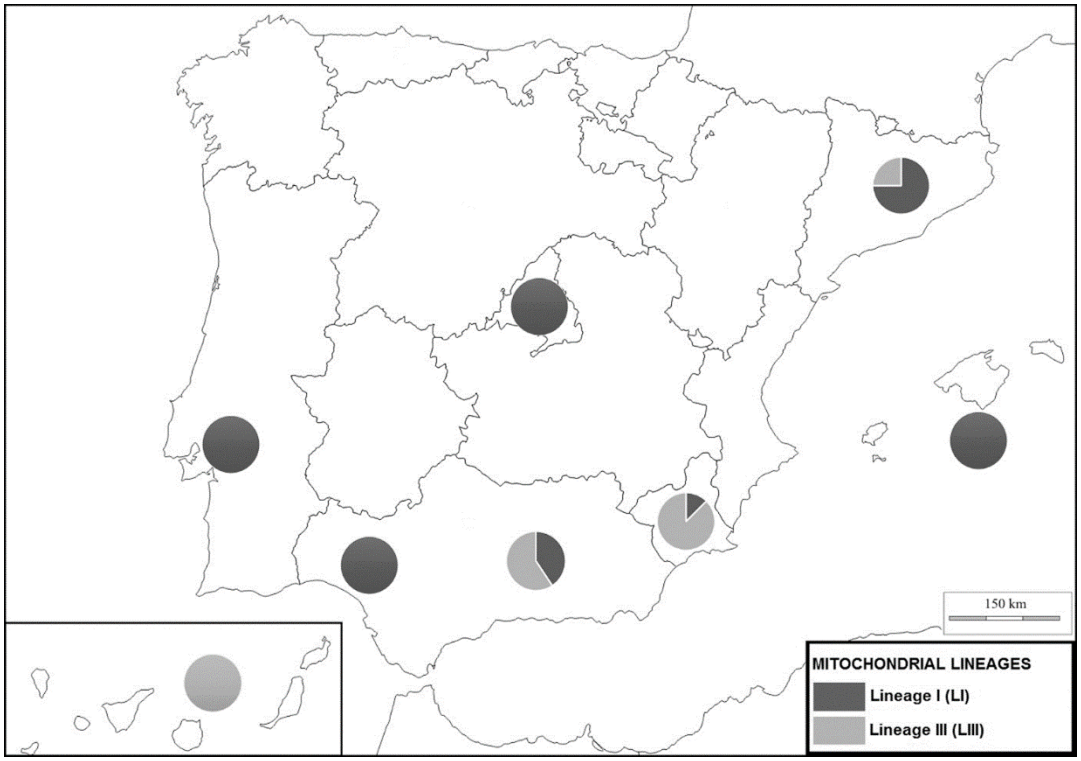

Figure 1. Geographical locations from which *Phlebotomus sergenti* was captured and molecularly identified showing the proportion for each mitochondrial lineage.

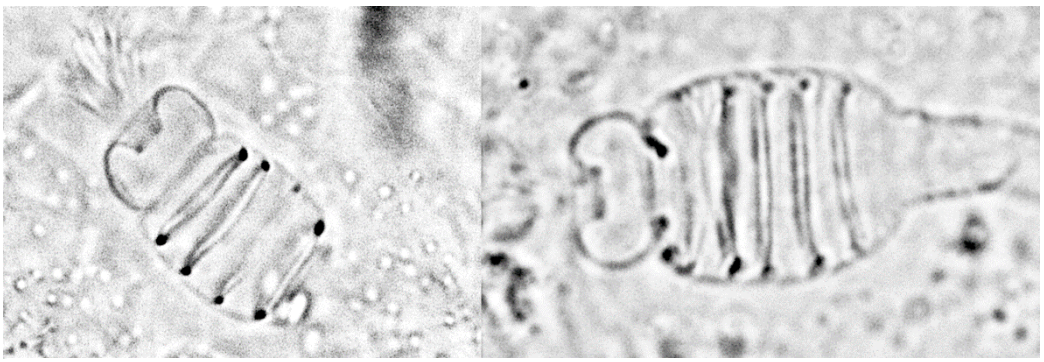

Figure 2. Detail of the spermathecae of *Phlebotomus sergenti* females from the Iberian Peninsula (left) and the Canary Islands (right).

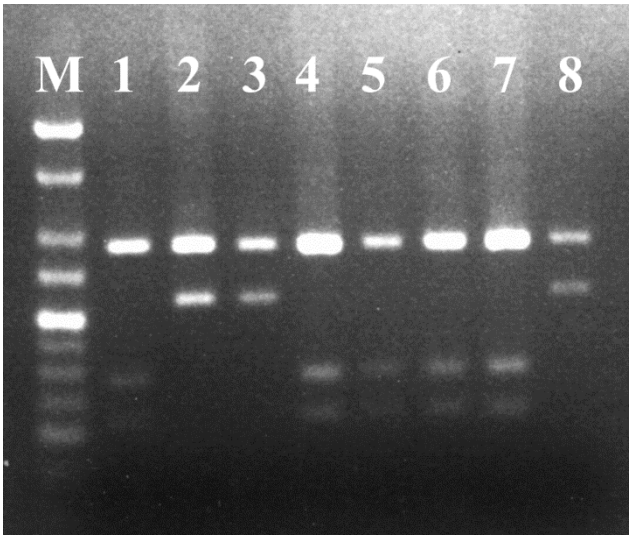

Figure 3. RFLP banding pattern obtained by Hae III digestion of the 500 bp cyt b fragment from the two mitochondrial lineages of *Phlebotomus sergenti* from southwestern Europe. M= Molecular marker (Hyperladder V, range 25-500 bp); 2, 3 and 8 are lineage I; 1, 4-7 are lineage III.

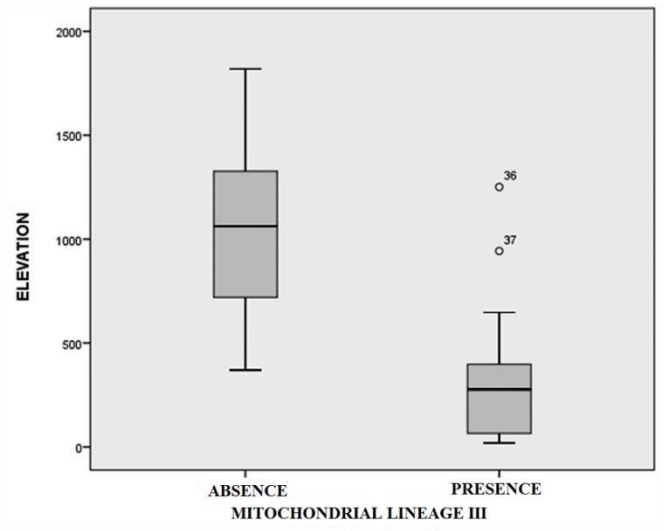

Figure 4. Box plots showing the association of presence of *Phlebotomus sergenti* Lineage III with altitude in areas where both mitochondrial lineages coexist. *Phlebotomus sergenti* Lineage I is distributed throughout the altitude range.

Locality	Province, Country	Year	Number of specimens	Coordinates	Lineage I	Lineage III
Guájar-Faragüit	Granada, Spain (SE)	2011	9	36° 51′ 00′′ N, 3° 33′ 00′′ W	3	6
Calahonda-Castell de Ferro	Granada, Spain (SE)	2011	4	36° 42′ 36′′ N, 3° 23′ 27.6′′ W	1	3
Gualchos_1	Granada, Spain (SE)	2008, 2011	12	36° 45′ 00′′ N, 3° 23′ 00′′ W	4	8
Gualchos_2	Granada, Spain (SE)	2008	5	36° 44′ 16.8′′ N, 3° 23′ 38.4′′ W	0	5
Melicena	Granada, Spain (SE)	2011	3	36° 45′ 10.8′′ N, 3° 14′ 13.2′′ W	0	3
Albuñol-Sorvilán	Granada, Spain (SE)	2011	3	36° 47′ 42′′ N, 3° 14′ 20.4′′ W	1	2
Polopos	Granada, Spain (SE)	2011	1	36° 48′ 10.8′′ N, 3° 17′ 52.8′′ W	1	0
Garnatilla	Granada, Spain (SE)	2011	2	36° 44′ 24′′ N, 3° 26′ 42′′ W	1	1
Güéjar Sierra	Granada, Spain (SE)	2008	5	37° 08′ 17.9′′ N, 3° 24′ 20.9′′ W	5	0
Otívar-Jete	Granada, Spain (SE)	2008	6	36° 47′ 19.9′′ N, 3° 40′ 12.3′′ W	2	4
Sorvilán	Granada, Spain (SE)	2008	2	36° 47′ 42′′ N, 3° 15′ 57.6′′ W	0	2
Albuñol-Albondón	Granada, Spain (SE)	2008	2	36° 52′ 12′′ N, 3° 12′ 32.4′′ W	0	2
Collado Chaquetas	Granada, Spain (SE)	2012	2	37° 02′ 23.6′′ N, 2° 17′ 11.4′′ W	2	0
Torvizcón	Granada, Spain (SE)	1999	14	36° 53′ 00′′ N, 3° 18′ 00′′ W	4	10
Tabernas	Almería, Spain (SE)	2005	1	37° 03′ 00′′ N, 2° 28′ 58.8′′ W	0	1
Los Gallardos	Almería, Spain (SE)	2005	1	37° 10′ 1.2′′ N, 1° 57′ 00′′ W	0	1
Benaoján	Málaga, Spain (SE)	2003	2	36° 43′ 00′′ N, 5° 15′ 00′′ W	2	0
Las Caballerizas	Málaga, Spain (SE)	2003	7	37° 02′ 00′′ N, 4° 43′ 00′′ W	7	0
Almáchar	Málaga, Spain (SE)	1999	5	36° 48′ 00′′ N, 4° 13′ 00′′ W	2	3
Riotinto	Huelva, Spain (SW)	2000	2	37° 41′ 00′′ N, 6° 33′ 00′′ W	2	0
Puerto de la Cadena	Murcia, Spain (SE)	1999	4	37° 54′ 00′′ N, 1° 07′ 58.8′′ W	1	3
Verdolay		1999	4	37° 56′ 00′′ N,	0	4

	Murcia, Spain (SE)			1° 08′ 00′′ W		
Àger	Lérida, Spain (NE)	2012	1	42° 01′ 2.3′′ N, 0° 44′ 39′′ E	1	0
Gisclareny	Barcelona, Spain (NE)	2012	1	42° 15′ 8.7′′ N, 1° 47′ 14.3′′ E	1	0
Sant Just Desvern	Barcelona, Spain (NE)	2002	5	41° 22′ 00″ N, 2° 04′ 00″ E	2	3
Agullana	Gerona, Spain (NE)	2012	3	42° 23′ 47.5′′ N, 2° 50′ 0.6′′ E	3	0
Montfullà	Gerona, Spain (NE)	2012	1	41° 58′ 0.3′′ N 2° 45′ 42.5′′ E	1	0
Sant Jaume de Llierca	Gerona, Spain (NE)	2002	1	42° 12′ 0″ N, 2° 37′ 0″ E	1	0
Calvià	Mallorca, Balearic Islands, Spain	2013	1	39° 33′ 53.6′′ N, 2° 30′ 37.8′′ E	1	0
Sant Joan	Mallorca, Balearic Islands, Spain	2013	2	39° 35′ 40.3′′ N, 3° 01′ 46.7′′ E	2	0
Santa Margalida	Mallorca, Balearic Islands, Spain	2013	1	39° 41′ 39.4′′ N, 3° 06′ 10.2′′ E	1	0
Porto Petro	Mallorca, Balearic Islands, Spain	2013	2	39° 21′ 50.8′′ N, 3° 12′ 43.7′′ E	2	0
Es Coll d'en Rabassa	Mallorca, Balearic Islands, Spain	2013	1	39° 32′ 42.8′′ N, 2° 42′ 22′′ E	1	0
Algaida	Mallorca, Balearic Islands, Spain	2013	1	39° 33′ 13.4′′ N, 2° 53′ 42.7′′ E	1	0
Artenara_1	Gran Canaria, Canary Islands, Spain	2013	1	27° 59′ 15′′ N, 15° 45′ 08′′ W	0	1
Artenara_2	Gran Canaria, Canary Islands, Spain	2013	1	27° 59′ 16′′ N, 15° 44′ 29′′W	0	1
Presa del Parralillo	Gran Canaria, Canary Islands, Spain	2013	1	27° 59′ 30′′ N, 15° 42′ 21′′ W	0	1
Era del Cardón	Gran Canaria, Canary Islands, Spain	2013	1	27° 50′ 02′′ N, 15° 26′ 56′′ W	0	1
Santa Lucía_1	Gran Canaria, Canary Islands,Spain	2013	2	27° 50′ 38′′ N, 15° 27′ 13′′ W	0	2
Santa Lucía_2	Gran Canaria, Canary Islands,Spain	2013	1	27° 54′ 50′′ N, 15° 32′ 40′′ W	0	1
Mirador de Guriete		2013	2	27° 51′ 54′′ N,	0	2

	Gran Canaria, Canary Islands, Spain			15° 32′ 40′′ W		
Ayacata	Gran Canaria, Canary Islands, Spain	2013	1	27° 57′ 21′′ N, 15° 36′ 59′′ W	0	1
Timagada	Gran Canaria, Canary Islands, Spain	2013	1	27° 56′ 30′′ N, 15° 36′ 59′′ W	0	1
Chinchón	Madrid, Spain (C)	2014	9	40° 11′ 1.3′′ N, 3° 27' 18.4′′ W	9	0
Lapa do Gato	Arrábida, Portugal (W)	2008	1	38° 30′ 00′′ N, 9° 00′ 00′′ W	1	0
	TOTAL		137		65	72

Table 1. List of the 45 geographical locations from which *Phlebotomus sergenti* which was captured and molecularly identified showing the number of specimens analyzed and the mitochondrial lineage identified. N= north, S=south, W=west, E=east, C= centre, SE= southeast, SW= southwest, NE= northeast.

Mitochondrial Lineage	**500 bp cyt b fragment**		**250 bp cytb fragment**	
	Number of fragments	Molecular weight of the fragments	Number of fragments	Molecular weight of the fragments
Lineage I	2	290 and 220	3	160, 34 and 22
Lineage II	2	290 and 260	2	194 and 22
Lineage III	3	290, 140 and 110	2	160 and 56
Lineage IV	2	330 and 220	2	194 and 22

Table 2. RFLP banding pattern obtained by Hae III digestion of the 500 bp and 250 bp Cyt b fragments from the four mitochondrial lineages of *Phlebotomus sergenti* from southwestern Europe.

Lineages	Cyt b Haplotypes	1	3	6	18	23	30	42	43	45	54	63	65	66	69	78	85	94	95	102	105	123	126	141	144	147	156	159	160	162	171	177	180	187	198	210	222	225	246	314	319	340
IV	M-J	A	T	T	T	C	A	C	C	T	T	C	A	G	T	T	C	A	T	C	A	T	T	T	G	C	A	A	G	T	C	T	T	T	T	C	C	T	C	T	G	A
II	M-G	A	C	C	T	C	A	C	T	T	C	C	G	G	T	T	T	A	T	C	A	T	C	C	A	C	A	A	G	C	T	A	C	C	C	T	C	T	C	T	G	A
	M-H	A	C	C	T	C	A	T	T	T	C	C	G	G	T	T	T	A	T	C	A	T	C	C	A	C	A	A	G	C	T	A	C	C	C	T	C	T	C	T	G	A
	M-I	A	C	C	T	C	A	C	T	T	C	C	G	G	T	T	T	A	T	C	A	T	C	C	A	T	A	A	G	C	T	A	C	C	C	T	C	T	C	T	G	A
III	M-A / S-F	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	A	T	C	C	A	C	A	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	M-B	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	T	A	T	C	C	A	C	A	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	M-C	A	C	C	T	T	T	C	C	T	C	T	A	A	C	C	T	G	T	T	A	T	C	C	G	C	A	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	M-D	A	C	C	C	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	A	T	C	C	A	C	A	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	M-E	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	A	T	C	C	A	C	T	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	M-F	A	C	T	T	C	T	C	C	T	T	T	A	A	C	C	T	A	T	C	G	T	C	C	A	C	A	A	A	T	T	C	C	T	C	T	C	A	T	T	A	A
	S-G	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	A	T	C	C	A	C	A	A	A	T	T	C	C	T	C	T	C	A	T	T	A	A
	S-H	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	G	C	C	C	A	C	A	A	G	T	T	C	C	T	C	T	C	A	T	T	A	A
	S-I	A	C	C	T	C	T	C	C	T	T	T	A	A	C	C	T	G	T	C	G	C	C	C	A	C	A	G	G	T	T	C	C	T	C	T	C	A	T	T	A	A
I	S-A	G	C	C	T	C	A	C	C	C	T	C	G	G	T	T	T	G	G	C	A	T	C	C	G	C	A	A	G	C	T	C	C	T	C	T	C	C	C	T	A	T
	S-B	G	C	C	T	C	A	C	C	C	T	C	G	G	T	T	T	G	G	C	A	T	C	C	G	C	A	A	G	C	T	C	C	T	C	T	T	C	C	T	A	T
	S-C	A	C	C	T	C	A	C	C	C	T	C	G	G	T	T	T	G	G	C	A	T	C	C	G	C	A	A	G	C	T	C	C	T	C	T	C	C	C	T	A	T
	S-D	G	C	C	T	C	A	C	C	C	T	C	G	G	T	T	T	G	G	C	A	T	C	C	G	C	A	A	G	C	T	C	C	T	C	T	C	C	C	C	A	T
	S-E	G	C	C	T	C	A	C	C	C	T	C	G	G	T	T	T	G	G	C	A	T	C	C	G	C	A	A	G	C	T	C	C	T	C	T	C	C	C	T	A	T

Table 3: Alignment of nucleotide characters at the polymorphic sites in the mtDNA Cytochrome b haplotypes of *Phlebotomus sergenti* with indication of the synapomorphic (highlighted) and the variable characters (underlined) for each lineage (modified from Barón et al., 2008). S-H and S-I are the new haplotypes from Canary Islands.

	LINEAGE I				LINEAGE III			
	Range (°C)	p-value	OR	95% CI	Range (°C)	p-value	OR	95% CI
Mean1	1.4-11.3	0.005	0.74	0.58-0.95	4.3-17.2	0.001	1.46	1.11-1.92
Mean2	1.9-12.2	0.007	0.74	0.57-0.96	5.3-17.5	0.0002	1.60	1.15-2.23
Mean3	3.7-14.2	0.023	0.751	0.57-0.99	7.4-18.3	0.0002	1.74	1.18-2.55
Mean4	5.6-16	0.168	0.83	0.62-1.11	9.7-18.7	0.001	1.63	1.13-2.33
Mean5	9.5-18.8	0.865	1.03	0.77-1.37	12.8-19.9	0.022	1.41	1.02-1.95
Mean6	13.6-22.9	0.052	1.34	0.98-1.84	15.9-23	0.116	1.26	0.93-1.71
Mean7	16.8-25.5	0.02	1.44	1.03-2.00	19-25.8	0.247	1.19	0.88-1.60
Mean8	16.4-25.9	0.057	1.33	0.97-1.82	19.2-25.9	0.134	1.25	0.92-1.68
Mean9	13.8-23.1	0.732	1.05	0.78-1.42	17.8-23.6	0.035	1.39	0.99-1.94
Mean10	9.1-19.1	0.096	0.80	0.60-1.07	12.9-22.4	0.002	1.52	1.10-2.11
Mean11	4.7-15	0.007	0.74	0.56-0.96	8.3-20.3	0.001	1.52	1.12-2.06
Mean12	1.9-12.2	0.005	0.75	0.58-0.95	5.2-18	0.001	1.45	1.10-1.89
Annual Mean	8.2-18	0.208	0.83	0.61-1.13	12.2-20.4	0.001	1.64	1.13-2.38
Max1	4.6-15.5	0.005	0.72	0.54-0.94	9-20.4	0.0001	1.70	1.20-2.42
Max2	5.5-16.6	0.01	0.72	0.53-0.96	10.5-20.7	0.0000	2.03	1.30-3.17
Max3	7.5-18.5	0.023	0.73	0.54-0.99	12.6-22.1	0.0000	2.09	1.32-3.30
Max4	9.8-20.6	0.286	0.86	0.63-1.16	15.3-22.5	0.0003	1.84	1.22-2.79
Max5	13.8-24	0.414	1.13	0.84-1.52	17.5-24.7	0.026	1.41	1.01-1.98
Max6	18.1-28.5	0.024	1.34	1.02-1.77	20-29.4	0.186	1.18	0.92-1.51
Max7	21.5-32.4	0.026	1.29	1.02-1.65	26.4-32.6	0.367	1.11	0.89-1.38
Max8	21-32.3	0.087	1.24	0.96-1.60	24-32.6	0.151	1.19	0.92-1.52
Max9	17.8-28.8	0.466	1.11	0.84-1.47	22.1-29.5	0.031	1.38	0.99-1.92

Max10	12.8-23.5	0.067	0.76	0.55-1.05	18.5-26.4	0.0002	1.87	1.23-2.85
Max11	8.1-19.2	0.01	0.72	0.53-0.96	13.2-23.3	0.0001	1.83	1.23-2.71
Max12	4.7-16.2	0.005	0.73	0.56-0.95	9.3-21.4	0.0001	0.73	0.56-0.95
Annual Max	12.1-22.5	0.345	0.87	0.63-1.19	17.9-24.1	0.0003	1.92	1.24-2.98
Min1	-1.8-8	0.007	0.78	0.63-0.96	-0.4-12	0.007	1.30	1.04-1.61
Min2	-1.7-8.7	0.008	0.78	0.62-0.96	0.1-14.4	0.003	1.35	1.07-1.72
Min3	-0.1-9.9	0.027	0.78	0.61-0.99	2.2-14.5	0.001	1.46	1.10-1.94
Min4	1.4-11.5	0.113	0.83	0.64-1.07	4.1-15	0.006	1.40	1.06-1.84
Min5	5.2-14.2	0.664	0.95	0.73-1.23	6.9-16.2	0.035	1.31	0.99-1.72
Min6	9.1-17.8	0.267	1.17	0.88-1.54	11-17.6	0.126	1.23	0.93-1.64
Min7	12.1-19.8	0.089	1.29	0.95-1.74	14.2-20.2	0.267	1.17	0.88-1.57
Min8	11.5-20.3	0.102	1.25	0.95-1.66	14.5-20.8	0.225	1.18	0.90-1.55
Min9	9.8-18.5	0.968	0.99	0.77-1.29	11.4-19.9	0.105	1.22	0.95-1.58
Min10	5.5-14.8	0.144	0.85	0.66-1.08	7.4-18.4	0.019	1.31	1.02-1.67
Min11	1.3-10.9	0.008	0.77	0.62-0.96	3.5-17.3	0.006	1.32	1.05-1.67
Min12	-0.8-8.7	0.007	0.77	0.62-0.96	1.1-14.7	0.006	1.32	1.05-1.67
Annual Min	4.3-13.4	0.163	0.84	0.65-1.09	6.3-16.7	0.012	1.36	1.04-1.78

Table 4. List of the monthly average temperatures and annual average temperature analysed for the two mitochondrial lineages of *Phlebotomus sergenti*. Range (°C) =suggested monthly temperature range. OR=Odds Ratio. CI=Confidence Interval. Mean=monthly mean temperature. Max=monthly maximum temperature. Min=monthly minimum temperature. 1-12=January to December.

High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (Oryctolagus cuniculus) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: Epidemiological consequences

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Abstract

Leishmania infantum infection has been reported in various host species, both domestic and wild, in some cases with high prevalence rates. However, until the recent Discovery of infected hares, no studies had provided clear evidence of any significant reservoir other than domestic dogs. Our focus was on another lagomorph, *Oryctolagus cuniculus* or wild rabbit. This species is native to the Iberian Peninsula and its presence and abundance gave rise to the name of Spain. In an endemic area for canine leishmaniasis in the southeast of Spain, 150 rabbits were captured over a period of three years. Samples of blood, bone marrow, liver, spleen, heart and skin were taken and analysed through parasitological, serological and molecular techniques in order to detect *Leishmania* and *Trypanosoma*. 20.7% of the rabbits were infected with *L. infantum* and 82.4% with *Trypanosoma nabiasi*, and 14.8% of mixed infections were detected. Both parasites were found in all the animal organs analysed, a factor which, along with the presence of serological

cross-reactions, must be taken into account in epidemiological studies on leishmaniasis. *O. cuniculus* is an abundant and gregarious species, with a long enough average lifespan to ensure *L. infantum* transmission. The presence of the parasite in the skin and blood of these rabbits with no acute manifestation of disease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit. The rabbit therefore seems to meet the most of conditions for being considered a reservoir host of *L. infantum*.

Keywords

Leishmania infantum, Trypanosoma nabiasi; Wild rabbits, Reservoir host

Introduction

Leishmania infantum is the species responsible for zoonotic visceral leishmaniasis (ZVL) in Mediterranean countries (both Southern Europe and Northern Africa), Middle East and certain parts of Asia, and was brought into Latin America from the Iberian Peninsula, where it adapted to the local permissive sandfly *Lutzomyia longipalpis* (Volf and Myskova, 2007; Alvar et al., 2012). In southern Europe, the incidence of ZVL in humans is relatively low (0.02–0.49/100,000 in the general population) (Alvar et al., 2012; Antoniou et al., 2013). However, out-breaks or recrudescence may occur periodically in foci like the new one in Spain, where incidences reached 56 per 100,000 (Molina et al., 2012; Arce et al., 2013). Dogs, which may suffer from severe disease (CanL), are the primary domestic reservoir hosts of ZVL. Furthermore, the infection has been described in various host species, both domestic and wild (Ashford, 1996; Quinnell and Courtenay, 2009). It has been found in marsupials, primates, rodents, carnivores, xenarthrans and bats (De Lima et al., 2008; Quinnell and Courtenay, 2009; Malta et al., 2010; Antoniou et al., 2013; De Araújo et al., 2013). The use of PCR in these studies confers great advantages such as higher sensitivity and the opportunity to identify the species involved. Several domestic species have now been shown to have a high prevalence of

infection in some areas. In particular, a number of European studies have shown a high prevalence in domestic cats; for example, 26% of 183 cats tested in a Spanish study were PCR positive (Martín-Sánchez et al., 2007). Infections have also been reported in horses in Europe (Fernández-Bellón et al., 2006). In wild animals, infections have been reported in red foxes, wolves, genets, lynxes, Egyptian mongooses, black rats, etc. (Criado-Fornelio et al., 2000; Portús et al., 2002; Di Bella et al., 2003; Ranieri et al., 2006; Dipineto et al., 2007; Sobrino et al., 2008). In studies of foxes, for example, 14.1% and 74% tested PCR positive in Spain (Criado-Fornelio et al., 2000; Sobrino et al., 2008) and 40% in southern Italy (Ranieri et al., 2006). The absence or low prevalence of lesions is a characteristic common to these studies. Infections have also been described in animals kept in captivity such as non-human primates (Malta et al., 2010), Barbary lions (Libert et al., 2012) and a Bennett's wallaby (Ramírez et al., 2012).

A mammal host responsible for the long-term maintenance of a population of infectious agents is called a reservoir host. In addition to the reservoir host(s) essential to the maintenance of parasite populations, there may be numerous incidental hosts that are irrelevant to long-term persistence. Occasionally, these incidental hosts may be responsible for some transmission. They may even become secondary reservoir hosts (Ashford, 1996). In addition to domestic dogs, the ability to transmit infection has been confirmed by xenodiagnosis in ground squirrels, Syrian hamster, black rats, domestic cats and recently, in hares (Gradoni et al., 1983; Maroli et al., 2007; Quinnell and Courtenay, 2009; Molina et al., 2012; Antoniou et al., 2013). An understanding of the reservoir system is important in the design of rational control measures (Ashford, 1996; Quinnell and Courtenay, 2009).

The domestic dog is the main reservoir host for *L. infantum* and as such, it is able to maintain the para-site indefinitely as its only host (Ashford, 1996); however, it is important to consider the potential importance of other hosts and its implications in the control of ZVL. Until recently, no study had provided clear evidence of any important ZVL reservoir other that the domestic dog, which does not mean to say

that they did not exist. Then Molina et al. (2012) demonstrated for the first time that apparently healthy naturally infected hares (*Lepus granatensis*) can be infectious to a competent *L. infantum* vector (*Phlebotomus perniciosus*), revealing that hares may be playing a role of active reservoirs in the recently reported leishmaniasis outbreak in the southwestern area of the Madrid autonomous community in Spain (Arce et al., 2013; Jiménez et al., 2013). The finding of positive hares in each of the six Spanish regions surveyed by Ruiz-Fons et al. (2013), suggests that the parasite is widespread in Spanish hare populations. These findings highlight the need for further research on this topic. Studies on the role of lagomorphs as reservoirs of leishmaniasis are very scarce worldwide. Our focus was on another lagomorph, the wild rabbit, which unlike the hare is a burrowing animal. Moreover, when the distribution of *Trypanosoma* and *Leishmania* species overlaps in the same geographical area, mixed infections can appear within the same host. In the Amazon Basin, for example, De Araújo et al. (2013) describe a triple mixed infection of *T. cruzi, T. rangeli* and *L. infantum* in the anteater *Tamandua tetradactyla*.

Trypanosomes are haemoflagellate protozoans (Kinetoplastida) found worldwide that infect a wide range of animals and man. Some species that parasitize animals are nonpathogenic under normal conditions, although they can produce fatal infections in young or immunologically compromised hosts (Molyneux, 1970). Nonpathogenic trypanosomes have strong host specificity, usually being infective for only one host species. Although the non-pathogenic trypanosome group nowadays includes at least 45 morphologically indistinguishable species, it is accepted that *T. nabiasi* is the species that infects the domestic and wild rabbit (Molyneux, 1970; Hamilton et al., 2005; Reglero et al., 2007). In spite of being included in the same family and sharing some antigenic and molecular characteristics, *Trypanosoma* (*Herpetosoma*) and *L. infantum* selected different life strategies in the course of their evolution. The former is transmitted by fleas, whilst *L. infantum* is transmitted to several mammal species by parasite regurgitation

during bloodmeals taken by infected sandflies, mainly belonging to the subgenera *Larroussius* and *Adlerius* in the Old World.

Our aim was to investigate natural *L. infantum* infection in wild rabbits (*Oryctolagus cuniculus*) and discuss their potential role as reservoirs in a recognized endemic area with ample epidemiological knowledge, as is the case of southeastern Spain. The sympatric and syntrophic presence of another kinetoplastid, *T. nabiasi*, was taken into consideration to avoid confusion.

Materials and methods

Specimens and capture area

From July 2009 to October 2011, on a private farm in the province of Granada in southeastern Spain (geographical coordinates: 37° 17′ 18′′ N, 3° 52′ 47′′ W), 150 wild rabbits (*O. cuniculus*) were captured. The farm has controlled hunting grounds and restricted access. Extending over 1000 ha, it is located in the Mesomediterranean bioclimatic level, at an altitude of 750–900 m above sea level. With an annual rainfall of approximately 600 mm, the summers are hot and dry. The terrain is undulating, with tree cover consisting mainly of olives in 45% of the estate, and 10% pine and holm oak on scrubland; the remaining 45% is destined to dry land crops such as wheat and barley and irrigated crops such as alfalfa. A stream runs through the estate. There is livestock, mainly sheep which are put out to pasture during the day and overnight in stables. There were 7 dogs, 3 of which were diagnosed with canine leishmaniasis by our group through serology and culturing. The ground is not too hard or stony, making burrowing easier. The density of the lagomorph population is high, at 11 rabbits per hectare, with an average life span of 2.5 years. The rabbits on the farm were genotyped as belonging to the subspecies *O. cuniculus algirus* and no repopulation measures have ever been taken; the rabbits from this farm are used to repopulate farms in other parts of Spain (Land agent of the state “La Torre”, personal communication).

Of the 150 rabbits, 107 were shot by hunters and 43 were captured live with ferrets. 52% (78) were male. Weight varied from 400 to 1200 g: 8.7% weighing ≤500 g, classified as small; 75.3% weighing > 880 g, classified as large and 16.0% classified as medium weight.

Obtaining and processing samples

Blood samples were collected from the recently shot rabbits (107) by cardiac puncture; this was the only type of sample analysed in this case. The rabbits captured live (43) were sacrificed by concussion in the laboratory and samples of blood, bone marrow, liver, spleen, heart and skin were taken. Some of the blood was reserved for separation of the serum with a view to detecting antibodies and the rest was mixed with EDTA as an anticoagulant to pre-serve the whole blood. As well as the whole blood, all the other types of samples were also divided into three parts for microscopy, culture and PCR. The PCR part was processed in a room exclusively destined to DNA extraction. Bone mar-row was extracted by passing RPMI medium supplemented with 20% Foetal Bovine Serum through the clean femur or tibia of the animal, and placed on a Petri dish. Due to the small amount of collected sample, the bone marrow was destined exclusively for culturing.

Laboratory techniques

Parasitological, serological and molecular techniques were all used to detect *Leishmania* and *Trypanosoma* infection.

1. *Microscopic techniques*

For detection of trypomastigotes both direct observation of a drop of blood placed between slide and coverslip and microhematocrit technique were performed.

Thin blood smears and liver and spleen imprints were made, and then fixed in pure methanol and Giemsa-stained in order to detect amastigotes.

Cultures were made with peripheral blood, bone mar-row and macerates of liver, spleen and heart, using a combination of EMTM solid phase made with rabbit blood and RPMI supplemented with 20% Foetal Bovine Serum and 5% human urine as the liquid phase. The cultures were kept for 6 months before being rejected as negative. In vitro sub-inoculations were performed weekly during the first two months and then every 15 days.

2. *Serum antibody testing by indirect fluorescent antibody test (IFAT)*

A suspension of 2 million/ml *L. infantum* zymodeme MON-1 = GR-1 promastigotes from strain MCAN/ES/91/DP204 or *T. nabiasi* epimastigotes from strain MLAG/ES/2010/DP543 were used as antigen in the IFAT. The antibody titre against *Leishmania* or *Trypanosoma* was determined in geometric dilutions from the serum obtained from each blood sample as previously reported for dogs; a starting dilution of 1/20 was used (Acedo-Sánchez et al., 1996). Rabbit anti-gamma globulin (ICN Biomedicals) was used as a conjugate at a concentration of 1/100 in Evans blue previously diluted at 1/104 with phosphate-buffered saline. A standard cut-off dilution for positivity in rabbits was not available for any of the parasites.

3. *DNA extraction*

DNA was obtained from the different samples using the REAL DNA SSS Extraction Kit (RBME01). Each extract was rehydrated in a final volume of 20 µl of sterile water. To make sure there was no contamination at this stage, extraction controls were carried out. These consisted of tubes of sterile water to which the whole extraction process was applied simultaneously with the biological samples. One control was used for every group of 7 biological samples. The extracted DNA was kept at −20 ºC until its amplification by PCR.

As a totally independent procedure to the DNA extraction from biological samples taken from wild rabbits, DNA was also extracted from *L. infantum* promastigotes (MCAN/ES/91/DP204) and *T. nabiasi* epimastigotes (MLAG/ES/2010/DP543) taken

from cultures. The parasites were washed and counted with a hemocytometer and adjusted to a final concentration of 1000 parasites/µl to be used as positive controls in the PCR. Various negative controls were used: (i) tube of PCR reagents without DNA, (ii) extraction controls, (iii) DNA from an uninfected domestic rabbit.

4. *PCR ELISA*

This technique is specific to the *L. infantum* species. It was performed following the protocol of Martín Sánchez et al. (2001) using kits PCR-ELISA DIG Labelling and PCR-ELISA DIG Detection (Roche Diagnostics GmbH. Mannheim, Germany). Every sample was analysed in duplicate, adding 2 and 3 µl of DNA in a final reaction volume of 25 µl. The results were read in a spectrophotometer at a λ of 405 nm. Samples returning absorbance values of ≥1 were considered positive, in accordance with the results obtained previously by Martín-Sánchez et al. (2001). When optical density was ≥0.5 and <1, amplification was repeated using 4 and 5 µl of DNA.

5. *PCR amplification assay ITS 1 fragment and restriction fragment length polymorphism (RFLP)*

ITS1 was amplified with primer pair LITSR/L5.8S and PCR conditions described elsewhere (El Tai et al., 2000, 2001; Schönian et al., 2003). The amplification products were tested by electrophoresis in 1.5% agarose gels in 1× TAE-buffer and visualized by staining with ethidium bromide. This technique permits differentiation between the *Trypanosoma* and *Leishmania* genera according to the size of amplified fragment. Digestion was carried out with restriction enzyme Hae III (BsuRI) (Thermo Scientific) for 3 h at 37 ◦C and the digestion products were controlled in 3% agarose gels.

6. *Isoenzymatic identification*

Starch gel electrophoresis was performed according to the method described by Rioux et al. (1990), using 15 enzyme systems. The studied strain was analysed along with diverse zymodeme reference strains and MHOM/FR/78/LEM75 *L. infantum* as the basic reference zymodeme.

7. *Statistical treatment of data*

The statistical analysis of data was conducted using the IBM SPSS Statistics 20 package. Significance level was set at 5%. Concordance between techniques was determined with the Kappa coefficient, interpreted in the following way: almost perfect (1.00–0.81), substantial (0.80–0.61), moderate (0.60–0.41), fair (0.40–0.21) and slight (0.20–0.0).

The existence of associations between *Leishmania* or *Trypanosoma* infection (dependent variable) and the characteristics of the rabbits (sex, weight or size) or their capture (capture period) and antibody response, was deter-mined by logistic regression analysis.

Results

L. infantum infection

1. *General results*

We were able to analyse all 150 of the rabbits captured by at least one of the techniques used to check for *L. infantum* infection. Using the whole array of techniques, the parasite was detected in peripheral blood, liver, spleen, heart, bone marrow and skin, returning a result of 31 infected rabbits (20.7%). An external visual inspection of the apparently healthy rabbits shot by hunters did not reveal the presence of clinical symptoms in any of the specimens. The 43 live rabbits which were transferred to the laboratory were more closely inspected and cutaneous lesions were found on 10 specimens (23.3%). The number of lesions

varied from 1 to 4, located on hind legs, ears or nose. Amastigotes in the lesions of two specimens were detected under microscope, both of which tested PCR positive for *L. infantum* and *Trypanosoma*; amastigotes were not detected in any other organ with the exception of the liver of one these two rabbits. A strain of *Leishmania* taken from the bone marrow of another rabbit was isolated in culture (MLAG72011/ES/DP568).

Leishmania infection in rabbits is not associated with the sex of the specimen ($p = 0.723$). It is however associated with size – large rabbits are more parasitized than small/medium ones ($p = 0.018$) – and with capture period ($p < 0.001$). The detection of the parasite is positively associated with the antibody titre determined using promastigotes as the antigen ($p = 0.003$; OR = 1.017), so as the antibody titre increases, the probability of detecting *Leishmania* by molecular techniques increases. In the multivariate model created with the three variables which turned out to be significant in the previous univariate models, the antibody titre lost significance and was therefore removed from the model, resulting in the structure displayed in Table 1. Thus when capture period is equal, the risk of *Leishmania* infection in large rabbits is 15 times higher than in smaller rabbits. Similarly, when size is equal, with respect to the figures for 2009, the risk of infection is 54 times higher in 2010 and 793 times higher in 2011. To rule out the possibility that capture method and the additional use of diagnostic methods was determining the influence of capture period, a parallel logistic regression analysis was conducted using the results of the blood diagnosis with PCR techniques as the dependent variable, confirming that the independent variable "capture period" continued to maintain its association and that its behaviour was similar.

2. Detection of *L. infantum* infection by PCR

20.7% (31/150) of the rabbits were found to be infected by *Leishmania* spp. on applying one of the two PCR techniques to the samples obtained.

The PCR-ELISA indicated the presence of *L. infantum* in 13.4% (20/149). The PCR of the ITS-1 fragment amplified a 300 bp fragment characteristic of the *Leishmania* genus in 10.9% of specimens (15/138); only two of the 15 positives were apt for digestion with restriction enzyme Hae III, giving unclear results for differentiation between *L. infantum* and *L. tropica* (in all other cases the band obtained was too weak to permit digestion).

Of the 10 rabbits with cutaneous lesions, for 2 of these no skin samples were reserved for molecular diagnosis, whilst 6 of the remaining 8 tested positive with these techniques on the skin lesions (6 positives with PCR-ELISA, of which 3 were also positive for *Leishmania* with PCR-ITS); all 6 specimens had *L. infantum* in other organs simultaneously. Of the two skin samples testing positive under microscope, one was positive with PCR-ELISA and the other could not be studied by molecular methods.

There are statistically significant differences between the results of the two PCR techniques ($p = 0.002$), with the PCR-ELISA returning a higher percentage of positive results. Its concordance expressed in terms of the kappa index is 31.4% ($k = 0.314$): the two PCR assays were positive for 7 animals and negative for 109.

3. Serology for L. infantum promastigotes

Antibody titres for the antigen made up of *L. infantum* promastigotes varied from 0 to 320: 71.4% of specimens had zero titres; 8.2% had titres of 20; 10.9% titres of 40; 6.8% titres of 80, 2.0% titres of 160 and 0.7% titres of 320. Thus 28.6% of the rabbits had antibody titres ≥20, and 20.4% had titres of ≥40. On comparing separately the positivity results obtained from these two different cut-off titres with those obtained with direct techniques, the resulting kappa index values were 0.563 ($p < 0.001$) and 0.372 ($p < 0.001$) respectively.

As stated previously, the detection of the parasite was positively associated with antibody titre ($p = 0.003$; OR = 1.017): the likelihood that rabbits with an antibody titre of 20 are infected with *Leishmania* is 1.7% higher than that of rabbits with a

titre of 0; in the case of rabbits with a titre of 40 is 1.72% higher, and 1.73% for animals with an antibody titre of 80, and so on.

4. *Specific identification of L. infantum*

Thirteen blood samples, 6 of spleen, 8 of liver, 3 of heart and 7 of skin, taken from a total of 20 rabbits, tested positive by PCR-ELISA, a technique which is specific to *L. infantum*. A strain taken from the bone marrow of one of the rabbits was also isolated in culture (MLAG72011/ES/DP568). DNA was extracted from the cultured parasite and submitted to PCR-ELISA with positive results indicative of *L. infantum*, and to PCR-RFLP of the ITS-1, giving a band pattern characteristic of *L. infantum*. Following mass culturing, it was submit-ted to isoenzyme electrophoresis for characterization and identified as *L. infantum* zymodeme MON-1. The specimen did not have skin lesions and amastigotes were not observed by direct techniques in any of the samples. The PCR of the ITS was negative in all organs analysed, whilst the PCR-ELISA returned positive results in liver, spleen, heart and blood. As with the rest of the specimens, no bone marrow was reserved for PCR.

Trypanosoma spp. infection

1. *General results*

Of the 150 rabbits studied, 142 were apt for use to determine the presence of *Trypanosoma* spp.; of these, 85.2% (121/142) were infected. The parasite was detected with the different techniques in peripheral blood, liver, spleen, heart, bone marrow and skin. In the peripheral blood, trypomastigotes were observed under microscope in 31.6% of cases (6/19); performing the microhematocrit technique did not increase diagnostic sensitivity. Through culturing we were able to visualize, and in most cases also isolate, epimastigotes from all the organ types except skin, as these cultures were contaminated with fungi. The positivity values obtained by culture were: 21.2% in spleen (7/33), 27.3% in liver (9/33), 21.1% in heart (4/19), 10.5% in blood (2/19), 30.3% in bone marrow (10/33). The overall percentage of rabbits testing positive with this technique was 45.5% (15/33).

Trypanosoma infection in rabbits is not associated with the sex of the specimen (p = 0.667). It is however associated with weight (p = 0.049): the risk of infection in small/medium rabbits is 7.8 times higher than in larger ones. It is also associated with capture period (p = 0.009), with a higher risk in July 2009 than in subsequent periods (Table 2).

2. *PCR of the ITS*

The PCR of the ITS-1 fragment amplified a 500 bp band which could not be digested with restriction enzyme Hae III in 82.4% of the rabbits (117/142), and another of 480 bp in 2.8% of cases (4/142). The two fragments were sequenced and their sequences compared with those of other species of Trypanosoma recorded in GenBank. This allowed us to confirm the identification of *T. nabiasi* in the rabbits of southern Spain.

3. Serology for T. nabiasi

The antibody titres for the antigen made up of *T. nabiasi* epimastigotes varied from 0 to 640: 28.9% of specimens had antibody titres equal to 0; 38.7% had titres of 20; 18.3% titres of 40; 9.2% titres of 80, 2.8% titres of 160; 1.4% titres of 320 and 0.7% titres of 640. Thus 71.1% of the rabbits had antibody titres of ≥20, and 32.4% a titre of ≥40. On comparing separately the positivity results obtained from these two different cut-off titres with those obtained by direct techniques, the kappa index values returned were −0.011 (p = 0.557) and −0.003 (p = 0.958) respectively. The detection of the parasite is negatively associated with the antibody titre determined using both epimastigotes (p = 0.05, OR = 0.994) and promastigotes (p = 0.007; OR = 0.985) as the antigen, meaning that as the antibody titre increases, the probability of detecting the parasite decreases.

Mixed infections

Mixed *Leishmania/Trypanosoma* infections were detected in 14.8% of specimens (21/142). Joint infection by the two *Trypanosoma* spp. haplotypes was found in 2.1% of the rabbits (3/142).

The correlation (Pearson correlation coefficient) between the antibody titres determined using the two types of antigen is 10.4% ($p = 0.216$).

Discussion

The wild rabbits taken from the endemic ZVL area that is southeastern Spain, with a CanL seroprevalence of 20.1% at the Mesomediterranean bioclimatic level (Martín-Sánchez et al., 2009) and PCR positivities of 54.5% (Morales-Yuste et al., 2012), were found to be infected by *L. infantum* in 20.7% of cases when direct techniques were used. The identity of the species was confirmed using a specific PCR (PCR-ELISA, Martín-Sánchez et al., 2001) and isoenzymatic characterization of a strain isolated in culture. It has become almost automatic that on detecting the parasite by techniques that do not permit specific identification, including PCR techniques which are only specific to genus, it is classified as *L. infantum* due simply to the fact that the area in question is endemic for ZVL (Criado-Fornelio et al., 2000; Di Bella et al., 2003; Dipineto et al., 2007; Helhazar et al., 2013). This lack of rigour in the expression of results can lead to errors, especially if there is a possibility that other species are present, not only in sympatry but also in syntrophy. The prevalence of infection of these rabbits by another kinetoplastid parasite, *T. nabiasi*, was very high, with values of up to 82.4%. Both parasites were detected in all the rabbit organs analysed, peripheral blood, liver, spleen, heart, bone marrow and skin. Finding these parasites in the heart probably reflects their presence in the blood. The amastigotes from both parasites are indistinguishable and culture forms of flagellated *Trypanosoma* initially were mistaken by these authors, despite their great experience with *Leishmania* culture. The PCR of the ITS1 proved useful for distinguishing between the two genera, although it was not sensitive enough

to allow specific identification of *L. infantum* through RFLP, and its diagnostic sensitivity for leishmaniasis was lower than that of the PCR-ELISA ($p = 0.002$). *Trypanosoma* acts as protection factor against *Leishmania* infection: the presence of *Leishmania* was 4.3 times greater in rabbits non-infected with *Trypanosoma* compared with the infected animals ($p < 0.05$).

The correlation between the antibody titres detected in the wild rabbits using the two types of antigen, *L. infantum* promastigotes and *T. nabiasi* epimastigotes, was low (10.4%), but we must take into account that this is not an indication of the reactivity for both antigens but it shows whether the two antibody titres have the same value. In general terms, the results reflect the existence of cross-reactions due to the presence of antigens shared by members of the same family, so caution must be exercised in the interpretation of serological results for lagomorphs when the studies do not include procedures that allow us to distinguish between the two kinetoplastids, such as that conducted by Moreno et al. (2013). In any case, on comparing the results obtained with direct and indirect diagnostic methods, taking titres 20 and 40 as threshold values for the latter, we find a moderate correlation in the diagnosis of leishmaniasis (56.3% and 37.2% respectively) and a very poor one in the case of trypanosomiasis (negative kappa values). In fact, as the antibody titre for *Leishmania* increases, the probability of the rabbit being infected by this parasite increases (1.7% with every increase in antibody titre, $p = 0.003$), whilst the association is negative in the case of *Trypanosoma*, whose likelihood of being present falls by 0.6% with every increase in antibody titre. A positive association between antibody titre and the presence of *L. infantum* is a characteristic found in dogs (Morales-Yuste et al., 2012), whilst in cats the association is negative (Martín-Sánchez et al., 2007); such negative association may suggest a possible protective role for the acquired immunity in infected animals.

1. L. infantum infection

Similarly to other countries in southwestern Europe, in Spain leishmaniasis is an endemic zoonosis and the parasite responsible in all forms, visceral, cutaneous and mucosal, is *L. infantum*, with the main reservoir encountered so far being the dog (Morillas et al., 1996; Fisa et al., 1999; Aliaga et al., 2003; Martín-Sánchez et al., 2004, 2009; Miró et al., 2008; Gálvez et al., 2010; Ballart et al., 2012a; Morales- Yuste et al., 2012).

Studies on the infection of *O. cuniculus* by *Leishmania* are very scarce worldwide. A study carried out in southeastern Spain suggests that wild rabbits have a very low risk of becoming infected with *L. infantum* (Chitimia et al., 2011).

The absence or low prevalence of lesions is another characteristic to highlight in studies on hosts other than dogs, such as cats or wild animals (Martín-Sánchez et al., 2007; Maia et al., 2008; Quinnell and Courtenay, 2009; Millán et al., 2011; Sherry et al., 2011). In our case, when we were able to inspect the animals more closely, we found that 23.3% (10/43) had skin lesions. In six of these specimens we have confirmed the presence of *L. infantum* in the skin; one of them was only infected by this kinetoplastid and no trace of *T. nabiasi* was detected in any of its tissues; the rest had mixed infections.

The specimen from which the *L. infantum* strain was isolated was only infected by this trypanosomatid and although the parasite was detected by PCR in other organs, we were only able to isolate it in the bone marrow. We cannot rule out the possibility that, in cultures with few passages taken from specimens infected with both trypanosomatids, mixed populations of L. infantum and *T. nabiasi* may be present, and that over time the former may disappear leaving behind only *T. nabiasi*, a parasite for which 23 isolations were achieved.

Certain ecological parameters of reservoir hosts are particularly important in the understanding of their role in reservoir systems. It has been shown that distribution at the levels of geography, biotope, habitat, and home is the first essential, and that the other most important parameters are those of age structure of

populations, their density, dispersion and movements, and social structure. Moreover, behavioural details may determine specific relationships with sandflies. A convincing model would depend on a close specific interaction between the putative reservoir host and the sandfly vector (Ashford, 1996).

O. cuniculus is native to the Iberian Peninsula and is widely distributed across the whole of southwestern, central and northern Europe. It has also been introduced into Australia, New Zealand, the Unites States, Chile, etc. It was the Phoenicians who, on reaching the Iberian Peninsula, named this country "Sphania", derived from the Greek Sphan meaning rabbit. This animal was considered to be a plague throughout Europe, until 1954–1955, when its population was decimated by myxomatosis. Since then, its numbers have gradually increased in areas where they find food and protection, and there is a rational control of hunting. The rabbit is found throughout Spanish territory, with relative density values of between 1.6845 (Community of Madrid, central Spain) and 0.0026 (Community of La Rioja, northeastern Spain); in the province of Granada (southeastern Spain) the relative density is 0.94. In any case, the absolute rabbit densities increase tenfold over the course of the year between the season of minimum and maximum abundance. The main biotope for the protection and reproduction of these lagomorphs is the warren, dug by the rabbits themselves in fertile soil. It is a network of branching tunnels and galleries, in which they build their nests before giving birth. This is a gregarious, territorial and nocturnal species. It is polygamous (one male for various females). The roaming area of the male is larger than that of the female, reaching up to 7 ha. They have litters of 2 –8 pups, with higher numbers in the hot months, at one month intervals from January to June. Sexual maturity is reached at 3–4 months. The average lifespan of the rabbit is long enough to ensure its survival during the non-transmission period of leishmaniasis; on the farm where the specimens were collected, the average lifespan was 2.5 years. The distribution of rabbits is very irregular, and it is common to find one farm with a highly dense population while on a neighbouring farm there are very low numbers (Burton,

1978; Ministry of the Environment, 1996). On the farm surveyed, the density was much higher than the average for the Granada province and other areas of Spain. *L. infantum* infection was detected in the rabbits throughout the entire capture period, from July 2009 to October 2011, with a sharp increase in infection prevalence which would be more indicative of an epidemic out-break than an endemic steady state. The 1.1% of infection detected in the month of July probably indicates infections acquired in the previous transmission period, given the phenology of the main vector in the area, *P. perniciosus* (Morillas-Márquez et al., 1983). Furthermore, infection is more frequent in larger rabbits, which have been exposed to the vector's bites for longer. All these points appear to indicate that the rabbits can be infected for a long time without displaying acute symptoms of disease, given that the only clinical manifestation detected was skin lesions in 23.3% of the specimens. The presence of the parasite in the skin and blood allows it to be presented to the vector sandfly. CDC traps were placed next to the warrens, hanging from trees, when available, or from a stick driven into the ground for this express purpose, allowing us to capture these dipterans in considerable densities (10.6 sandflies/trap, collecting a total of 1812 sandflies). The most abundant species was *P. perniciosus* (72.3%), which is precisely the main vector of leishmaniasis in the area (Barón et al., 2011; Martín-Sánchez et al., 1994), followed by *S. minuta* (20.1%), *P. papatasi* (6%), *P. ariasi* (0.94%), which also acts as a vector (Ballart et al., 2012b; Morillas et al., 1996), and P. sergenti (0.71%) (data not shown).

2. T. nabiasi infection

T. nabiasi has been reported in *O. cuniculus* from various European countries (UK, France, Italy, Portugal and Spain) and in domestic *O. cuniculus* outside Europe. Little is known about the identification and distribution of *T. nabiasi* in Spanish rabbit populations. In a study conducted in southwestern Spain, Reglero et al. (2007) found that *Trypanosoma* was prevalent in the blood of rabbits, but this was the only type of sample analysed. Prevalence was 23.81% in young rabbits, 84.85%

in juveniles and 47.37% in adults. The prevalence and abundance of parasites in the blood was higher in females than in males, and higher in juvenile rabbits, followed by adults and lastly, young rabbits. The figures were also higher in fenced nuclei than in open nuclei. In our case, no differences were found between males and females, whilst we were able to con-firm the lower parasitization levels in larger rabbits, i.e. adults; young rabbits have an eight times higher risk of infection than adults. Effective self-cure of infection and a role for acquired immunity has been suggested as an explanation for this, which would be in keeping with the negative association between antibody titres and parasite presence reported in this study. The prevalence figures we found are clearly higher than those given by Reglero et al. (2007), and they remained high throughout the duration of the study. These differences may be due to these authors using only blood and direct observation methods to detect the parasites, as opposed to the variety of samples and diagnostic techniques which we used. Moreover we cannot rule out the potential influence of our survey site being a fenced farm, i.e. a closed nucleus.

Conclusion

In order to determine the role of a given host in a reservoir system, it is far from sufficient to simply discover infected individuals. Despite the fact that in southwestern Europe *L. infantum* antibodies and DNA have been found in a wide array of domestic and wild animals, and that in some cases it has been possible to isolate the parasite and submit it to isoenzymatic characterization, no clear evidence has ever been found of their involvement as reservoirs, with the possible exception of the recent study on hares. Without a doubt, the impossibility of finding an area where there are no dogs further hinders the process of evaluating the possible contribution of these other hosts to the epidemiology of leishmaniasis. In the systems that have been adequately described, the reservoir host is abundant, forming a large proportion of the mammalian biomass and it is

often a gregarious species, all characteristics which are true of the wild rabbit to an even greater extent than the dog. Furthermore, an effective reservoir host can be expected to be long-lived, at least surviving through any non-transmission season. Although the average lifespan of rabbits is shorter than that of dogs, it is long enough to ensure the transmission of *L. infantum*. If a rabbit reaches adulthood it may live for 4–5 years, clearly a much lower figure than the estimated average 14-year lifespan of dogs. The presence of the parasite in high proportions in the skin and peripheral blood of these rabbits with no apparent signs of acute dis-ease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit. Therefore, the rabbit appears to fulfil the most of conditions which would justify it being considered a reservoir host of L. infantum and it would be interesting to conduct xenodiagnostic experiments using the local phlebotomine vectors. The sympatric and syntrophic presence of *T. nabiasi* must be taken into account in order to avoid any confusion.

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Variable	N	P	*p*	OR
Size	150	20,7%	0,05	-
Small/Medium rabbits (Ref)	37	5,4%	-	-
Large rabbits	117	25,7%	-	15,220
Capture period	150	20,7%	<0,001	-
July 2009 (Ref)	86	1,1%	-	-
October 2009	21	9,5%	0,165	-
October 2010	24	50,0%	<0,001	54,205
October 2011	19	84,2%	<0,001	792,711

Table 1. Factors associated with *Leishmania infantum* infection in rabbits. Multivariate model obtained by logistic regression. N is the number of rabbits, P is the prevalence of infection in each category, p shows the level of significance, OR shows the risk of infection with respect to the reference category, ref is the reference category.

Variable	N	P	*p*	OR
Size	142	85,2%	0,049	-
Large rabbits (Ref)	107	97,1%	-	7816
Small/Medium rabbits	35	81,3%	-	-
Antibody titres for the antigen made up of *Trypanosoma* epimastigotes	134	85,2%	0,05	0,994
Capture period	142	85,2%	0,009	-
July 2009 (Ref)	80	95,0%	-	-
October 2009	19	75,0%	0,009	0,147
October 2010	24	68,4%	0,008	0,158
October 2011	19	73,7%	0,002	0,114

Table 2. Factors associated with *Trypanosoma* infection in rabbits. Univariate models obtained by logistic regression. N is the number of rabbits, P is the prevalence of infection in each category, *p* shows the level of significance, OR shows the risk of infection with respect to the reference category, ref is the reference category.

Leishmania infantum in wild rodents: reservoirs or just irrelevant incidental hosts?

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Abstract

Wild rodents constitute a very large biomass of potential reservoirs for *Leishmania* spp. Therefore, an epidemiological study was carried out in a well-known focus of canine leishmaniasis from southern Spain, with the objective of detecting and characterizing *Leishmania infantum* infection in wild rodents. Blood, liver, spleen, bone marrow, and skin from 37 rodents (24 *Apodemus sylvaticus*, 9 *Rattus rattus*, and 4 *Mus musculus*) were analysed by optical microscopy, culture, and two different polymerase chain reactions. *L. infantum* DNA was found in 27 % (10 out of 37) of the trapped rodents, in a variety of tissues: bone marrow, spleen, or healthy skin (ear lobe). High prevalences of *L. infantum* infection were found in the three investigated rodent species. The presence of other trypanosomatids was also evidenced. These rodent species are abundant, widely distributed in Europe, and have a long enough lifespan to overcome the low sandfly activity season. They live in a suitable habitat for sandflies and serve as blood sources for these insects, which can become infected when induced to feed on *Leishmania*-infected animals.

Whether they are reservoirs or just irrelevant incidental hosts, it is clear that the epidemiology of *L. infantum* is more complex than previously thought, and so is its control. The classic epidemiological cycle dog-sandfly-human is turning into a network of animal species that collaborate with the dog in the maintenance of the parasite under natural conditions and probably showing local differences.

Keywords

Leishmania infantum, Wild rodents, Epidemiology, Reservoirs, Incidental hosts

Introduction

In southwestern Europe, leishmaniasis is an endemic zoonosis, and the parasite responsible for all clinical forms, visceral, cutaneous, and mucosal, is *Leishmania infantum*, with the main reservoir encountered so far being the dog (Acedo-Sánchez et al. 1996; Morillas et al. 1996; Fisa et al. 1999; Aliaga et al. 2003; Martín-Sánchez et al. 2004, 2009; Miró et al. 2008; Gálvez et al. 2010; Ballart et al. 2012; Morales-Yuste et al. 2012; Alvar et al. 2012; Antoniou et al. 2013). In addition, *L. infantum* infection has been reported in various host species, both domestic and wild, in some cases with high prevalence rates (Criado-Fornelio et al. 2000; Portús et al. 2002; Di Bella et al. 2003; Fernández-Bellón et al. 2006; Dipineto et al. 2007; Martín-Sánchez et al. 2007; Sobrino et al. 2008). However, until the recent discovery of infected lagomorphs, no studies had provided clear evidence of any significant reservoir other than domestic dogs. Currently, hares and wild rabbits are thought to meet all the conditions to be considered reservoirs of *L. infantum* (Molina et al., 2012; Díaz-Sáez et al., 2014; Jiménez et al., 2014).

Elucidating the role of these animals in the life cycle of *L. infantum*, whether these are common reservoirs or just incidental hosts, is crucial to design control and

prevention strategies for leishmaniasis. Current strategies of early diagnosis and treatment of the dogs together with protection measures against the vector bite are not really correct, as control measures should be taken together against dogs, vectors, and other possible new wild reservoirs in the foci of zoonotic leishmaniasis. Molecular methods provide new opportunities for a better collection and clarification of transmission patterns and are being widely used for the detection of the parasite in wildlife (Millán et al. 2014); however, using them alone or choosing an inappropriate PCR method might not answer the raised questions.

Wild rodents constitute a very large biomass of potential reservoirs for *Leishmania* (Ashford, 1996; Quinnell and Courtenay, 2009). The collection of wild animals for detection of a possible parasitic infection seems to be the first step for reservoir host identification. Therefore, an epidemiological study was carried out in a well-known focus of canine leishmaniasis by *L. infantum* in southern Spain (Martín-Sánchez et al. 2009), with the objective of detecting and characterizing *L. infantum* infection in wild rodents.

Materials and methods

Capture area and rodent captures

Wild rodents were trapped alive, in 2012 in Granada province, southeastern Spain, by using rodent traps placed at burrow entrances. These metallic traps were placed in a variety of biotopes, defined by the presence of slopes drilled with burrows and flow tunnels over brooks, surrounded by blackberry bushes and crops. Prevailing crops in this capture stations, localized at 37° 21′ 13″ N and 3° 19′ 14.4″W with an altitude of 1310 m are cereals and olive trees in rainfed terrain and orchard in irrigation terrain. Vegetation in non-cultivated area was common in Supra-Mediterranean bioclimatic level: broom, hawthorn, blackberry bushes, and other shrubs (rosemary, gorse, thyme, and esparto grass). There are some fruit trees like

cherry tree, apple tree, or walnuts and as wild tree species include poplars, rods, oak, and pine reforestation.

Rodent handling, sacrifice, sampling, and processing for diagnosis

Trapped rodents were sacrificed by introducing the animal in a hood with 75 % carbon dioxide and 25 % oxygen. This method of euthanasia does not produce any stress to the rodent and prevents the onset of apnoea. Rodents were examined for the existence of skin lesions and samples of blood, liver, spleen, bone marrow, and skin (ear lobe and, when available, injured skin lesion) were obtained for parasite detection. All the samples were divided into three parts for microscopy, culture, and PCR. In some animals, samples were not available from all sites for every diagnostic procedure. In these cases, culture and PCR were prioritized over microscopy and the bone marrow was destined exclusively for culture.

Parasitological analysis

A part of each sample was smeared onto a glass slide, fixed with methanol, stained with Giemsa, and examined by microscopy. A second part was inoculated on a combination of Evans' Modified Tobie's Medium solid phase made with rabbit blood and RPMI supplemented with 20 % foetal bovine serum and 5% human urine as the liquid phase. The cultures were incubated at 24 °C and observed every week, at first, and then every 2 weeks, for 4 months, due to the long time required by some *Trypanosoma* spp. to be evidenced. For blood samples, also direct observation of a drop placed between the slide and coverslip was performed to demonstrate the possible existence of *Trypanosoma* spp.

Molecular analysis

DNA from tissues was obtained using the REAL DNA SSS Extraction Kit (RBME01). Each extract was rehydrated in a final volume of 20 µL of sterile water. DNA was

tested for Kinetoplastid infection by internal transcribed spacer 1 (ITS1) PCR and L. infantum PCR-ELISA.

As a totally independent procedure to the DNA extraction from biological samples taken from rodents, DNA was also extracted from *L. infantum* promastigotes (MCAN/ES/91/DP204) and used as reference strain.

ITS1 was amplified with primer pair LITSR/L5.8S and PCR conditions described by Schönian et al. 2003. The amplification products were tested by electrophoresis in 1.5 % agarose gels in 1× TAE-buffer and visualized by staining with ethidium bromide. This technique permits the differentiation between the *Trypanosoma* and *Leishmania* genera according to the size of amplified fragment. Positive ITS PCR products were analysed by sequencing using the same primers as those used for the PCR.

PCR-ELISA is specific to the *L. infantum* species. It was performed following the protocol of Martín-Sánchez et al. (2001) using kits PCR-ELISA DIG Labeling and PCR-ELISA DIG Detection (Roche Diagnostics GmbH. Mannheim, Germany). In the PCR, primers 9 (forward): 5′ CAAAAGTCCCCACCAATCCC-3′ and 83 (reverse): 5′-AAACCCTGGTCTGGAGGCTTAG-3′ amplify a 75 bp fragment belonging to the variable region of the *L. infantum* kDNA minicircle. The amplified fragment will be sprinkled with digoxigenin due to the use of dUTP labeled with this hapten. The amplified fragment was detected on a streptavidin-coated microtiter plate, through hybridization with the oligonucleotide probe specific to *L. infantum* 5′-CCAAACAGGGCAAAAACC-3′, labeled at the 5′ end with biotin, followed by ELISA using a peroxidase-labeled anti-digoxigenin antibody and ABTS as substrate. The results were read in a spectrophotometer at a λ of 405 nm.

Bioinformatics analysis

Sequences were aligned using the multiple alignment program Clustal 1.81 and manually adjusted. Taxonomic analysis was performed using NJ and UPGMA methods of clustering as implemented by the PHYLIP version 3.65. Robustness of

the internal branches was tested by bootstrap analysis from 1000 bootstrap replications using the heuristic search option and retaining groups compatible with the 50 % majority rule consensus tree.

Results

Rodent captured and clinical examination

Between October 2012 and March 2013, 37 animals were captured: 24 *Apodemus sylvaticus* (16 female and 8 male, 8.7–32.6 g weight, 15.2 g average); 9 *Rattus rattus* (6 female and 3 male, 33.7–221.2 g weight, 106.1 g average); and 4 *Mus musculus* (2 female and 2 male, 10.5–18.0 g weight, 13.2 g average).

Physical examination of animals did not indicate the presence of lesions or other cutaneous signs, except for two of them (a female *A. sylvaticus* and a female *R. rattus*), both of them with alopecia on the head.

Parasitological analysis

Observation of fresh blood samples through optical microscopy did not show the presence of trypomastigotes, this result was confirmed later by blood smear examination. Amastigotes were found in the liver of two *R. rattus* and two *A. sylvaticus*. Spleen smears were carried out in only six of the animals, and all of them turned out to be negative, as partially showed in Table 1. A specimen of *A. sylvaticus* was positive for spleen and bone marrow cultures, showing epimastigotes after 7 days of culture (see Table 1, rodent n° 21). *Leishmania* was not isolated in any of the 37 animals.

Molecular analysis

PCR-ELISA confirmed the presence of *L. infantum* in 10 rodents (Table 1): 3 *R. rattus* (detected either in the blood, bone marrow, or ear lobe), 5 *A. sylvaticus* (two in the bone marrow, one in spleen, and two in the ear lobe), and 2 *M. musculus* (one in

the bone marrow and blood, one in the ear lobe). These positive specimens were captured between November and April: three in November, one in January, two in February, three in March, and one in April.

Using PCR-ITS1, a 500-bp fragment was amplified in the liver, spleen, and ear lobe from different *A. sylvaticus* specimens; also, a 450-bp fragment was amplified in bone marrow or ear lobe from two *A. sylvaticus* specimens. A 1000-bp fragment was amplified in the spleen, liver, or bone marrow from 2 *M. musculus* and 11 *A. sylvaticus*.

The 450, 500, and 1000 bp fragments were sequenced. Their sequences were compared with a 300-bp fragment which was amplified from a reference *L. infantum* strain (MCAN/ES/91/DP204). Then, they were compared with ITS1 sequences of a number of Trypanosoma species (GenBank) as well (Fig. 1). Also, a BLAST analysis was performed in order to compare the sequences with those already published in GenBank. The 500-bp fragment turned out to belong to *Trypanosoma* spp. and showed 100 % identity to the ITS1 fragment amplified from epimastigotes isolates from the spleen and bone marrow of the *A. sylvaticus* specimen no. 21 (Table 1). Figure 1 shows, as a tree, the taxonomic relationship between the isolated *Trypanosoma* spp. strain (DP573) and other species belonging to the same genus. On the other hand, the 450- and 1000-bp fragments amplified from 2 to 13 animals (Table 1), respectively, belonged to fragments of chromosomes 9 and 13 from their hosts.

Discussion

L. infantum DNA was found in 27 % (10 out of 37) of the rodents analysed, in a variety of tissues: bone marrow, spleen, or healthy skin (ear lobe). The infection was found in the three investigated species with prevalences of 33.3 % in *R. rattus*, 20.8 % in *A. sylvaticus*, and 50.0 % in *M. musculus*. These prevalence values are very high, particularly taking into account that this is the bioclimatic level with the

lowest endemicity in southern Spain: seroprevalence of canine leishmaniasis in villages was 1.1 % (0 to 7.1 %) and no changes have occurred over two decades (Martín-Sánchez et al. 2009). Sandfly density in areas between villages as the sampling station where rodents were trapped was 46.5 individuals/m2 and specifically in regard to the vector species, values were 4.4 *Phlebotomus perniciosus*/m2 and 0.6 *Phlebotomus ariasi*/ m2 (Barón et al. 2011).

Parasitological methods detected amastigotes in another four animals with low parasite loads (one amastigote per smear); one of them turned out to be *Trypanosoma* spp., as confirmed by culture and sequencing of the ITS1 fragment. *Trypanosoma* spp. infection prevalence in *A. sylvaticus* was estimated in 8.3 % (2 out of 24 animals); this infection was not found in either of the rodent species investigated.

The *Trypanosoma* spp. strain isolated from specimen no. 21 (*A. sylvaticus*, Table 1) is more closely related to *Trypanosoma lewisi* than to any of the *Trypanosoma* species (Fig. 1), including *Trypanosoma grosi*, a species which is usually found in *A. sylvaticus*. Homology found between the new *Trypanosoma* strain and those species is not close enough to be confident that it is included in any of them (93 and 92 % identity with *T. lewisi* and *T. grosi*, respectively). This lack of identity with *T. lewisi* was confirmed by the unsuccessful experimental infection of a laboratory rat (*Rattus norvegicus*) with 46 million epimastigotes intraperitoneally, which showed no parasitemia after 32 days, and what is more, PCR analysis on the liver, spleen, and bone marrow was negative (data not shown).

The trypanosomes of rodents like *T. lewisi, Trypanosoma musculi*, or *T. grosi* divide in the epimastigote stage in the peripheral blood stream. In contrast, other *Trypanosoma* species such as *Trypanosoma microti* and *Trypanosoma nabiasi* divide in the amastigote stage; they are found in the tissues, and they never exhibit dividing forms in the peripheral blood stream (Molyneux 1970). In this case, the parasite was not detected either by microscopy or PCR in blood, conversely, it was

found in other tissues, skin among them. The data suggest that this parasite divides in amastigote stage and show the limitation of parasitological diagnostic methods. Rodents are well known for their role as reservoir of other *Leishmania* species in other zones of the world: *Leishmania major* typically uses rodents as reservoir hosts, additionally, parasites of several *Leishmania* species have been carried in several rodent species, in both the Old World and the New World (Ashford 1996; Desjeux 2001; Di Bella et al. 2003; Svobodová and Votýpka 2003; Mohebali et al. 2004; Oliveira et al. 2005; Motazedian et al. 2010).

To date, in Mediterranean Europe, amastigotes, parasite DNA, or antibodies have been detected in *R. rattus, R. norvegicus, M. musculus, Mus spretus, Glis glis, Elyomis quercinus*, and *A. sylvaticus* (Rioux et al., 1968; Gradoni et al., 1983; Morillas-Márquez et al., 1985; Portús et al., 2002; Di Bella et al., 2003; Papadogiannakis et al., 2010; Psaroulaki et al., 2010; Helhazar et al., 2013; Millán et al., 2014). In addition to the selection of a suitable focus, the selection of appropriate biological tissues and sensitive diagnostic techniques is also important for the successful detection of pathogens in wildlife. In this case, five biological samples were analysed by optical microscopy, culture, and two different PCR's. According to Svobodová and Votýpka (2003), ear tissue is the most suitable tissue sample for *Leishmania* diagnosis in rodents, and its analysis was included in this work.

Regarding serological methods, it is crucial to take into account that the presence of other trypanosomatids has previously been proved to lead to cross-reactions, as reported by this research group in a previous work (Díaz-Sáez et al. 2014), reason why these methods were not carried out. The PCR technique performed in this type of study must be contrasted in other trypanosomatids, and the use of a *L. infantum*-specific PCR is strongly advisable, as a *L. infantum* infection cannot be identified with genus-specific PCR. The amplification of unspecific fragments, belonging to the host in this case, must be highlighted, given the wide use in the

diagnosis of leishmaniasis of the ITS1 fragment, stressing the necessity of confirmation by sequencing in the light of these results.

Five *L. infantum* strains have been isolated from *R. rattus* in Italy where the prevalence was 1 to 2 % of infected rats (Gramiccia et al. 1982; Gradoni et al. 1983) and southern Spain—unknown prevalence (Morillas-Márquez et al. 1985). In contrast, despite the detection of low levels on anti-*Leishmania* antibodies in eight rodents (0/15 *R. rattus*, 7/69 *M. spretus*, 0/9 *M. musculus* and 1/28 *A. sylvaticus*), Portús et al. (2002) did not detect any parasitized animal in northeastern Spain. Similar results were also obtained in studies performed in the Cèvennes focus of leishmaniosis in France (Rioux and Golvan 1968). Culture methods do not yield good results. In general, when two or more diagnostic techniques were used, their results are not in accordance, for example, serology and PCR of the spleen (Di Bella et al. 2003), or microscopy and PCR of the liver or spleen (Helhazar et al. 2013). This disagreement might partially be due to the presence of other trypanosomatids, as reported by this group and other authors (Rioux et al. 1968; Díaz-Sáez et al. 2014).

Studies carried out in Italy and Portugal showed that 45.0 % of *R. rattus* and 33.3 % of *M. musculus* were positive to *Leishmania* by PCR (Di Bella et al. 2003; Helhazar et al. 2013), similar values to these found in this work. Moreover, Zanet et al. (2014) have recently shown that on the island of Montecristo (Italy) where no dogs or other carnivores are present, up to 15.5 % of black rats are infected by *L. infantum*. However, those authors did not take into account the presence in that island of wild rabbits, which have recently been identified as reservoirs of the parasite (Díaz-Sáez et al. 2014; Jiménez et al. 2014); in any way, this fact clearly reveals the maintenance of the parasite in the absence of the dog.

Rioux et al. (1968) experimentally infected several species of wild rodents, and the infection was successfully evidenced by parasitological methods (smear and culture) in *A. sylvaticus, R. norvegicus, Glis glis*, and *Eliomys quercinus. R. rattus* experimentally infected with *L. infantum* by Gradoni et al. (1983) displayed a very

low number of amastigotes which persisted in the animal for a long period. According to these authors, *P. perniciosus*, the most important vector of *L. infantum*, was induced to feed on infected rat, became infected, and is readily attracted to, and fed on, the black rat. Data about *M. musculus* is not available; nevertheless, the use of Balb/c mice as an experimental laboratory model for leishmaniasis must be taken into account. Furthermore, Svobodová and Votýpka (2003) demonstrate the transmission of *Leishmania tropica* to mice by the bite of *Phlebotomus sergenti*.

These three rodent species, ranging from a very small size (*M. musculus*) to big size (*R. rattus*) are extremely prolific animals (Burton 1978). *A. sylvaticus* is one of the most abundant rodents in Spain, with densities in Europe from 1 to 50 animals per hectare, which would justify its predominant capture in our study. Moreover, (a) these rodents are widely distributed in Europe, being even cosmopolitan; (b) they dig burrows and they also adapt to holes in trees, which establish a suitable habitat for sandflies (Díaz-Sáez et al. 2014) and they certainly serve as one of the blood sources for sandfly populations: based on the results of blood meal analyses, it is known that *P. perniciosus* is an opportunist that feeds on those animals to which it has easiest access (De Colmenares et al. 1995; Bongiorno et al. 2003; Rossi et al. 2008) and a significant number of *P. perniciosus* was found engorged with rodent blood (27/31) by Maia et al. (2013); (c) although they have an average lifespan of between 12 (*M. musculus*) and 18 months (*A. sylvaticus* and *R. rattus*), some of them can live up to several years; in any case, this life expectancy is long enough to maintain the parasite availability at least for 1 year, even during low sandfly activity season, as showed by the fact of capturing these 10 infected specimens during this time of the year.

Incidental hosts, defined as hosts that are capable of becoming infected but that are not required for disease maintenance, are most often considered irrelevant to the long-term persistence of disease. Current molecular techniques are so sensitive that detecting a PCR-positive individual does not necessarily mean that it is

involved in *Leishmania* transmission. However, rodent species such as *R. rattus, A. sylvaticus*, and possibly *M. musculus* seem to display consistent characteristics expected for a reservoir. It is clear that the epidemiology of *L. infantum* is more complex than previously thought, and so is its control. The classic epidemiological cycle dog-sandfly-human is turning into a network of animal species that collaborate with the dog in the maintenance of the parasite under natural conditions and probably showing local differences.

Conclusion

The results of this research, analysed in the context of the contributions from other authors and eco-biological data of rodents, support its role as wild reservoirs in zoonotic leishmaniasis foci. Nevertheless, more research is necessary to definitely confirm this fact.

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Rodent species and number of specimens	Rodent nº	PCR analysis: ITS/kDNA					Parasitological analysis			
		Blood	Liver	Spleen	Bone marrow	Ear lobe	Blood G/S	Liver S/C	Spleen S/C	CBone marrow
Rattus rattus *N*= 9	*1*	N/N	N/N	N/N	N/nd	N/nd	N/N	*P*/nd	N/nd	nd
	2	N/N	N/N	N/N	N/N	N/N	N/N	*P*/N	nd/N	N
	3	N/N	N/N	N/N	N/P	N/N	N/N	N/N	N/N	N
	5	N/P	N/N	N/N	N/nd	N/N	N/N	N/N	nd/N	N
	7	nd/nd	N/N	N/N	N/N	N/P	N/N	N/N	nd/N	nd
Apodemus sylvaticus *N*=24	13	N/N	N/N	N/N	450/N	N/N	N/N	N/N	nd/N	N
	15	nd/nd	N/N	N/N	1000/N	N/N	N/N	nd/N	nd/N	N
	16	nd/nd	N/N	N/N	N/N	N/N	N/N	*P*/N	nd/N	N
	18	1000/N	N/N	1000/N	N/P	N/N	N/N	N/N	nd/N	N
	20	1000/N	1000/N	N/N	N/N	N/N	N/N	N/N	nd/N	N
	21	nd/nd	500/N	500/N	N/N	N/N	nd/nd	*P*/N	nd/P[a]	P[a]
	22	nd/nd	N/N	N/P	N/N	N/N	nd/nd	N/N	nd/N	N
	23	nd/nd	1000/N	N/N	N/N	N/P	nd/nd	N/N	nd/N	N
	24	N/N	N/N	1000/N	N/N	N/N	N/N	N/N	nd/N	N
	25	N/N	N/N	1000/N	N/N	N/N	N/N	N/N	nd/N	nd
	26	nd/nd	N/N	N/N	N/N	N/P	N/N	N/N	nd/N	N
	27	1000/N	N/N	N/N	N/nd	N/N	N/N	N/N	nd/N	nd
	28	N/N	N/N	1000/N	N/N	N/N	N/N	N/N	nd/N	N
	29	N/N	N/N	1000/N	N/nd	500/N	N/N	N/N	nd/N	N
	30	nd/nd	N/N	1000/N	N/N	450/N	N/N	N/N	nd/N	N
	33	nd/nd	1000/N	1000/N	N/P	N/nd	N/N	N/N	N/N	N
Mus musculus *N*=4	34	1000/P	1000/N	N/N	1000/P	N/N	N/N	N/N	nd /N	N
	35	N/N	N/N	1000/N	1000/N	N/P	N/N	N/N	nd /N	N

Figure 1. Results obtained from molecular and parasitological analysis. Only rodents with some positive results with any of the techniques are shown. *Leishmania infantum*-infected rodents are shown in light shading. *Trypanosoma* infected rodents are shown in dark shading. The presence of amastigotes is highlighted in italics. *S*=smear, *C*=culture, *P*=positive result, *N*=negative result, *nd*=not done, *ITS*=amplification of ITS fragment, *1000*=1000 bp ITS fragment, *500*=500 bp ITS fragment, *450*=450 bp ITS fragment, *kDNA*=amplification of a specific kDNA *L. infantum* fragment. [a]Epimastigotes were observed. 450 and 1000-bp fragments belong to rodent host.

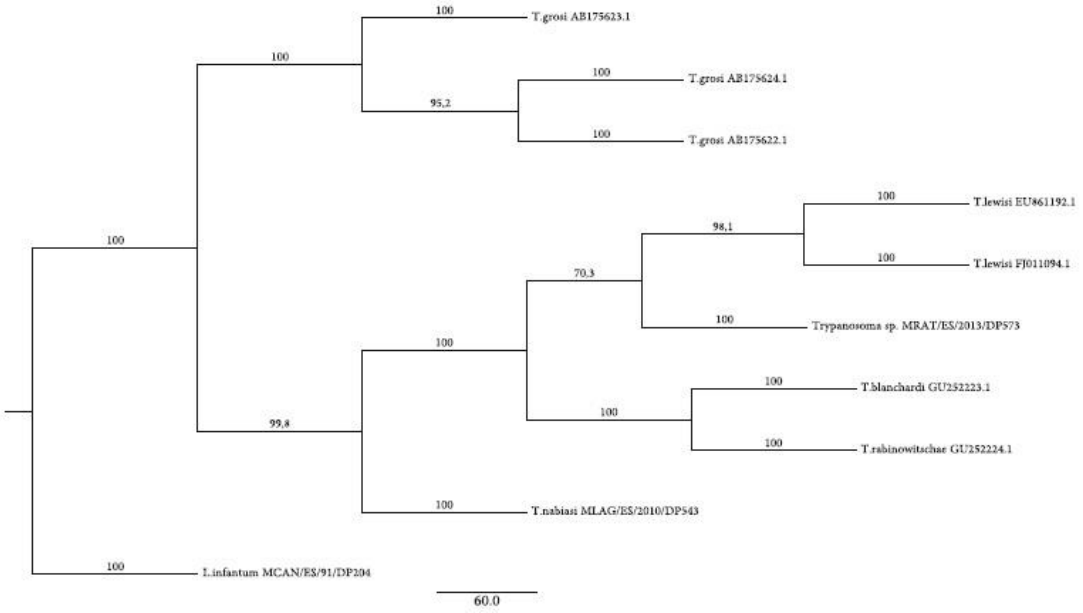

Figure 2. UPGMA tree based on DNA ITS1 sequences. *Leishmania infantum* is used as outgroup. The numbers above the branches are bootstrap percentages (1000 replications) for branches supported above the 50 % level.

Genetic variability and infective ability of the rabbit trypanosome, *Trypanosoma nabiasi* Railliet 1895, in southern Spain

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Abstract

Trypanosomes are widespread haemoflagellate protozoans, commonly found in all groups of vertebrates and usually transmitted by arthropods. Non-pathogenic species are those that cause little or no apparent negative effects in the host and it is accepted that *Trypanosoma nabiasi* is the species that infects the domestic and wild rabbit, *Oryctolagus cuniculus*. Knowledge about genetic variability, *in vitro* cultivation and infectivity of this parasite is very scarce, so the aim of this study was to provide an insight on them. The parasite was detected in all the type of samples of 121 wild rabbits. Epimastigotes were visualized and isolated from all the organ cultures types except from skin, and twenty-six strains were isolated and grown in mass. Epimastigote infectivity was assessed *in vitro* and *in vivo*. Amastigotes were obtained in infected macrophages from cultured epimastigotes. Furthermore, trypomastigotes were found in the peripheral

bloodstream of an experimentally infected naïve domestic rabbit with cultured epimastigotes at the fourth day after infection. The rising titre of antibodies led to the disappearance of the parasite from blood. In addition, this study reports the existence of two *T. nabiasi* genetic lineages in southern Spain. Phylogenetic analysis places *T. nabiasi* in the same clade as *T. lewisi* and other rodent trypanosomes of the subgenus *Herpetosoma*.

1. Introduction

Trypanosomes are widespread haemoflagellate protozoans (Kinetoplastida), commonly found in all groups of vertebrates (Hoare, 1972) and usually transmitted by arthropods. Some species are the causative agents of diseases in humans and livestock (Barrett et al., 2003; Hamill et al., 2013) and the non-pathogenic species cause little or no apparent negative effects in the host (Mansfield, 1977; Thompson et al., 2014).

Although the last group nowadays includes at least 45 morphologically indistinguishable species, it is accepted that *Trypanosoma nabiasi* is the only one that infects the domestic and wild rabbit, *Oryctolagus cuniculus* (Hamilton et al., 2005; Molyneux, 1970; Reglero et al., 2007). Jolyet and Nabias (1891) were the first to discover a trypanosome in the blood of a rabbit and Railliet (1895) named it *T. nabiasi*.

Trypanosomes of mammals are divided into two biological groups according to their developmental pattern in the vector and the transmission mode to their host. The salivarian trypanosomes are developed in the mid-gut of an insect vector and, following migration to salivarian glands or proboscis, they are injected directly into a host during feeding (Brun et al., 1998; Hoare, 1972). The stercorarian trypanosomes are developed in the hind gut of an invertebrate vector and they are deposited in faeces during feeding. *T. nabiasi* belongs to the last group and its

vector is the rabbit flea, *Spilopsyllus cuniculi*, (Hoare, 1972); rabbits ingest fleas and flea faeces when grooming, as they use the mouth and tongue to clean their toes after scratching (Channon and Wright, 1927; Hamilton et al., 2005). Some non-pathogenic trypanosomes have strong host specificity, usually being infective to only one host species, rarely involving new hosts in their life cycle (Hoare, 1972; Smith et al., 2005). Experimental evidence suggests that *T. nabiasi* is restricted to lagomorphs whereas laboratory rodents, such as guinea pigs, rats or mice, are not susceptible to infection (Channon and Wright, 1927; Grewal, 1957; Petrie, 1905). Vertical transmission between generations of rabbits is not believed to occur (Hamilton et al., 2005; Mansfield, 1977; Molyneux, 1970; Reglero et al., 2007).

Trypanosoma nabiasi has been reported in *O. cuniculus* from various European countries, such us France, Italy, Portugal, UK and Spain (Grewal, 1957; Reglero et al., 2007); and also in Australia (Hamilton et al., 2005). Rabbits are a key species in the Mediterranean shrub lands of Spain, Portugal and southern France and their conservation and restocking in natural habitats is a primary management objective as a target prey for medium-sized predators of Spanish Mediterranean habitats, particularly the Iberian Lynx (*Lynx pardinus*) and the Iberian Imperial Eagle (*Aquila adalberti*) (Delibes-Mateos et al., 2007; Moreno et al., 2004). However, in Australia and New Zealand, rabbits are considered a pest and they are vigorously suppressed (Cooke, 2008; Hamilton et al., 2005; Thompson et al., 2014).

Nevertheless, this is not the only trypanosomatid described in lagomorphs. In endemic areas of leishmaniasis, rabbits and hares are often infected by *Leishmania infantum* and play a role as reservoirs (Díaz-Sáez et al., 2014; Jiménez et al., 2014). Moreover, when *Trypanosoma* and *Leishmania* species are present in the same geographical area, mixed infections can appear within the same host (Bastrenta et al., 2003; De Araújo et al., 2013; Díaz-Sáez et al., 2014), what causes problems in their diagnosis.

Although this parasite is worldwide distributed and high infection rates have been reported across the world, knowledge about *T. nabiasi* is very scarce.

Additionally, few authors have succeeded in maintaining *T. nabiasi* in culture (Channon and Wright, 1927; Grewal, 1957; Mohamed and Molyneux, 1987; Petrie, 1905). Therefore, the aim of this study was to provide an insight into the genetic variability of this parasite, its *in vitro* cultivation and infectivity, and finally investigate the course of infection in its host (*O. cuniculus*).

2. Materials and Methods

2.1. Specimens capture and biological sample collection

One hundred and forty two wild rabbits (*O. cuniculus*) were either captured alive using ferrets or shot by hunters in a private farm in the province of Granada (southern Spain).

2.2. Culture media

Evans' modified Tobie's medium (EMTM) solid phase was made with 10% anticoagulated rabbit blood and added to slant tubes and culture flasks. RPMI-1640 was purchased from Sigma (Madrid, Spain), prepared according to the manufacturer's instructions and supplemented with 2 g/L $NaHCO_3$, 10 to 20% Foetal Bovine Serum (FBS) and 0 to 5% human urine. A modified EMTM medium was employed using EMTM solid phase and RPMI-1640 supplemented with 20% FBS and 5% human urine as the liquid phase.

2.3. Parasitological analysis

For the detection of trypomastigotes, direct observation of a drop of blood placed between slide and coverslip was performed. Additionally, Giemsa-stained blood smears were made in order to detect parasite. An average of 100 fields were observed.

Cultures were carried out using peripheral blood, bone marrow samples, and liver, spleen, heart and skin macerates in modified EMTM medium. The primary

cultures were passaged at day 7 and then every 15 days. They were kept at 24 ºC for 6 months before they were rejected as negative.

2.4. In vitro infectivity of T. nabiasi

L929 fibroblasts (ECACC: 85011425) were purchased from the Cell Bank of University of Granada (Granada, Spain). They were grown in RPMI-1640 medium supplemented with 10% FBS and 200 U/mL penicillin at 37 °C in 5% CO_2. These cells were used for obtaining L929 Cell Conditioned Medium (LCCM). Bone Marrow-Derived Macrophages (BMDM) were obtained as undifferentiated bone marrow cells from bone marrow of healthy ICR (CD-1) mice. They were differentiated into macrophages, according to Zamboni and Rabinovitch (2003), by culturing in RPMI-1640 supplemented with 20% FBS, 30% LCCM and 200U/L penicillin at 37 °C 5% CO_2. For *in vitro* experiments, macrophages were cultured in RPMI-1640 supplemented with 20% FBS, 5% LCCM and 200 U/L penicillin at 37 °C 5% CO_2.

BMDM were used as an infection model (Corpas-López et al., 2015; Zauli-Nascimento et al., 2010). They were counted and distributed (4×10^5 cells/well) in 24-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with round coverslips. Macrophages were left a day to allow them to stick to the coverslips. Then, they were infected with *T. nabiasi* epimastigotes from a stationary-phase culture in a 10:1 parasite:macrophage ratio for 4, 24 and 72 h, then washed to remove non-interiorized parasites and incubated at 37 ºC 5% CO_2 up to 72 h. The coverslips with macrophages were washed, fixed with methanol, and stained with Giemsa. The infection level was quantified by optical microscopy. Macrophages were considered infected when at least one amastigote was observed inside.

2.5. In vivo experimental infection

A two-month-old naïve domestic rabbit was inoculated through intraperitoneal route with 2.6 x 10^7 stationary-phase epimastigotes in a final volume of 5 mL of physiological saline solution (0.9% NaCl). The rabbit was examined periodically to determine the presence of trypanosomes through direct observation of a drop of blood placed between slide and coverslip and Giemsa-stained blood smears. For this purpose, a 26G needle was used to draw the blood from the ear vein rabbit. In addition, blood samples were taken in order to perform antibody detection by IFAT. At day 99 post-infection, euthanasia was performed by concussion. Macerates of spleen, liver, bone marrow and peripheral blood were inoculated in modified EMTM medium and they were examined under the same conditions previously indicated at 2.3. A portion of liver, spleen and peripheral blood was saved for PCR.

2.6. Immunofluorescence antibody test (IFAT)

Antibodies specific to *T. nabiasi* in the rabbit sera were measured by IFAT against a suspension of 2×10^6 acetone-fixed *T. nabiasi* epimastigotes. The antibody titre was simultaneously determined in the rabbit serum at each time point. Geometric dilutions of the sera were used with a starting dilution of 1/20, as previously reported (Acedo Sánchez et al., 1996). Goat anti-rabbit IgG (ICN Biomedical, Ohio, United States) was used at a concentration of 1/100 in Evans Blue previously diluted to $1/10^4$ with phosphate buffered saline.

2.7. DNA extraction for molecular characterization

A commercially available kit was used (DNA SSS Extraction Kit: REAL Durviz S. L., Valencia, Spain), according to the manufacturer's instructions. The DNA was resuspended in 20 µL of bidistilled water and kept at -20 °C until use.

2.8. Molecular identification of T. nabiasi

2.8.1. 18S and ITS-1 rDNA PCR

The small ribosomal subunit 18S rDNA was amplified as described previously by Maslov et al. (1996) and Hamilton et al. (2005). The Internal Transcribed Spacer 1 (ITS-1) was amplified according to Schönian et al. (2003). For both PCR various negative controls were used: i) tube of PCR reagents without DNA, ii) extraction controls, iii) DNA from an uninfected domestic rabbit. Amplicons were resolved in ethidium bromide-stained (1.5%) agarose gels and their sizes determined by comparison with HyperLadder IV (Bioline, London, UK).

The ITS-1 PCR permits the differentiation between the *Trypanosoma* and *Leishmania* genera according to the size of amplified fragment: 500 bp for *Trypanosoma* spp. and 300-350 bp for *Leishmania* spp. (Díaz-Sáez et al., 2014).

2.8.2. DNA sequencing

Amplified PCR products were eluted from agarose gel using Real Clean Spin kit (REAL Durviz S. L., Valencia, Spain), according to the manufacturer's protocol. Direct cycle sequencing of the PCR product was performed in both directions by an automated sequencer (3130XL from Applied Biosystems) using the primers used for DNA amplification.

18S and ITS-1 sequences were aligned using the multiple alignment program CLUSTALX 1.81 (Thompson et al., 1997) and manually adjusted, and they were subjected to BLAST analysis to find the most similar sequences in the GenBank database. Phylogenetic analysis was performed using PHYLIP version 3.65 (Felsenstein, 1989) (http://evolution.genetics.washington.edu/phylip) with three different methods: maximum likelihood (ML), maximum parsimony (MP) and distance matrix analysis; for the latter we used the F84 model of nucleotide substitution (the default method) with both neighbor-joining (NJ) and unweighted pairgroup method using an arithmetic average (UPGMA) method of clustering. The F84 model incorporates different rates of transition and transversion, and different

frequencies of the four nucleotides. We carried out MP analysis using the DNAPARS program, which searches for the most parsimonious tree via a heuristic algorithm; in this analysis, codon positions were unweighted and transitions and transversions are also equally weighted. For ML analysis the model was chosen with jModelTest version 2.1.8 (Darriba et al., 2012): GTR + I + G with empirical nucleotide base frequencies; a four-category gamma distribution was used. Robustness of the internal branches was tested by bootstrap analysis from 1000 bootstrap replications using the heuristic search option and retaining groups compatible with the 50% majority rule consensus tree. For the 18S phylogenetic analysis, the sequences analysed by Hamilton et al. (2005) were included in the present study.

2.9. Ethical Issues

All experiments were approved by the Ethics Committee of Animal Experimentation of the University of Granada, in accordance with European Parliament and of the Council of 22 September 2010 (2010/63/UE and RD 1201/2005).

3. Results

3.1. Detection of the parasite in rabbit samples

Trypanosoma spp. was detected using different techniques in peripheral blood, liver, spleen, heart, bone marrow and skin of 121 wild rabbits. Giemsa-stained blood films showed trypanosomes with a prominent kinetoplast, pointed posterior end and a long free flagellum (Fig. 1a).

Epimastigotes were visualized and isolated from all the organ cultures types except from skin, which were contaminated with fungi. The percentage of positive cultures were 21.2% in spleen (7/33), 27.3% in liver (9/33), 21.1% in heart (4/19), 10.5% in blood (2/19) and 30.3% in bone marrow (10/33). The overall percentage

was 51.5% (17/33). The culture lag phase varied from 7 to 97 days. Although the most frequent form was the epimastigotes stage (Fig. 1b), amastigote-like forms, spheromastigotes and trypomastigotes were also observed sporadically. Twenty-six strains were isolated and grown in mass from 16 rabbits.

3.2. In vitro infectivity of T. nabiasi

Trypanosoma nabiasi amastigote-like forms were found inside BMMs after different infection times (4, 24 and 72h). These amastigote-like forms had a single nucleus and a single kinetoplast (Fig. 1c). Mean infection rates and 95% confidence intervals (CI) were 8.02±1.8 [CI= 6.14-9.90], 38.84±9.7 [CI= 28.76-48.92] and 44.08±10.6% [CI= 32.96-55.21] for the different infection times (4, 24 and 72h, respectively). In addition, trypomastigote-like forms were also found in these preparations showing its typical morphology as described above.

3.3. In vivo experimental infection

Trypanosomes were first detected in the rabbit blood on the fourth day after inoculation. The number of trypanosomes per field increased steadily until reaching a maximum by the fifteenth day, when the parasite load accounted for an average of 1.9 trypomastigotes/field (Fig. 2). Then, there was an abrupt fall in parasitemia, which was absent until day 43, when a single trypomastigote was found in a smear. No parasites were found afterwards (day 50 to day 99). The antibody titre followed the same trend, since the evolution of parasitemia reached a maximum by the fifteenth day (IFAT titre = 160). This value remained for 20 days, diminishing at the following analysis (day 43, IFAT titre = 80), when the parasite was detected in blood for the last time. The antibody titre remained at 80 until the end of the experiment.

The experimentally infected rabbit gained weight and was very active during the course of the experiment. However, on day 15 post-infection an intense leukocytosis was observed, but it disappeared afterwards.

After euthanasia, none of the spleen, liver, bone marrow and peripheral blood cultures were positive after a 6-month follow-up. The samples of liver, spleen and peripheral blood were negative for ITS-1 PCR.

3.4. Molecular identification of T. nabiasi

A 560 bp fragment was amplified through 18S rDNA PCR from all the *Trypanosoma* strains isolated in culture. Sequences were obtained and compared with *Trypanosoma nabiasi* species (AJ843896 from UK and AJ620568 from Australia) and other *Trypanosoma* species from GenBank. All trypanosome sequences from Spanish rabbits, UK fleas, Australian rabbits and fleas were identical.

A 500 bp fragment was amplified through ITS-1 PCR in 96.7% of the *Trypanosoma* infected wild rabbits (117/121) and in 73.3% of *Trypanosoma* strains isolated from cultures (11/15). Another 480 bp fragment was amplified in 3.3% of infected animals (4/121) and 26.7% of parasites from culture (4/15). Sequences were obtained from two ITS 1 amplified fragments from 2 wild rabbits and 15 cultured *Trypanosoma* strains and they were compared with those of other *Trypanosoma* species recorded in GenBank. No published record of the *T. nabiasi* ITS-1 fragment sequence was found.

A comparative analysis of the *T. nabiasi* sequences revealed the presence of 4 haplotypes from southern Spain: Nab-A, Nab-B, Nab-C and Nab-D (Genbank accession number LT575228 to LT575231 respectively). Among Nab-A, Nab-B and Nab-C haplotypes, all of them 500 bp long, 3 different bases were found; whereas between them and Nab-D (480 bp) 31 differences were detected: 13 substitutions and 18 deletions (Table 1). Twelve 500-bp sequences belong to Lineage I: 66,7% (8) were identified as Nab-A, 16,7% (2) were identified as Nab-B and 16,7% (2) as Nab-C haplotypes. All 480-bp sequences (5) belong to Lineage II Nab-D haplotype

(100%). A BLAST search with the four sequences revealed similarity with *T. otospermohili* (87-89%) and *T. kuseli* (87-88%).

Topologies of the 18S and ITS-1 phylogenetic trees, obtained by ML and MP analysis and phenetic trees, generated by NJ or UPGMA, turned out to be similar. *Trypanosoma nabiasi* is included in the same clade as *T. lewisi* (bootstrap support: 74% ML, 48% MP, 100% UPGMA from ITS-1 trees) (Fig. 3b) and it is included in a subclade with those trypanosomes whose division stage is the amastigote in both 18S and ITS-1 trees (Fig. 3). The Spanish rabbit trypanosome fell in the same branch that the Australian and UK rabbit trypanosomes in 18S trees (bootstrap support: 99% ML, 96% MP, 99% UPGMA) (Fig. 3a). Additionally, haplotypes Nab-A, Nab-B and Nab-C are closely related among them, constituting the Lineage I, which is well separated from haplotype Nab-D, which integrates the Lineage II (bootstrap support: 98% ML, 63% MP, 100% UPGMA) (Fig. 3b).

4. Discussion

The prevalence of *T. nabiasi* infection in wild rabbits from southern Spain is very high, with values higher than 80% (Díaz-Sáez et al., 2014) and higher than the values found by other authors (Reglero et al., 2007). In the present study, epimastigotes of this trypanosomatid were detected in all rabbit organ cultures analysed except for skin, showing a lag phase of up to 97 days. Mass culture of the parasite was achieved, allowing for the demonstration of the transformation of epimastigotes to amastigotes in the *in vitro* infections and the transformation of epimastigotes into trypomastigotes in the *in vivo* experiment. The ITS-1 PCR proved useful to detect *T. nabiasi* in all types of rabbit samples, including skin. This study was complemented with sequence analysis, which allowed for the identification of two genetic lineages, with 3 and 1 haplotypes, respectively.

There have been reported several cases of infections due to non-pathogenic *Trypanosoma* species in humans, such as *T. lewisi*-like trypanosomes (Sarataphan

et al., 2007; Vanhollebeke et al., 2006; Verma et al., 2011). These parasites are not usually infectious to humans because they undergo immediate lysis due to the trypanolytic activity of the human-specific apolipoprotein L-1 (ApoL1). ApoL1 is naturally present in the human blood and it is not a response of the triggered immune system by infection. Vanhollebeke et al. (2006) showed that the human infection with *T. evansi* in India was linked to the lack of the trypanolytic ApoL1 protein in the patient's serum.

Nevertheless, evidence for the pathogenicity of *T. nabiasi* is not clear. According to Jolyet and Nabias (1891), the health of the animal does not seem to be affected by the presence of this trypanosome, even though it is present in peripheral blood showing high parasitemia. Petrie (1905) found that blood infected with *T. nabiasi* was harmful, killing two out of of ten rabbits inoculated, while Grewal (1957) did not detect pathogenic effects in 24 experimentally infected laboratory rabbits and two naturally infected wild rabbits. An external visual inspection of all the rabbits captured in this study did not reveal the presence of clinical symptoms in any of the specimens. The infected naïve rabbit did not show any clinical symptom either, except for the transitory leucocytosis episode on day 15 post-infection.

Little is known about the identification and distribution of *T. nabiasi* in Spanish rabbit populations. During a restocking program in southwestern Spain, Reglero et al., (2007), found a *Trypanosoma* spp. prevalence of 23.81% in young rabbits, 84.85% in juveniles and 47.37% in adults, being higher in females. Díaz-Sáez et al., (2014) found no differences in prevalence between males and females and young/juvenile rabbits showed a risk 8 times higher of becoming infected than adults. Effective self-cure of infection and a role for acquired immunity has been suggested to explain this finding. Kroó (1936) has pointed out that rabbits remain immune to re-infection after recovering from the infection. Grewal (1957) inoculated twice three rabbits that had overcome a prior infection to assess whether they could be re-infected, but they remained immune to further infection.

A negative association between antibody titres and *T. nabiasi* presence was reported by Díaz-Sáez et al. (2014). In our experimentally infected rabbit, the rising titre of antibodies led to the disappearance of the parasite from blood, suggesting a protective role of these antibodies. In the present study, IgG antibody titres were evaluated throughout the experimental infection, detecting a rise in IgG titre at day 4 post-infection and reaching the peak at day 15 post-infection. Although unusual, a recent study has described an early increase in IgG titre after the infection of cows with *T. vivax* (Uzcanga et al., 2016), similar to the rise of IgM titre that was observed at day 7 post-infection as well.

Giemsa-stained blood films showed trypanosomes with a prominent kinetoplast, pointed posterior end and a long free flagellum, similar to the description of the rabbit trypanosome, *T. nabiasi* (Grewal, 1957; Hamilton et al., 2005; Mansfield, 1977; Molyneux, 1970; Reglero et al., 2007).

Trypanosoma nabiasi is the most challenging *Trypanosoma* to grow *in vitro*. Petrie (1905) cultured it only for 16 days in rabbit blood-agar medium, during which he detected some dividing forms that disappeared quickly. Channon & Wright (1927) failed to cultivate this trypanosome in NNN medium at room temperature in the dark. Grewal (1957) tried unsuccessfully to cultivate blood in NNN and Hartley's Broth Digest medium. Mohamed and Molyneux (1987) did not achieve to grow *T. nabiasi* in the following media: Schneider's Drosophila, Mitsuhashi and Maramorosch, and RPMI 1640. They were only able to obtain a very slight growth in Grace's insect tissue culture. According to these authors, when cultures are maintained at 25-27 ºC, trypanosomes undergo transformation into epimastigote forms that are indistinguishable from those in the gut of their vector fleas. In our case, we were able to visualize and isolate epimastigotes in our combination of EMTM and supplemented RPMI 1640 medium from all the organ cultures types except for skin due to fungal contamination. Cultures were maintained at 24ºC for extended periods up to 6 months. In addition, twenty six strains were isolated and grown in mass from 16 rabbits. Although the most

frequent form was epimastigotes, amastigote-like forms, spheromastigotes and trypomastigotes were sporadically observed as well.

Amastigotes-like forms were obtained after the infection of BMDM with epimastigotes from culture. Trypomastigote-like forms were also found *in vitro* as a result of the transformation of intracellular amastigotes. These amastigotes had a single nucleus and a single kinetoplast. Some authors have also found amastigotes with 4 nuclei and 4 kinetoplasts (Grewal, 1957) and even larger forms with 8 nuclei and 8 kinetoplasts (Molyneux, 1969).

An experimental infection of a rabbit with culture-derived epimastigotes was accomplished as well. We were able to detect trypomastigotes in peripheral blood since the fourth day post-infection, in agreement with the incubation period of 7 days obtained by Kroó (1936) and 5-12 days obtained by Grewal (1957). The number of trypanosomes reached a maximum by the fifteenth day, after which a steady fall in parasitemia began. The parasitemia ended on the fiftieth day after epimastigote inoculation, with the disappearance of all trypanosomes from blood. At day 99 post-infection, the rabbit was sacrificed and none of the samples were positive with any of the techniques, which demonstrated the disappearance of the infection. The duration of infection was within the ranges of 52-213 days described by Kroó (1936) and 73-136 days obtained by Grewal (1957).

According to Grewal (1957), after the inoculation of metacyclic trypanosomes from infected fleas or blood forms, *T. nabiasi* amastigotes multiply by binary fission in the spleen capillaries. This author reported the highest parasitemia on days 6-10, similar to the parasitemia peak found on day 15 in the present study, gradually decreasing afterwards. These transform into trypomastigote forms, which are shed into the peripheral blood, where they change into the typical adult trypomastigotes. Our results support these data and indicate that the formation and multiplication of intracellular amastigotes seems to occur in greater variety of organs than previously reported.

Several authors have tried to infect laboratory rabbits through the inoculation of blood from infected wild rabbits (Mohamed and Molyneux, 1987; Petrie, 1905; Watson and Hadwen, 1912) but only Grewal (1957) succeeded. As reported by Channon and Wright (1927) and Mohamed and Molyneux (1987), infection with macerated fleas was more reliable. In addition, Channon & Wright (1927) were only able to infect rabbits by intravenous inoculation. Grewal (1957) was able to produce infection using trypomastigotes through intraperitoneal route. In the present study, an infection was established using a higher infective dose (2.6×10^7 epimastigotes), which was possible due to the successful mass culture.

Aiming at resolving the relationship between Spanish isolates of rabbits and published *T. nabiasi* sequences, we carried out comparative analyses of the 18S and ITS1 rDNA fragments from Trypanosomes of the subgenus *Herpetosoma* (Hamilton et al., 2005; Maia da Silva et al., 2010; Votýpka et al., 2015). The topologies of the 18S trees are similar to that obtained by Hamilton et al. (2005) and Maia da Silva et al. (2010), both using SSU rDNA sequences (Fig. 3a). This allowed us to confirm the identification of *T. nabiasi* in the rabbits of southern Spain. The topologies of ITS-1 trees support these findings. Additionally, the differences detected between the obtained sequences of 500 and 480 bp from ITS-1 fragment appear to indicate the existence of two genetic lineages: Lineage I, which is the best represented, with Nab-A haplotype being the most frequent followed by Nab-B and Nab-C haplotypes, and Lineage II with the Nab-D haplotype (Fig. 3b).

In the resulting 18S and ITS-1 phylogenetic trees, the rabbit trypanosomes fell in the same clade of *T. lewisi* (Fig. 3). All trypanosomes included in this clade are from mammals and the only known vectors are fleas. Furthermore, this clade is also divided into two subclades: subclade 1 contains trypanosomes from two rodent families, such as Muridae (*T. blanchardi, T. grosi*, and *T. lewisi*) and Gliridae (*T. rabinowitschae*); while subclade 2 contains *T. nabiasi*, and two trypanosomes from squirrels: *T. otospermohili* and *T. kuseli* (Fig. 3), which are similar to those

obtained by Hamilton et al. (2005). This division is in agreement with development in the vertebrate host: all trypanosomes from subclade 1 divide as epimastigotes in the bloodstream while those from subclade 2 divide as amastigotes (Grewal, 1957).

5. Conclusions

The successful isolation and mass culture of *T. nabiasi* from all the rabbit organs was accomplished using modified EMTM medium, allowing for conducting *in vitro* and *in vivo* infection experiments. These epimastigotes are infective to both primary macrophages and naïve domestic rabbits, transforming into amastigotes and trypomastigotes respectively. The formation and multiplication of intracellular amastigotes seems to occur in a greater variety of rabbit organs than previously reported. The genetic variability and relationship of *T. nabiasi* with other trypanosomatids were investigated. Two lineages and four haplotypes of *Trypanosoma nabiasi* were identified in southern Spain. In the phylogenetic trees, *T. nabiasi* fell in the same clade of *T. lewisi* and other rodent trypanosomes, and it is included in a subclade with those trypanosomes whose division stage is the amastigote.

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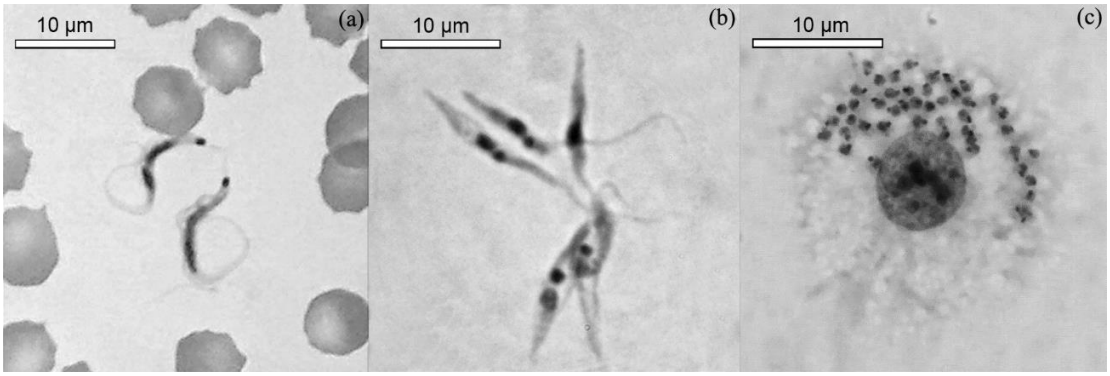

Figure 1: Preparations of *Trypanosoma nabiasi* stained with Giemsa (1000x). (a) Bloodstream trypomastigotes. (b) Culture epimastigotes. (c) Amastigotes inside macrophages.

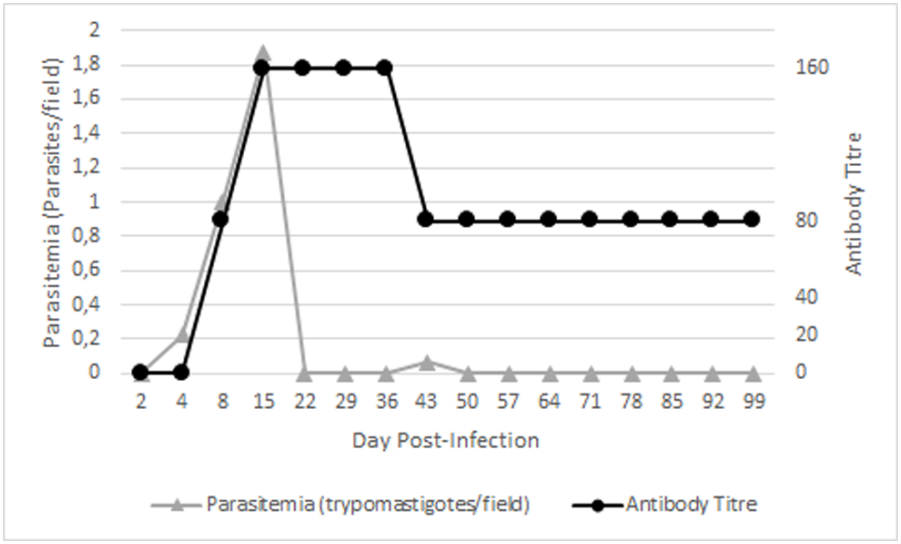

Figure 2: Blood parasite load and IgG titre over time during the course of the experimental infection of the rabbit.

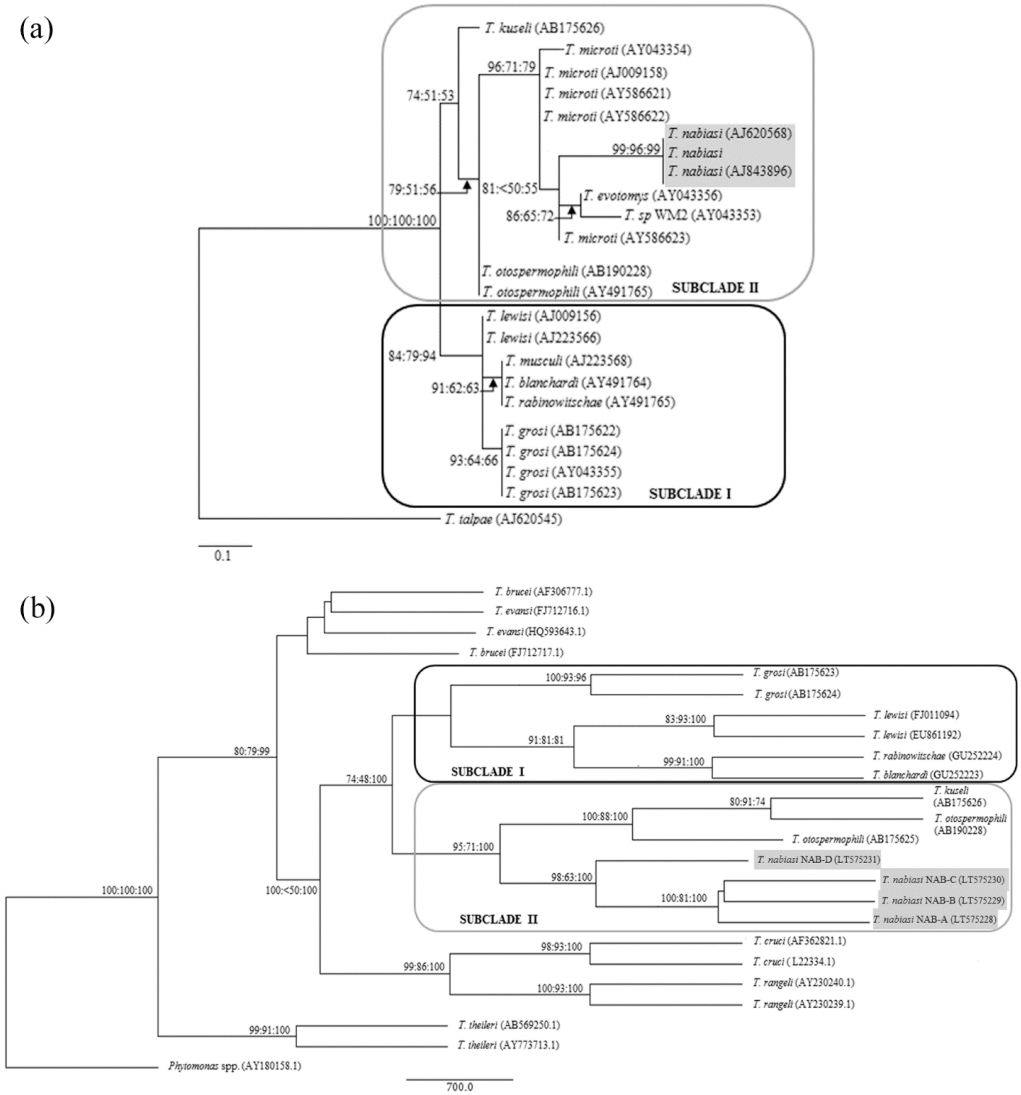

Figure 3: (a) Maximum-likelihood tree based on an alignment of 18S rDNA gene sequences. Values at nodes are bootstrap values (%) in order: ML, MP and distance analysis. Genbank codes are in parentheses. (b) Maximum Parsimonia (MP) tree based on an alignment of ITS-1 rDNA gene sequences. Values at nodes are bootstrap values (%) in order: ML, MP and distance analysis. Genbank codes are in parentheses.

Lineages	ITS-1 Haplotypes	9	1 4	1 9	3 4	3 8	9 0	2 2 8	2 2 9	2 3 0	2 3 1	2 3 2	2 3 3	2 3 4	2 3 5	2 3 6	2 3 7	2 3 8	2 3 9	2 4 0	2 4 1	2 4 2	2 4 3	2 6 7	2 9 0	3 5 1	3 5 6	3 5 7	4 5 0	4 5 2	4 5 5	4 5 8	4 6 2	4 6 5	4 8 6
I	Nab-A	T	C	A	T	A	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	T	T	A	T	G	G	G	G	G	G	C
	Nab-B	T	C	A	T	A	G	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	T	T	A	T	G	G	G	G	G	G	C
	Nab-C	T	C	A	T	A	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	G	C	T	A	T	G	G	G	G	G	G	C
II	Nab-D	C	T	T	C	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	C	-	-	T	T	A	A	A	A	A

Table 1: Alignment of nucleotide characters at the polymorphic sites in the ribosomal DNA Internal Transcribed Spacer 1(ITS 1) haplotypes of *T. nabiasi* with indication of the synapomorphic (highlighted) and the variable characters (underlined) for each lineage.

V. Discusión general

Un método válido para el diagnóstico de la leishmaniosis debe ser lo suficientemente sensible para detectar el parásito, eficaz en la identificación de las especies de *Leishmania* y tan simple como sea posible en su realización. Incluso, se recomienda la utilización de más de una técnica diagnóstica tanto para estudios epidemiológicos sobre leishmaniosis como para la práctica clínica (Morales-Yuste et al., 2012; Mouttaki et al., 2014). Los signos y síntomas clínicos de la leishmaniosis no son patognomónicos en ninguna de las formas clínicas de la enfermedad de forma que sólo inducen a la sospecha de la misma, siendo de vital importancia que el clínico esté familiarizado con ellos. Así por ejemplo, en zonas endémicas, la presencia de lesiones en zonas expuestas del cuerpo como la cara o miembros superiores o inferiores, de varios meses de evolución y sin respuesta al tratamiento con antibióticos o antifúngicos induce a la sospecha de LC que debe ser confirmada correctamente, generalmente mediante los métodos parasitológicos clásicos. Sin embargo, estos métodos poseen una sensibilidad diagnóstica media-baja (Akkafa et al., 2008; Lemrani et al., 2009; Mouttaki et al., 2014), con valores que oscilan entre 17-83% para la observación microscópica de improntas de tejido (Andresen et al., 1996; Aviles et al., 1999; Matsumoto et al., 1999) y entre 17-85% para el cultivo (Aviles et al., 1999; Khademvatan et al., 2011; Matsumoto et al., 1999), lo que pone de manifiesto la influencia que tienen en este tipo de diagnóstico tanto las especies de *Leishmania*, la carga parasitaria y la experiencia del personal técnico (Ricciardi and Ndao, 2015). Cabe esperar que la PCR se convierta en la técnica de referencia, o "*gold standard*", para el diagnóstio de la leishmaniosis, dados los avances en los protocolos de extracción de ADN, la celeridad de la técnica y la variedad de tipos de muestras que pueden utilizarse (Vega-López, 2003).

Se han descrito en la literatura numerosas técnicas de PCR y aunque todas representan una mejora considerable en la sensibilidad y especificidad respecto a los métodos convencionales (Al-Jawabreh et al., 2006; Bensoussan et al., 2006; Marfurt et al., 2003; Schönian et al., 2003), la escasez de estudios comparativos y la falta de estandarización entre laboratorios complican la selección de las técnicas más relevantes. En este estudio hemos podido verificar la eficacia diagnóstica de 4 técnicas de PCR utilizando muestras cutáneas de dos áreas endémicas de LC con diferentes características epidemiológicas, España y Marruecos. Tres de las PCR que hemos utilizado permiten diferenciar entre los agentes etiológicos más importantes de la LC en el Viejo Mundo: *L. tropica, L. major* y *L. infantum*, en función de sus valores de T_m (PCR con cebadores JW13/JW14) o el patrón de bandas generado directamente con la PCR (cebadores Lmj4/Uni21) o tras la

digestión con enzimas de restricción (PCR ITS-1). La cuarta técnica de PCR es específica para *L. infantum*, la especie con mayor distribución geográfica y la única responsable de leishmaniosis visceral, cutánea y mucosa.

En este estudio, el 37,5% de los pacientes del hospital marroquí (PHM) con resultado parasitológico negativo resultaron positivos con al menos una de las técnicas de PCR realizadas. De manera similar, 10 de los 21 (47,6%) de los pacientes de hospitales españoles (PHE) en los que no se sospechaba LC y, por lo tanto, no se les realizó el análisis parasitológico clásico, fueron positivos con la PCR, incluso con 3 técnicas de PCR. Estos resultados indican que la PCR debe ser utilizada de forma rutinaria en pacientes con lesiones cutáneas compatibles con LC. Sin embargo, ninguna de las PCR llevadas a cabo en este estudio permite el diagnóstico de todos los casos de LC por sí sola, por lo que es aconsejable combinar el uso de dos técnicas de PCR.

La PCR con los cebadores Lmj4/Uni21 (Anders et al., 2002) mostró la mejor sensibilidad con PHM (77,3% vs métodos parasitológicos convencionales y 84,2% vs 2 PCRs positivas), pero no proporcionó resultados positivos con PHE, a pesar de que algunos de estos pacientes eran positivos con otras de las técnicas de PCR utilizadas en el estudio. Por el contrario, aunque la PCR en tiempo real con los cebadores JW13/JW14 mostró buena sensibilidad con PHE (100 y 81,8%), no fue tan buena con PHM (52,6 y 58,8%).

En cuanto a la PCR-ELISA específica de *L. infantum* (Martin-Sanchez et al., 2001), se obtuvo una sensibilidad de 83,3 y 90,9% con PHE pero su utilidad en PHM es limitada dado que en Marruecos, la LC se debe principalmente a *L. tropica* y *L. major*, mientras que *L. infantum* sólo se detecta esporádicamente (Ajaoud et al., 2013).

El formato de PCR anidada o *"nested"* ITS-1 mostró mayor sensibilidad con PHE (50 y 72,7%) que con PHM (55 y 61,1%). Cabe resaltar que esta es la técnica más utilizada para el diagnóstico de la leishmaniosis, especialmente en la región mediterránea, así como para la identificación de casos importados (Al-Jawabreh et al., 2006; Harms et al., 2003; Rhajaoui et al., 2007). Sin embargo, se obtuvieron resultados falsos negativos en el 19% de PHE debido a hibridaciones inespecíficas de los cebadores del fragmento ITS-1 de *Leishmania* con el cromosoma 1 de humano, amplificando el gen RABGAP1L que codifica la proteína activadora RAB GTPasa de tipo 1. Esta proteína regula varios procesos del transporte intracelular de proteínas a través de membranas, incluyendo la formación de vesículas, el

movimiento de estas vesículas a lo largo de las redes de actina y tubulina y su fusión a membranas (http://www.ebi.ac.uk/QuickGO/GProtein?ac=Q5R372).

Aunque la PCR ITS-1 permite la identificación de casi todas los especies de importancia médica del género *Leishmania* tanto del Viejo como del Nuevo Mundo, con el uso de una sola enzima de restricción (HaeIII) para la digestión del amplicón (Ben Abda et al., 2011; Roelfsema et al., 2011; Schönian et al., 2003), la identificación de la especie se ve dificultada cuando la carga parasitaria es baja y el patrón de restricción tiene baja definición (Bousslimi et al., 2014; Roberts et al., 2015). En este estudio se encontraron algunos problemas con HaeIII para distinguir *L. infantum* y *L. tropica*, que se resolvieron cambiando la enzima de restricción por MnlI (Asmae et al., 2014) o mediante la secuenciación del fragmento amplificado. En el amplicon ITS-1, la enzima MnlI genera dos fragmentos en *L. tropica* mientras que queda intacto en *L. infantum*.

La optimización de los procedimientos de extracción de ADN a partir de biopsias de tejido embebidas en parafina está permitiendo el uso de este tipo de muestras para el diagnóstico de la LC (Huijsmans et al., 2010; Laskay et al., 1995). En este estudio, la PCR del fragmento ITS-1 no fue suficientemente sensible con este tipo de muestras del grupo PHE tal como ha sido indicado por Schönian et al. (2003) y Yehia et al. (2012); por el contrario la PCR-ELISA específica de *L. infantum* resultó ser una prueba muy sensible incluso en lesiones crónicas, cuando otros métodos no detectaron el parásito.

Cabe destacar que se detectó el parásito en 10 PHE para los que no se sospechó leishmaniosis, a pesar del carácter endémico de *L. infantum* en España y, por tanto, de la supuesta familiaridad del personal clínico con ésta enfermedad. Se obtuvieron resultados positivos con al menos dos PCRs en 4 de estos pacientes sin sospecha de LC del grupo PHE y en 7 pacientes de PHM con resultados negativos con las técnicas parasitológicas convencionales. Un resultado positivo obtenido simultáneamente por dos PCR diferentes refuerza el diagnóstico de LC en un paciente, en particular cuando el análisis parasitológico es negativo o no se realiza. Sin embargo, un resultado positivo obtenido de una única PCR no debe considerarse un falso positivo si esta técnica está suficientemente validada y la extracción de ADN se ha realizado cuidadosamente para evitar riesgos de contaminación. Por otro lado, son muchos los factores que pueden haber influido en los resultados negativos de todas las técnicas de PCR en 3 pacientes de PHM en los que se detectaron amastigotes mediante el análisis parasitológico convencional: un falso positivo, una falta de amplificación debido al polimorfismo

en el fragmento diana de ADN o la baja carga parasitaria en la muestra sometida al diagnóstico molecular (Van der Auwera et al., 2014).

En definitiva, ninguna de las PCRs utilizadas en este estudio permite por si sola el diagnóstico de todos los casos de LC en ninguno de los dos escenarios epidemiológicos analizados. Aunque en España, el uso de una técnica específica de *L. infantum* con buena sensibilidad como es la PCR-ELISA podría ser suficiente, la existencia de casos importados (en este estudio se detectaron 6 casos de LC por *L. major*) aconseja disponer de otra técnica capaz de detectar otras especies de *Leishmania.*

Todo esto nos ha llevado a diseñar una nueva técnica de PCR capaz de diferenciar entre las tres especies analizadas en este trabajo y con un formato de PCR en tiempo real, caracterizado por su mayor simplicidad y rapidez de realización. Esta nueva PCR está en proceso de registro de marca y patente.

La nueva PCR se ha utilizado en un estudio en el que analizamos muestras cutáneas granulomatosas que no tienen diagnóstico (LGOC: Lesiones Granulomatosas sin Origen Conocido), es válida para ser utilizada en biopsias embebidas en parafina y puede diferenciar entre *L. infantum, L. tropica* y *L. major.* Para ello, aplicamos la nueva PCR en un estudio retrospectivo en el que se recogen muestras de 44 pacientes procedentes de hospitales españoles y establecemos las siguientes categorías: casos control con otras patologías diferentes de leishmaniosis (9), casos de leishmaniosis confirmados histológicamente (16), casos con histología sospechosa de leishmaniosis (4) casos LGOC (15). Sus resultados se comparan con los obtenidos al aplicar la PCR-ELISA específica de *L. infantum* (Martín-Sánchez et al., 2001). Los 9 casos negativos y los 16 positivos fueron confirmadas como tales con las 2 técnicas de PCR. Igualmente, se comprueba la infección por *Leishmania infantum* en los 4 pacientes que forman el grupo con sospecha de leishmaniosis. Una mención especial requiere el grupo de pacientes LGOC, en el que se confirma la infección por *L. infantum* en el 73,33% (11/15) de los casos. Los 4 restantes son negativos. En el examen clínico del grupo LGOC se sugirieron diagnósticos como dermatofibroma, sarcoidosis, dermatitis o síndrome de Melkersson-Rosenthal. La sensibilidad de la nueva PCR para detectar *L. infantum* es inferior a la de la PCR-ELISA, como hemos podido comprobar al utilizarlas en el grupo LGOC pero ofrece un formato de realización más simple y fácil de implementar en nuestros hospitales.

Por otro lado, nuestro grupo de investigación tiene una amplia trayectoria en estudios epidemiológicos de la leishmaniosis que se han centrado en gran medida, aunque no de forma exclusiva, en la provincia de Granada. La epidemiología de la leishmaniosis se viene beneficiando de la aportación que realizan las técnicas moleculares desde hace décadas y en este trabajo de tesis hemos seguido esta tendencia, intentando resolver con ellas diversos aspectos epidemiológicos que nos han parecido de interés, como la posible introducción en España de otras especies de *Leishmania* o la existencia de otros reservorios de *L. infantum* distintos del perro.

La introducción de la leishmaniosis en una nueva zona requiere una población bien establecida de las especies que actúan como vectores del parásito. En este sentido, varias zonas del centro y sur de España se consideran puntos calientes potenciales para el establecimiento de un hipotético foco autóctono de *L. tropica* (Barón et al., 2013). Efectivamente, *Phlebotomus sergenti* se encuentra ampliamente distribuido en la península ibérica, alcanzando densidades de hasta 35,3 flebotomos/m2 en algunas zonas, lo que es suficiente para actuar como vector (Barón et al., 2013); ya que en focos marroquíes donde la LCA se considera emergente, se han encontrado densidades de 4-16 flebotomos/m2 (Ramaoui et al., 2008), mientras que en un foco típico de LCA en las zonas áridas de Túnez, se ha descrito una densidad de 1,8 flebotomos/m2 (Rioux et al., 1986).

Aunque entre los casos de LC diagnosticados en este trabajo de tesis doctoral no se ha detectado ningún paciente infectado por *L. tropica,* el flujo de inmigrantes procedentes de regiones endémicas del norte de África y, en menor medida, de Oriente Medio es bien conocido y se tiene constancia de la importación de casos de LCA producidos por *L. tropica* en otros países europeos (Antinori et al., 2005; Gramiccia and Gradoni, 2005; Grimm et al., 1996; Morizot et al., 2007). Por el contrario, el establecimiento de un foco de LC zoonótica por *L. major* se considera menos probable al no existir en nuestro país los roedores que actúan como reservorios.

La posible emergencia de *L. tropica* en España estaría por tanto sustentada en la densidad de su vector, *Phlebotomus sergenti* y además habría que tener en cuenta sus características genéticas. Esta especie se caracteriza por presentar una elevada diversidad genética y se clasifica en al menos veinte haplotipos englobados en cuatro líneas mitocondriales (Barón et al., 2008; Yahia et al., 2004). El polimorfismo del Cyt B descrito en los ejemplares del norte de África es mayor que en los del suroeste de Europa, donde sólo están presentes dos de las cuatro líneas

mitocondriales detectadas, concretamente la línea I y la línea III y algunos de los haplotipos se encuentran presentes en ambos continentes. Este hecho es coherente con la hipótesis generalmente aceptada de la dispersión de *P. sergenti* (Depaquit et al., 2002, 2000). Mientras que la línea mitocondrial I está presente únicamente en el suroeste de Europa, la línea III está ampliamente representada en todos los focos marroquíes de leishmaniosis debidos a *L. tropica*, de forma endémica en el centro-sur del país y epidémico-emergente en el norte (Barón et al., 2008; Yahia et al., 2004).

Resultaba así de gran interés disponer de una técnica que facilitara el análisis del Cyt B en las poblaciones de *P. sergenti* y, por tanto, permitiera analizar la distribución de ambas líneas mitocondriales en el suroeste de Europa sin necesidad de recurrir a la secuenciación. Con ese fin se ha diseñado una nueva PCR-RFLP que permite diferenciar las líneas mitocondriales del Cyt B del vector por su patrón de digestión con la enzima Hae III. Además, se han diseñado y utilizado unos nuevos cebadores para la amplificación de un fragmento interno de Cyt B para aquellos ejemplares en los que la amplificación de Cyt B con las condiciones originales descritas por Esseghir et al. (1997) es desfavorable, como por ejemplo en los casos en los que se analizan flebotomos en mal estado de preservación. La enzima de restricción Hae III se aplica tanto para la digestión del fragmento mitocondrial de Cyt B de 550 pb como del fragmento interno de 250 pb.

El estudio de las poblaciones de *P. sergenti* con esta nueva técnica nos ha mostrado una distribución distintiva de ambas líneas mitocondriales en el suroeste de Europa, probablemente derivado de las diferentes características climáticas preferenciales de cada línea. La línea III se limita a las zonas peninsulares del Mediterráneo y a las Islas Canarias, mientras que la línea I está ampliamente extendida en la península ibérica y es la única presente en las Islas Baleares. Además, al encontrarla presente también en Portugal, sería aconsejable denominarla la línea Ibérica.

La Línea I parece tener ventajas adaptativas, dada su tolerancia a temperaturas mínimas y máximas extremas (desde -1.8 a 32,4 °C), su preferencia por zonas lluviosas (306-983 mm) (Fig. 22), su rango altitudinal más amplio (de 19 a 1819 m sobre el nivel del mar o ms.n.m.) y su extensa distribución geográfica. Por el contrario, la línea III se encuentra restringida a zonas con temperaturas cálidas (12,2-20,4 °C) (Fig. 23), con menos precipitaciones (150-609 mm) y menor altitud (19-1251 ms.n.m.), características típicas de zonas del Mediterráneo.

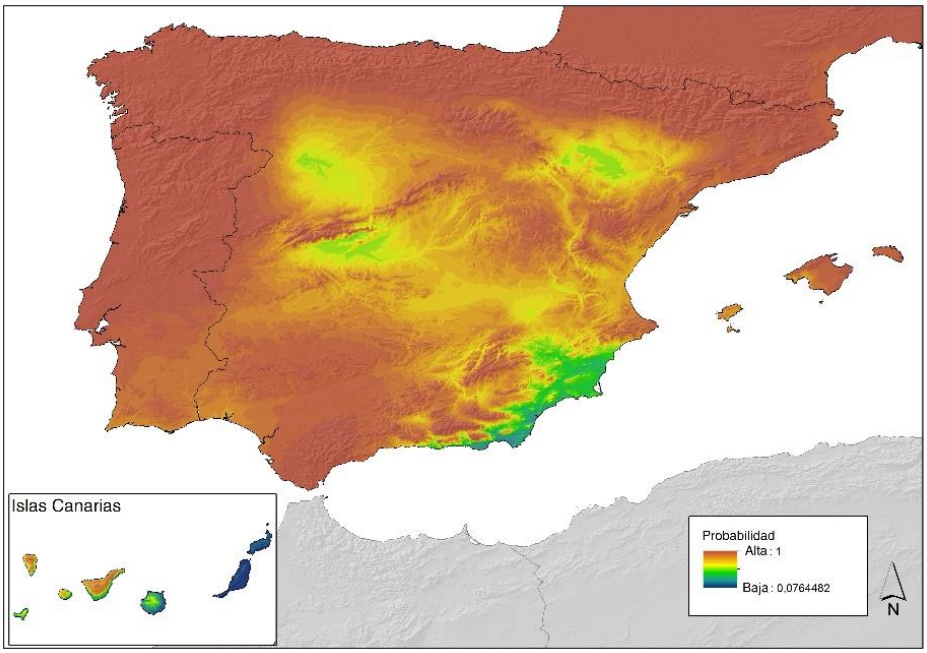

Figura 22. Mapa que predice la distribución geográfica de la Línea I de *Phlebotomus sergenti* basado en la precipitación total anual, de acuerdo con la ecuación probabilidad Línea I = exp(eta)/(1+exp(eta)) donde eta=-3.45676+0.010606*

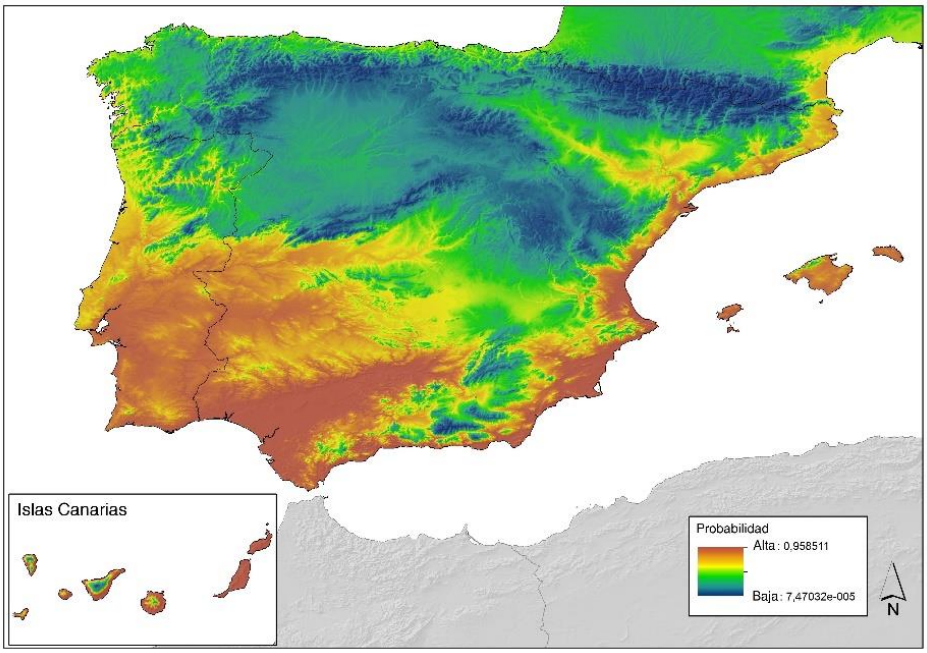

Figura 23. Mapa que predice la distribución geográfica de la Línea III de *Phlebotomus sergenti* basado en la temperatura media anual, de acuerdo con la ecuación probabilidad Línea III = exp(eta)/(1+exp(eta)) donde eta=-7.27971+0.493823*

Según Depaquit et al. (2000; 2002) la distribución occidental de *P. sergenti* sugiere una migración a lo largo del borde sur del mar de Tethys a través de Líbano, Egipto y el norte de África. Desde Marruecos, habría colonizado Madeira y algunas de las islas Canarias. Antes de la apertura del estrecho de Gibraltar, hace unos 5 millones de años (Buffetaut and Le Loeuff, 1998; De Jong, 1998), la unión entre África y Europa le habría permitido llegar a España, donde se encuentra hoy en día, seguido de las islas Baleares, Córcega y el sur de Francia (Fig. 24).

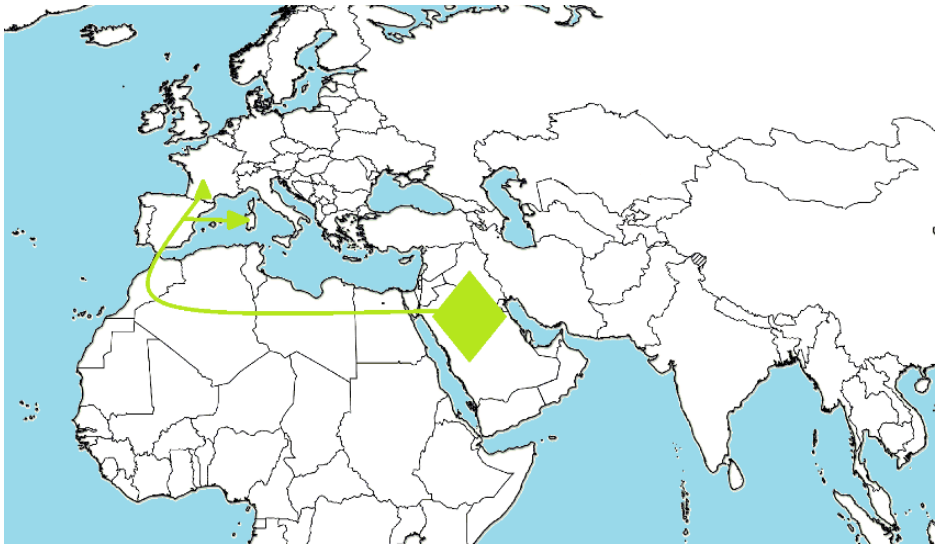

Figura 24. Mapa que muestra el origen y la dispersión de *Phlebotomus sergenti* hacia el continente europeo, basado en Depaquit et al. (2000, 2002).

La utilización de la calibración del reloj molecular sugerido por Esseghir et al. (1997) nos ha permitido plantear la hipótesis de que la línea III podría haber originado al resto de líneas mitocondriales. Las primeras líneas en dividirse serían las líneas III y IV hace 3,8 a 2,2 millones de años, mientras que la línea II se originaría más tarde a partir de línea III (hace 2,9-1,7 millones de años). La división entre las líneas mitocondriales I y III podría haber ocurrido hace 2,4-1,4 millones de años en la península ibérica, de modo que la línea I o Ibérica habría derivado de los primeros antecesores que habrían pertenecido a la línea III. Sin embargo, en las islas Canarias no se habría producido esta mutación.

El conocimiento actual acerca de la ecología de muchas enfermedades parasitarias, como en el caso de la leishmaniosis, ha requerido mucho trabajo durante el último siglo para identificar los posibles reservorios involucrados en la epidemiología de esta enfermedad en las diferentes regiones geográficas en las que se encuentra presente. Tradicionalmente, se utilizaban tanto métodos directos como indirectos

para el diagnóstico de la leishmaniasis pero la llegada de las técnicas moleculares, como la PCR y la gran diversidad de marcadores genéticos, incrementó la sensibilidad en la detección del parásito. En el Viejo Mundo, el perro doméstico (*Canis lupus familiaris*) es el principal reservorio de *L. infantum* y es capaz de mantener al parasito indefinidamente como su único hospedador vertebrado (Ashford, 1996). Sin embargo, es importante considerar la importancia de otros reservorios potenciales y sus implicaciones en el control de la enfermedad. Hasta hace poco, ningún estudio había proporcionado evidencias claras de la existencia de cualquier otro reservorio importante a parte del perro doméstico, lo que no significa que no pudiesen existir. El muestreo de animales para la detección de una posible infección parasitaria es el primer paso para la identificación de nuevos reservorios. Hasta la fecha, *Leishmania* se ha identificado y aislado en más de 70 especies de animales domésticos y salvajes pertenecientes a una gran variedad de familias.

De modo que llevamos a cabo un estudio epidemiológico en un foco bien conocido de leishmaniosis canina (LCa) (Martín-Sánchez et al., 2009) en el que capturamos conejos (*Oryctolagus cuniculus*) y roedores silvestres con el fin de investigar la infección natural por *L. infantum* y su papel como posibles reservorios.

Detectamos la presencia de *L. infantum* en el 20,7% (31/150) de los conejos. El parásito se encontró en todos los tipos de tejido ensayados y la identidad de la especie se confirmó mediante PCR-ELISA específica de *L. infantum* (Martín-Sánchez et al., 2001).

Es importante destacar que la detección del parásito debería conllevar la identificación a nivel de especie, ya que se tiende a generalizar que la especie responsable es *L. infantum* simplemente por el hecho de que el área de estudio en cuestión sea endémica y, por ello, al uso de técnicas que no permiten la identificación de la especie, incluyendo técnicas de PCR que son sólo específicas del género (Criado-Fornelio et al., 2000; Di Bella et al., 2003; Dipineto et al., 2007; Helhazar et al., 2013). Esto supone que los resultados puedan conducir a errores, especialmente si existe la posibilidad de otras especies se encuentren presentes, ya sea en simpatría y/o sintrofia. En nuestro caso, hemos encontrado en estos conejos una prevalencia de infección del 82,4% por otro parásito kinetoplástido, *Trypanosoma nabiasi* y detectamos ambos parásitos en todos los órganos de conejo analizados. Nuestros resultados sugieren que *Trypanosoma* actúa como factor de protección frente a la infección por *Leishmania*: la presencia de

Leishmania fue 4,3 veces mayor en conejos no infectados con *Trypanosoma* en comparación con los animales infectados (p <0,05).

La PCR del ITS-1 (Schönian et al., 2003) fue de gran utilidad para distinguir entre *L. infantum* y *T. nabiasi*, aunque no fue lo suficientemente sensible como para identificar específicamente a *L. infantum* mediante la digestión del amplicón con la enzima de restricción HaeIII, al igual que nos ha sucedido en el diagnóstico de las muestras cutáneas de PHE. Su sensibilidad diagnóstica fue menor que la de la PCR-ELISA (p=0,002).

Los resultados del análisis del título de anticuerpos en conejos utilizando como antígenos promastigotes de *L. infantum* y epimastigotes de *T. nabiasi* reflejan la existencia de reacciones cruzadas debido a la presencia de antígenos compartidos por miembros de la misma familia, por lo que se debe tener cuidado a la hora de interpretar los resultados serológicos con lagomorfos cuando en los estudios no se incluyen procedimientos que permitan distinguir entre los dos kinetoplástidos, como ocurre en el estudio realizado por Moreno et al., (2014). En nuestro caso, al comparar los resultados obtenidos mediante los métodos de diagnóstico directos e indirectos, encontramos una correlación moderada en el diagnóstico de leishmaniosis (56,3% y 37,2% respectivamente) y muy pobre en el caso de la tripanosomiasis (valores kappa negativos). De hecho, a medida que aumenta el título de anticuerpos frente a *Leishmania*, la probabilidad de que el conejo esté infectado por este parásito aumenta (1,7% con cada aumento en el título de anticuerpos, p=0,003), mientras que la asociación es negativa en el caso del *Trypanosoma*, cuya probabilidad de estar presente en el conejo se reduce en 0,6% con cada aumento en el título de anticuerpos. Una asociación positiva entre el título de anticuerpos y la presencia de *L. infantum* es una característica en perros (Morales-Yuste et al., 2012), mientras que en gatos la asociación es negativa (Martín-Sánchez et al., 2007). Por lo que tal asociación negativa podría sugerir una posible inmunidad adquirida frente a *Trypanosoma* en animales infectados.

En los estudios sobre posibles reservorios destaca la baja prevalencia o ausencia de lesiones (Maia et al., 2008; Martín-Sánchez et al., 2007; Millán et al., 2011; Quinnell and Courtenay, 2009a; Sherry et al., 2011). Cuando inspeccionamos a los conejos, encontramos que el 23,3% (10/43) tenía lesiones cutáneas. En seis de ellos confirmamos la presencia de *L. infantum* en la piel (Fig. 25) y, además, en 5 de ellos identificamos infecciones mixtas (Fig. 26). No podemos descartar la posibilidad de que al realizar los cultivos con las muestras obtenidas a partir de ejemplares infectados con ambos tripanosomátidos puedan existir poblaciones mixtas de *L.*

infantum y *T. nabiasi* y que, con el tiempo, las primeras acaben desapareciendo dejando sólo *T. nabiasi*, para el cual se lograron 23 aislamientos; mientras que sólo logramos un aislamiento de *L. infantum* a partir de médula ósea y caracterizada como MON-1 mediante la electroforesis de isoenzimas.

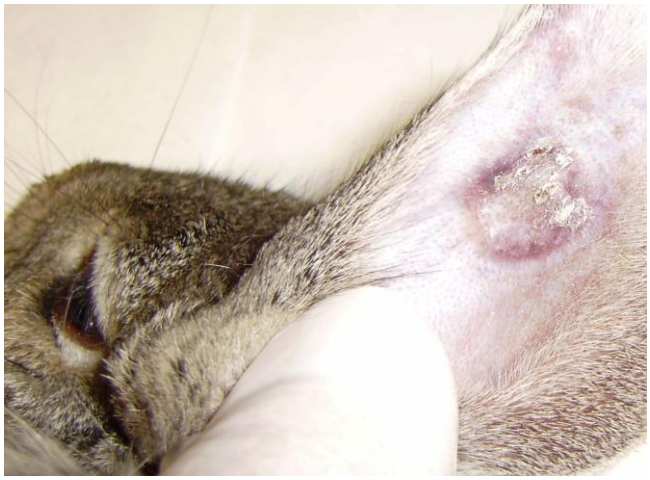

Figura 25. Lesión cutánea en oreja de conejo.

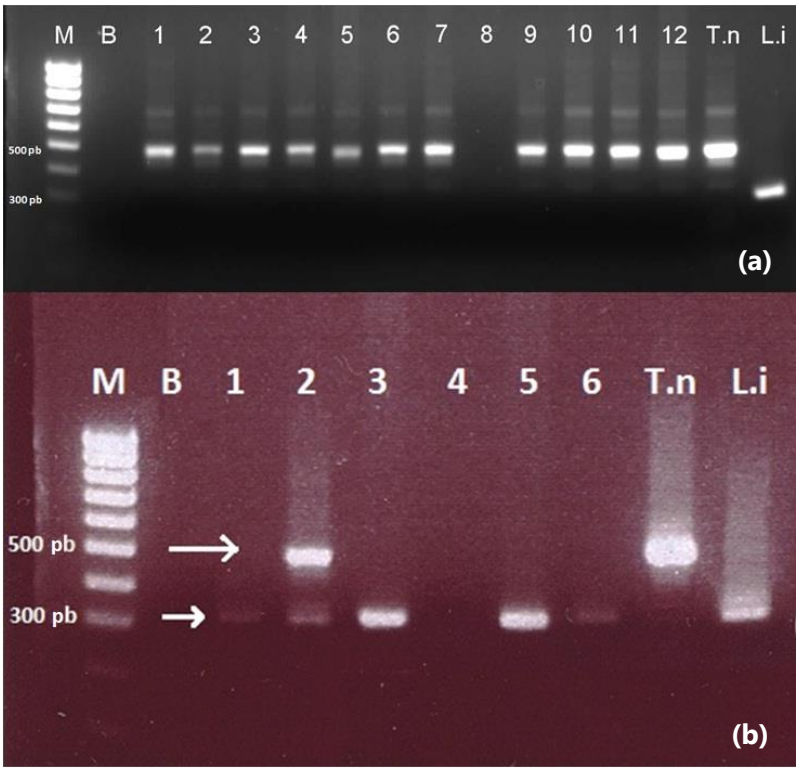

Figura 26. (a) Gel en el que se observa la infección por *T. nabiasi* en casi todas las muestras. (b) Gel en el que se observa infección mixta por *T. nabiasi* y *L. infantum* en pocillo 2.

Los parámetros ecológicos de los reservorios, tales como biotopo, hábitat, estructura social, densidad y dispersión, son particularmente importantes para comprender su papel en el mantenimiento del parásito. Además, su modelo de comportamiento puede determinar la interacción específica con los flebótomos (Ashford, 1996).

El conejo (*O. cuniculus*) es nativo de la Península Ibérica y se encuentra ampliamente distribuido en todo el suroeste, centro y norte de Europa. Fue introducido en otros países como Australia, Nueva Zelanda, Estados Unidos, etc. Está presente en todo el territorio español, con valores de densidad relativa entre 1.6845 (Comunidad de Madrid, centro de España) y 0.0026 (Comunidad de La Rioja, en el noreste de España); en la provincia de Granada (sureste de España) la densidad relativa es de 0,94. El principal biotopo de estos lagomorfos son las madrigueras que excavan en suelo fértil. Se trata de una especie gregaria, territorial, nocturna y polígama. Tienen camadas de 2-8 crías y la madurez sexual se alcanza a los 3-4 meses. Su esperanza de vida es suficientemente larga para asegurar su supervivencia durante el período en el que no se produce transmisión de la leishmaniosis; de hecho el promedio de vida de los conejos capturados en este estudio fue de 2,5 años.

Detectamos conejos infectados por *L. infantum* durante todo el período de captura, con un fuerte aumento de la prevalencia de la infección que indicaría un brote epidémico. Las infecciones detectadas en el mes de julio probablemente indican que se adquirieron en el período previo de transmisión. Además, los conejos más grandes son más susceptibles a la infección al estar más expuestos a las picaduras del vector. Todo ello podría indicar que los conejos pueden estar infectados durante bastante tiempo sin presentar síntomas agudos de enfermedad, ya que la única manifestación clínica que detectamos fueron lesiones cutáneas en el 23,3% de los conejos. La presencia del parásito en la piel y en la sangre permite que se presente más accesible al flebotomo. Las trampas CDC se colocaron cerca de la madrigueras y se capturaron flebotomos en densidades considerables (10.6 flebotomos/trampa, recolectando un total de 1812 ejemplares). La especie más abundante fue *P. perniciosus* (72,3%), que es precisamente el principal vector de leishmaniosis en la zona (Barón et al., 2011; Martín-Sánchez et al., 1994; Morillas Márquez et al., 1983), seguido por *S. minuta* (20,1%), *P. papatasi* (6%), *P. ariasi* (0,94%), que también actúa como vector (Ballart et al. 2012; Morillas et al. 1996) y *P. sergenti* (0,71%).

Los roedores salvajes constituyen una gran biomasa de reservorios potenciales para *Leishmania* (Ashford, 1996; Quinnell and Courtenay, 2009), como en el caso de *L. major* en el que el gran jerbo (*Rhombomys opimus*) es su principal reservorio. Además, varias especies de *Leishmania* se han expandido geográficamente gracias a los desplazamientos de roedores, tanto en el Viejo como en el Nuevo Mundo (Ashford, 1996; Desjeux, 2001; Di Bella et al., 2003; Mohebali et al., 2004; Motazedian et al., 2010; Svobodová and Votýpka, 2003).

Detectamos la presencia de *L. infantum* en el 27% (10/37) de los roedores: 33,3% (3/9) en *Rattus rattus*, 20,8% (5/24) en *Apodemus sylvaticus* y 50,0% (2/4) en *Mus musculus*. El parásito se encontró en todos los tipos de tejido ensayados y la identidad de la especie se confirmó mediante PCR-ELISA específica de *L. infantum* (Martín-Sánchez et al., 2001). Estudios llevados a cabo en Italia y Portugal han mostrado que el 45,0% de *R. rattus* y el 33,3% de *M. musculus* fueron positivos a *Leishmania* por PCR (Di Bella et al., 2003; Helhazar et al., 2013), valores similares a los encontrados en este trabajo. Además, Zanet et al. (2014) han demostrado recientemente que en la isla de Montecristo (Italia), donde no hay perros ni otros carnívoros, hasta el 15,5% de las ratas negras están infectadas por *L. infantum*. Sin embargo, estos autores no tuvieron en cuenta la presencia en esta isla de conejos silvestres, recientemente identificados como reservorios del parásito (Díaz-Sáez et al., 2014; Jiménez et al., 2014). De alguna manera, este hecho revela claramente el mantenimiento del parásito en ausencia del perro.

Al igual que en el caso de los conejos, detectamos *Trypanosoma* sp. en uno de los ejemplares de *A. sylvaticus*, de modo que la prevalencia de infección fue del 8,3% (2/24), pero no se observaron positivos en las otras dos especies analizadas. Cuando comparamos la secuencia con las bases de datos comprobamos que posee un 93% de homología *Trypanosoma lewisi* y 92% *Trypanosoma grosi*, una especie que se encuentra generalmente en *A. sylvaticus*, lo que no es lo suficientemente cercana como asegurar que se corresponde con una de ellas. Además, confirmamos esta falta de identidad tras una infructuosa infección experimental de una rata de laboratorio (*Rattus norvegicus*): no se observó parasitemia durante la infección y los análisis de PCR en el hígado, el bazo y la médula ósea fueron negativos. Los tripanosomas de roedores como *T. lewisi*, *T. musculi* o *T. grosi* se dividen como epimastigotes en el torrente sanguíneo. En contraste, otras especies de *Trypanosoma* como *T. microti* y *T. nabiasi* se dividen como amastigotes en los tejidos y nunca muestran formas divisorias en el torrente sanguíneo (Molyneux 1970). En nuestro caso, el parásito se detectó tejidos, la piel

entre ellos, lo que nos sugiere que se divide como amastigote y pone de manifiesto la limitación de los métodos de diagnóstico parasitológico clásicos.

Cabe destacar que al utilizar la PCR del ITS-1 (Schönian et al., 2003) hemos detectado la amplificación de fragmentos inespecíficos que pertenecen al ADN del hospedador en este caso; al igual que los resultados obtenidos en las muestras cutáneas del grupo de pacientes PHE obteniéndose hibridaciones inespecíficas con ADN humano, en concreto con el cromosoma 1. Dada la amplia utilización de esta técnica en el diagnóstico de leishmaniosis en todo el mundo, es necesario la confirmación de la infección en roedores mediante secuenciación a la luz de los resultados obtenidos.

Los roedores se encuentran ampliamente distribuidos en Europa. Viven en madrigueras y oquedades de los árboles, lo que proporciona un hábitat adecuado a los flebotomos (Díaz-Sáez et al., 2014) y en ellas sirven como fuentes de ingesta de sangre para las poblaciones de flebotomos; ya que *P. perniciosus* es un oportunista que se alimenta de animales con acceso fácil (Bongiorno et al., 2003; De Colmenares et al., 1995; Rossi et al., 2008), hecho que comprobaron Maia et al. (2013) al encontrar sangre de roedor en el interior de individuos de *P. perniciosus*. Son extremadamente prolíficos y su esperanza de vida se encuentra comprendida entre los 12 (*M. musculus*) y 18 meses (*A. sylvaticus* y *R. rattus*), de modo que mantendrían disponible a los parásitos incluso durante la temporada baja de actividad de los flebotomos, como lo demuestra el hecho de capturar estos 10 roedores infectados desde noviembre a abril. La densidad de flebotomos en las zonas de muestreo en las que se capturaron los roedores fue de 46,5 individuos/m2 y con respecto a las especies vectoras, los valores fueron de 4,4 *P. perniciosus*/m2 y 0,6 *P. ariasi*/m2 (Barón et al., 2011).

La infección de *T. nabiasi* ha sido descrita en *O. cuniculus* en varios países europeos (Reino Unido, Francia, Italia, Portugal y España) y fuera de Europa, aunque poco se sabe acerca de la identificación y distribución de este protozoo en las poblaciones españolas de conejos. En un estudio realizado en el suroeste de la península, Reglero et al. (2007) encontraron que *Trypanosoma* spp. estaba presente en la sangre de conejos, pero éste fue el único tipo de muestra analizada. La prevalencia que obtuvieron fue del 23,81% en conejos jóvenes, 84,85% en juveniles y 47,37% en adultos, siendo estos valores más altos en las hembras que en los machos, y mayor en conejos juveniles, seguidos por adultos y, por último, conejos jóvenes. En nuestro caso, no encontramos diferencias entre machos y hembras y pudimos confirmar los niveles de parasitación más bajos en conejos más grandes, es decir,

los adultos. Las cifras de prevalencia que encontramos se mantuvieron elevadas durante todo el estudio y son claramente superiores a las obtenidas por Reglero et al. (2007). Estas diferencias pueden deberse a que estos autores utilizan sólo sangre y métodos de observación directa para detectar los parásitos, en contraposición a la variedad de muestras y técnicas de diagnóstico que hemos utilizado en el estudio. Por otra parte, no podemos descartar la posible influencia de nuestro lugar de muestreo al tratarse de una finca cercada, es decir, un núcleo cerrado.

La evidencia sobre la patogenicidad de *T. nabiasi* en su hospedador no está clara. Jolyet & Nabias (1891) y Grewal (1957) no detectaron signos clínicos en la salud del animal, mientras que Petrie (1905) encontró que la sangre infectada con *T. nabiasi* era nociva tras la muerte de dos de los diez conejos inoculados con sangre de conejos infectados. Tras la inspección visual de todos los conejos capturados en este estudio no se apreció ninguna sintomatología en los ejemplares. El conejo que infectamos no mostró ningún síntoma clínico, excepto el episodio de leucocitosis transitoria el día 15 tras la infección.

Los conejos jóvenes mostraron un riesgo ocho veces mayor de infección que los adultos. De modo que podríamos encontrar que estos animales se curan por si solos y adquieren inmunidad frente a *T. nabiasi*, lo que concuerda con la asociación negativa entre los títulos de anticuerpos y la presencia de parásitos descritos en este estudio. Kroó (1936) y Grewal (1957) señaló que los conejos permanecen inmunes a la re-infección tras recuperarse de la infección anterior, lo que sugiere un papel protector de estos anticuerpos. Además, se evaluaron los títulos de anticuerpos IgG a lo largo de la infección experimental, detectando un aumento del título IgG el día 4 post-infección (pi) y alcanzando un pico el día 15 pi. Aunque no es lo usual, un estudio reciente ha descrito un aumento temprano en el título de IgG similar al aumento del título de IgM el día 7 pi en la infección de vacas con *T. vivax* (Uzcanga et al., 2016).

Los frotis de sangre teñidos con Giemsa mostraron parásitos con un kinetoplasto prominente, un extremo posterior puntiagudo y un flagelo libre largo, similar a la descripción de *T. nabiasi* (Grewal, 1957; Hamilton et al., 2005; Mansfield, 1977; Molyneux, 1970; Reglero et al., 2007).

Las condiciones de cultivo de *T. nabiasi* in vitro son muy exigentes y son pocos autores los que han conseguido un crecimiento ligero del parásito, que acabó por desaparecer rápidamente (Channon and Wright, 1927; Grewal, 1957; Mohamed

and Molyneux, 1987; Petrie, 1905). En nuestro caso, hemos sido capaces de visualizar y aislar epimastigotes en nuestra combinación de medio EMTM y suplementado con RPMI 1640 para el cultivo de todos los tipos de órganos, excepto de piel debido a la contaminación por hongos. Los cultivos se mantuvieron a 24 °C durante períodos prolongados hasta 6 meses. Además, se aislaron 26 cepas y se cultivaron en masa a partir de 16 conejos. Aunque la forma que visualizamos más frecuente fue la de epimastigotes, también se observaron esporádicamente amastigotes, esferomastigotes y tripomastigotes.

Se obtuvieron amastigotes al infectar macrófagos derivados de la médula ósea con epimastigotes procedentes de cultivo y tripomastigotes *in vitro* como resultado de la transformación de amastigotes intracelulares mantenidos en las condiciones adecuadas. Estos amastigotes tenían un solo núcleo y un solo kinetoplasto. Algunos autores también han encontrado amastigotes con 4 núcleos y 4 kinetoplastos (Grewal, 1957) e incluso formas más grandes con 8 núcleos y 8 kinetoplastos (Molyneux, 1969). A continuación, conseguimos infectar de forma experimental un conejo de laboratorio con epimastigotes de cultivo: pudimos detectar tripomastigotes en sangre periférica desde el cuarto día tras la infección, lo que concuerda con el período de incubación de 7 días obtenido por Kroó (1936) y 5-12 días obtenidos por Grewal (1957). El número de parásitos alcanzó un máximo el decimoquinto día, después del cual comenzó la caída constante de la parasitemia hasta el día 50 tras la inoculación en el que desaparecieron todos los tripanosomas en sangre. El día 99 tras la infección, el conejo fue sacrificado y ninguna de las muestras fue positiva con ninguna de las técnicas realizadas, lo que demostró la desaparición por completo de la infección. La duración de la infección estaba dentro de los intervalos de 52-213 días descritos por Kroó (1936) y 73- 136 días obtenidos por Grewal (1957). Según este último, después de la inoculación de los tripanosomas metacíclicos a partir de pulgas infectadas o de las formas sanguíneas, los amastigotes de *T. nabiasi* se multiplican por fisión binaria en los capilares del bazo. Este autor describió el pico de parasitemia en los días 6-10, similar al pico encontrado el día 15 en el presente estudio y que fue disminuyendo gradualmente. Éstos se transforman en formas tripomastigotes que llegan a la sangre periférica, donde se transforman en tripomastigotes adultos típicos. Nuestros resultados apoyan estos datos e indican que la formación y multiplicación de amastigotes parece ocurrir en una mayor variedad de órganos de la que se tenía constancia.

Varios autores han tratado de infectar conejos de laboratorio a través de inoculación de sangre a partir de conejos silvestres infectados (Mohamed and Molyneux, 1987; Petrie, 1905; Watson and Hadwen, 1912) pero sólo Grewal (1957) tuvo éxito. Según describieron Channon & Wright (1927) y Mohamed & Molyneux (1987), la infección con pulgas maceradas era una forma más fiable. Además, Channon & Wright (1927) sólo fueron capaces de infectar conejos mediante inoculación intravenosa. Grewal (1957) consiguió producir una infección utilizando tripomastigotes por vía intraperitoneal. En el presente estudio, la infección se estableció gracias a una dosis infectiva alta ($2{,}6 \times 10^7$ epimastigotes), lo que fue posible gracias al cultivo en masa.

Con el objetivo de resolver la relación entre los aislados de los conejos españoles silvestres y las secuencias de *T. nabiasi* publicadas, llevamos a cabo análisis comparativos de los fragmentos de ADN ribosómico (ADNr) 18S e ITS-1 de con otros tripanosomas del subgénero *Herpetosoma* (Hamilton et al., 2005; Maia da Silva et al., 2010; Votýpka et al., 2015). Las topologías de los árboles del fragmento 18S son similares a los obtenidos por Hamilton et al. (2005) y Maia da Silva et al. (2010). Esto nos permitió confirmar la identificación de *T. nabiasi* en los conejos del sur de España. Las topologías de los árboles con el fragmento ITS-1 apoyan estos hallazgos. Además, las diferencias encontradas entre las secuencias obtenidas de 500 y 480 pb del fragmento ITS-1 parecen indicar la existencia de dos linajes genéticos: la línea I, que es la mejor representada siendo el haplotipo Nab-A el más frecuente, seguido de los haplotipos Nab-B y Nab-C, y Lla línea II con el haplotipo Nab-D. En los árboles filogenéticos resultantes de los fragmentos 18S e ITS-1, el tripanosoma del conejo está incluido en el mismo clado que *T. lewisi*. Todos los tripanosomas incluidos en este clado pertenecen a mamíferos y sus únicos vectores conocidos son las pulgas. Además, este clado se divide en dos subclados; subclado 1, que incluye tripanosomas de dos familias de roedores, tales como Muridae (*T. Blanchardi, T. grosi* y *T. lewisi*) y Gliridae (*T. rabinowitschae*), mientras que el subclado 2 incluye a *T. nabiasi* y dos tripanosomas de las ardillas: *T. otospermohili* y *T. kuseli*. Esta división es similar a la obtenida por Hamilton et al. (2005) y que concuerda con el desarrollo en el huésped vertebrado: todos los tripanosomas del subclado 1 se dividen como epimastigotes en el torrente sanguíneo mientras que los pertenecientes al subclado 2 se dividen como amastigotes (Grewal, 1957).

En resumen, en este estudio hemos podido comprobar que las técnicas de PCR aplicadas a muestras cutáneas están influenciadas por la especie que las produce

ya que las lesiones debidas a *L. infantum* presentan una carga parasitaria más baja que las debidas a *L. tropica* o *L. major,* lo que va en consonancia con el método de recogida de muestra siendo biopsias de tejido en el caso de *L. infantum* o aspiración de exudado/raspado de la lesión en el caso de *L. tropica* o *L. major*. Es recomendable recurrir al uso de dos técnicas de PCR o, si no es posible, confirmar el diagnóstico parasitológico clásico con una de las PCR con eficiencia contrastada en función de la especie endémica de la zona de estudio. A su vez, hemos podido demostrar que línea mitocondrial I de *Phlebotomus sergenti* muestra una mejor correlación a la diversidad bioclimática del suroeste de Europa, mientras que la línea III (la común entre Marruecos y España) prefiere temperaturas más cálidas y menos precipitaciones, características típicas de zonas del Mediterráneo. De modo que la línea I sería la más adecuada para expandirse hacia el resto de Europa, lo que unido a la importación de casos desde zonas endémicas y las continuas modificaciones ambientales en el marco del cambio climático ofrecen mejores oportunidades para la posible expansión geográfica de los vectores de *L. tropica.* Por otro lado, está claro que la epidemiología de *L. infantum* es más compleja de lo que se pensaba anteriormente y también su control. El clásico ciclo epidemiológico perro-flebotomo-humano se está convirtiendo en una red de especies animales, a las que hay que sumar conejos, liebres, otros cánidos y varias especies de roedores, que colaboran con el perro en el mantenimiento del parásito en condiciones naturales y probablemente mostrando diferencias locales. Cabe destacar que existe la posibilidad de que otras especies parásitas puedan estar presentes en estos animales silvestres, ya sea en simpatría y/o sintrofia. La prevalencia de infección de *Trypanosoma* junto con *L. infantum* ha sido alta tanto en los conejos como en los roedores muestreados. La aplicación de los métodos moleculares en estos estudios, como el uso de la PCR, confieren grandes ventajas como el incremento de la sensibilidad, la identificación de la especie responsable de la enfermedad, mejor comprensión sobre los patrones de transmisión y están siendo ampliamente utilizados para la detección del parásito en la vida silvestre. Sin embargo, su utilización sin complementarlos con otras técnicas o la elección de un método inapropiado de PCR podría no responder a las preguntas planteadas. Además, gracias al éxito alcanzado al cultivar en masa a *T. nabiasi*, hemos logrado infectar de forma experimental a un conejo en el que pudimos demostrar que este protozoo se divide en más tejidos de los que se tenía constancia. Asímismo, confirmamos que la especie que infecta a los conejos españoles es *T. nabiasi* mediante el análisis filogenético de los marcadores 18S e ITS-1 y describimos la existencia de dos líneas genéticas compuestas por 4 haplotipos, siendo el Nab-A el más abundante.

VI. Conclusions

1. The combination of parasitological and molecular methods, using at least two different PCRs, is a reliable and accurate procedure for the diagnosis of CL, allowing the simultaneous identification of the parasite.
2. The selection of these PCR techniques will be influenced by the epidemiological scenario: in areas where *L. infantum* is endemic, the use of the PCR-ELISA joint with JW13/JW14 PCR or ITS-1 seems an appropriate choice whereas in areas like Morocco, where *L. tropica* and *L. major* are the main CL causative agents with sporadic CL cases due to *L. infantum*, PCR-ELISA is the least useful while Lmj4/Uni21 and ITS-1 provide satisfactory results.
3. The possibility of using FFPE samples for DNA extraction and PCR allows discarding CL in patients with skin lesions of unknown etiology for which leishmaniasis was not suspected.
4. A high percentage of GLUO are due to *L. infantum* infection. Given that the success of direct microscopy analysis is associated to the experience of the analyst, we suggest the generalized implementation of a PCR technique, such as those indicated: these show high sensitivity and specificity and could help overcome the drawbacks of direct diagnosis. Additionally, we recommend that all granulomatous cutaneous lesions from *L. infantum* endemic areas should be systematically investigated in by these techniques.
5. *Phlebotomus sergenti* lineage I shows a better correlation to the bioclimatic diversity in southwestern Europe. Conversely, *P. sergenti* lineage III prefers warmer temperatures and less precipitation, which are typical of the Mediterranean. Therefore, lineage I would seem more suitable to lead a potential geographical expansion towards the rest of Europe.
6. Importation of ACL cases had previously been verified in other European countries and seems likely especially now through the flow of refugees from North Africa and the Middle East to Northern Europe as a result of armed conflict.
7. Continued environmental modifications in the current climate change will provide better opportunities for the geographical expansion of *L. tropica* vectors. Integrated regional vector surveillance and control programs must be set up by local and European authorities in areas that meet *P. sergenti* lineage I environmental requirements.
8. In order to determine the role of a given host in a reservoir system, it is far from sufficient to simply discover infected individuals. Despite the fact that in southwestern Europe *L. infantum* antibodies and DNA have been found in a wide array of domestic and wild animals, and that in some cases it has

been possible to isolate the parasite and submit it to isoenzymatic characterization, no clear evidence has ever been found of their involvement as reservoirs, with the possible exception of the recent study on hares.

9. Without a doubt, the impossibility of finding an area where there are no dogs further hinders the process of evaluating the possible contribution of these other hosts to the epidemiology of leishmaniasis. In the systems that have been adequately described, the reservoir host is abundant, forming a large proportion of the mammalian biomass and it is often a gregarious species, all characteristics which are true of the wild rabbit to an even greater extent than the dog.
10. An effective reservoir host can be expected to be long-lived, at least surviving through any non-transmission season. Although the average lifespan of rabbits is shorter than that of dogs, it is long enough to ensure the transmission of *L. infantum.* If a rabbit reaches adulthood it may live for 4–5 years, clearly a much lower figure than the estimated average 14-year lifespan of dogs.
11. The presence of the parasite in high proportions in the skin and peripheral blood of these rabbits with no apparent signs of acute dis-ease ensures its contact with the vector, which finds in their warrens a suitable biotope to inhabit.
12. The rabbit appears to fulfil the most of conditions which would justify it being considered a reservoir host of *L. infantum* and it would be interesting to conduct xenodiagnostic experiments using the local phlebotomine vectors. The sympatric and syntrophic presence of *T. nabiasi* must be taken into account in order to avoid any confusion.
13. The results of this research, analysed in the context of the contributions from other authors and eco-biological data of rodents, support its role as wild reservoirs in zoonotic leishmaniasis foci. Nevertheless, more research is necessary to definitely confirm this fact.
14. The successful isolation and mass culture of *T. nabiasi* from all the rabbit organs was accomplished using modified EMTM medium, allowing for conducting in vitro and in vivo infection experiments. These epimastigotes are infective to both primary macrophages and naïve domestic rabbits, transforming into amastigotes and trypomastigotes respectively. The formation and multiplication of intracellular amastigotes seems to occur in a greater variety of rabbit organs than previously reported.
15. The genetic variability and relationship of *T. nabiasi* with other trypanosomatids were investigated. Two lineages and four haplotypes of *T.*

nabiasi were identified in southern Spain. In the phylogenetic trees, *T. nabiasi* fell in the same clade of *T. lewisi* and other rodent trypanosomes, and it is included in a subclade with those trypanosomes whose division stage is the amastigote.

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