Infection and immune response against *Leishmania infantum* in healthy dogs and horses

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Hugo Fernández Bellon

Directed by:

Dr. Antoni Ramis Salvà

Dept. de Sanitat i d'Anatomia Animals
Universitat Autònoma de Barcelona

Dr. Jordi Alberola Domingo

Departament. de Farmacologia, de Terapèutica i de Toxicologia
Universitat Autònoma de Barcelona

Programa de Doctorat en Medicina Veterinària

Departament de Sanitat i Anatomia Animals,
Facultat de Veterinària
Universitat Autònoma de Barcelona

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El Dr.Antoni Ramis Salvà, Titular d'universitat numerari del Departament de Sanitat i d'Anatomia Animals de la Universitat Autònoma de Barcelona, i el Dr. Jordi Alberola Domingo, Titular d'unversitari numerari del Departament de Farmatologia, de Terapèutica i de Toxicologia de la Universitat Autònoma de Barcelona,

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Prefaci

Resulta inevitable, en atansar-me a la culminació (potser hauria de dir liquidació) d'un projecte com aquesta tesi, aixecar el cap, fer una ullada al voltant i copsar allò que m' ha envoltat i envolta, i que potser no havia observat tot i haver-ho vist. Al llarg de catorze anys pots acumular molt al teu voltant. I he estat de sort, he estat envoltat d'amistat, d'amor, d'ensenyança, de fraternitat, de lleialtat i de suport. M'han crescut amistats, una família, companys, i fins i tot, espero, un xic de seny. Tinc l'esperança d'haver sabut reciprocar.

Agraïments? Es clar, molts més, i a molta més gent, del que pugui arribar a expressar. Entre d'altres, dec molt a:

Laia i Alhe, ja sabeu que aquesta tesi hagués estat impossible sense vosaltres.

Jordi i Toni, la (enorme) paciència que m'heu demostrat en aquests anys no eclipsa el seguiment, suport i confiança que m'heu donat.

La gent d'AP i l'embrió de CreSA dels meus anys de laboratori: vaig passar anys molt feliços i profitosos amb vosaltres.

Mar, Xavi i Manel, companys i artífexs de la supervivència d'aquesta tesi.

Mis padres, m'heu fet el que soc.

Càrol, simplement gràcies per ser-hi.

Vivim un moment de la història de la humanitat i en una part del mon privilegiats. És una fortuna que no tenim cap dret a malbaratar. S'exigeix combatre la pseudociència, la incultura, la ignorància i la superstició, que fomenten els poderosos, i que ens deshumanitzen.

> “If we can't think for ourselves, if we're unwilling to question authority, then we're just putty in the hands of those in power. But if the citizens are educated and form their own opinions, then those in power work for us.”
>
> Carl Sagan, *The demon-haunted world*

A mis abuelos,

A mis chigüines

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CHAPTER 1

INTRODUCTION

“La vida es un proceso que implica, necesariamente, conocimiento.”

Antonio López Campillo, *La ciencia como herejía*

LEISHMANIA IN MAMMALS

The term leishmaniosis encompasses a number of diseases with distinct clinical symptoms, epidemiological cycles, vector and host species, and geographical distributions caused by flagellate protozoans of the genus *Leishmania*. *Leishmania* sp. (Ross, 1903) are digenean obligate parasites of mammals transmitted by hematophagous sandflies, *Phlebotomus* sp in the Palearctic ecoregion, and *Lutzomya* spp in the Neotropical ecoregion (Herwaldt, 1999). The *Leishmania* genus comprises two subgeni which in turn include several species complexes, the taxonomy of lesser known species still being controversial (Bañuls et al., 2007; Antinori et al., 2012). The most relevant species in either animal or public health, *L.* (*Leishmania*) *donovani*, *L.* (*L*) *infantum*, *L.* (*L*) *major* and *L.* (*Viannia*) *braziliensis*, show marked genetic, morphological, pathogenic, and epidemiological differences, and are each linked to specific presentations of disease in mammals (including humans) (Desjeux, 2004a; Baneth & Solano-Gallego, 2012).

Mammalian infection by *Leishmania* follows regurgitation of *Leishmania* promastigotes (the motile phase of the parasite) by an infected sandfly into its host's dermis (Killick-Kendrick, 1999). Once in the mammalian host, the promastigotes infect phagocytic immune cells wherein they transform into amastigotes (Solbach & Laskay, 2000). Survival of phagocytosed amastigotes and subsequent propagation of infection can lead to different diseases, or patterns of disease, (Herwaldt, 1999) known as leishmanioses - or leishmaniases, (Kassai, 2006).

Leishmanioses affect human beings living in tropical and temperate areas of the world. Human leishmanioses (HuL) are mainly rural and surburban zoonotic diseases, with domestic or peridomestic mammals acting as reservoir hosts. Instances of anthroponotic leishmanioses, where animal hosts do not partake in the epidemiology are a rare exception (Ashford, 2000). Indeed, humans are generally considered marginal, or even dead-end hosts in epidemiological cycles that usually involve several mammalian hosts, one or two of which are implicated as main reservoirs. Rural cycles of leishmanioses can involve domestic and peridomestic animals, such as dogs and rats (*L. infantum* in South America and the Mediterranean ecoregions) (Ashford, 2000; Baneth & Solano-Gallego, 2012) or putatively equines (*L. donovani* in Sudan) (Mukhtar et al., 2000) or L braziliensis in South America (Brandão-Filho et al., 2003). Sylvatic reservoirs for other instances of HuL have also been identified, as with *L. major* in North Africa (*Psammomys obesus*) (Ghawar et al., 2011)or in Iran (*Rhombomys optimus*) (Akhavan et al., 2010). Other instances of human

involvement are restricted to sporadic infection from strictly sylvatic epidemiological cycles of *Leishmania*, such as *L. panamensis*, maintained by sloths (Ashford, 1996).

Although human infections by *Leishmania* spp. are a significant global public health concern, the different forms of HuL have lower morbidity and mortality than notable infectious diseases such as tuberculosis. *Leishmania* ranks below pathogens such as *Treponema*, *Clostridium*, or *Morbillivirus* in terms of mortality or social impact (DALYs) (World Health Organization, 2004). Moreover, HuL are mainly tropical diseases, mostly impinging upon impoverished social groups in underdeveloped areas of the world, and are considered neglected diseases by the World Health Organization (World Health Organization, 2007). However, *Leishmania* sp. and their infection of vertebrate hosts are more extensively studied than their ranking and neglected disease status might suggest [1].

The scientific interest in leishmanioses is not only due to their impact on public health. Several factors combine to enhance the scientific relevance of leishmanioses. Perhaps most importantly, *Leishmania* infection is a key model to study vertebrate immunology. Immune responses by vertebrate hosts to *Leishmania* sp. are varied and complex, and key in determining the course and outcome of infection (Herwaldt, 1999) Efforts to understand these responses have made *Leishmania* infection an unequalled source of information on mammalian immunity, to the point that the differentiated T-helper lymphocyte responses, which led to the development of the paradigm of Th1-Th2 adaptive immune responses in the late nineteen eighties, were identified in the experimental infection of laboratory mice with *L. major* (Sacks & Noben-Trauth, 2002).

Besides their key role in immunology, research in leishmanioses is also stimulated by other factors. For one, leishmanioses are more likely to receive attention in the developed world. Unlike other tropical diseases, leishmanioses are endemic in Mediterranean countries of the European Union (Gállego, 2004). This enhances their “visibility” for the scientific community, particularly in comparison to relatively obscure (for us first-world dwellers) diseases such as trachoma or lymphatic filariosis. Furthermore, HuL is an emerging (or re-emerging) disease (Ashford, 2000; Shaw, 2007), critically, through HIV-coinfection in developed countries in the Mediterranean area (Alvar et al., 2008). Both facts help to make research in leishmanianioses more likely to attract

1 Whereas *Mycobacterium* and *Plasmodium* are responsible for thirty- and twenty-five-fold as many deaths as *Leishmania* respectively (World Health Organization, 2004), these ratios drop dramatically in counts of published research. Pubmed searches for these pathogens only yield slightly over four (*Mycobacterium*) or two (*Plasmodium*) times as many articles as for *Leishmania* (NCBI).

funding, than other tropical or neglected diseases.

A final reason for which the number of *Leishmania*-related scientific publications is further compounded is the relative feasibility of investigating natural and experimental *Leishmania* infection, even on limited or crude resources. Natural infection is a plentiful source of epidemiological, immunological, pathological and parasitological data, given the variety of species (parasites, vectors, and hosts) involved, and ecological, sociological and epidemiological settings of leishmanioses. Furthermore, since experimental infections by *Leishmania* spp can be readily induced in many hosts (Garg & Dube, 2006), they have led to the further "models of disease" of varying sophistication, popularity and validity. To add insult to injury, heterogeneity and even contradictions in the data obtained from natural and experimental infections have only helped to spawn further research into the different models, but only a few attempts at integrative research.

AIMS

As mentioned earlier, natural infection by *Leishmania* is shaped by innumerable variables. Although this provides plentiful data for descriptive studies, it severely hampers elucidation of cause-effect relations between observations and results in studies of the *Leishmania*-host relation. In this thesis, we attempt to present data that help advance a coherent picture of the host-parasite relationship in natural infections of mammalian hosts of *Leishmania infantum* in NE Spain. We have attempted to avoid a reductionist approach, and instead focus on the broad picture evaluating indicators of how healthy hosts respond to infection. The specific aims of this thesis are:

1. To evaluate the significance and how to interpret commonly used assays of specific cellular immunity against *Leishmania*, which is a key determinant of the outcome of infection.

2. To evaluate the impact of the *Leishmania* transmission season on indicators of infection and immune response in healthy hosts.

3. To describe cutaneous leishmaniosis by *L. infantum* in horses.

4. To investigate the status of domestic horses as hosts for *L. infantum.*

5. To advance an integrative interpretation of the significance of commonly used indicators of infection by *Leishmania* and immunity in susceptible hosts.

LITERATURE REVIEW

The large numbers of mammalian host, phlebotomine vector, and *Leishmania* species involved in *Leishmania* epidemiology yield a heterogeneous set of pathological and epidemiological processes affecting most mammalian taxa. Different combinations of sandlfy vectors, mammalian hosts, and *Leishmania* sp. constitute *nosodemiological units* (Ashford, 2000); unique systems of parasite maintenance involving a concrete set of hosts and vectors and displaying a defined pattern of nosological presentations. HuL can be ascribed to several such units, greatly defined by the *Leishmania* species involved and the clinical presentation of disease (Ashford, 2000).

HuL display largely dichotomic clinico-pathological patterns of disease, characterized by either cutaneous or systemic involvement (Cohen & Warren, 1982; Herwaldt, 1999). *Leishmania* was first described as the causative agent of a severe systemic disease known as kala-azar, or visceral leishmaniosis (HuVL), by Leishman and Donovan in the nineteenth century (Gibson, 1983). Other *Leishmania* sp were subsequently linked to focal benign skin lesions (cutaneous leishmaniosis, HuCL) as well as other, more rare, clinical presentations (Murray et al., 2005; David & Craft, 2009).

Human visceral leishmaniosis is a severe disease, officially accounting for 59000 deaths worldwide every year, although this may be a gross underestimate (Desjeux, 2004a; Bern et al., 2008; Reithinger, 2008). In the classical manifestation of HuVL, severe systemic clinical signs (including fever, splenomegaly, lymphadenopathy, pancytopenia and hypergammaglobulinemia) evolve over a variable time course, and ultimately lead to death if untreated (Gállego, 2004; Murray et al., 2005).

HuCLis far more prevalent in humans than HuVL (World Health Organization, 2004), but comparatively benign. It normally consists of single self-healing skin lesions which evolve over prolonged periods of time (Reithinger et al., 2007). Despite its benign nature, HuCL has a significant impact on affected populations because of its high prevalence (1-1.5 million new worldwide cases yearly), inherent social stigma and the potential for evolving to severe disease (Desjeux, 2004a; Reithinger, 2008).

Both HuCL and HuVL are also epidemiologically distinct, and each comprises different sets of *nosodemiological units*. Most instances of HuVL are caused by the *L.* (*donovani*) complex (*L. infantum* and *L. donovani*), which is distributed across a broad swath of tropical and temperate areas

in South America, Africa, Europe and Asia (Desjeux, 2004a; Reithinger, 2008). HuCL, on the other hand is caused by a different set of *Leishmania* species (*L. major*, *L. tropica*, *L. braziliensis*, and *L. mexicana*) found over a wider geographical range than those causing HuVL. HuCL is most prevalent in different countries than HuVL (Desjeux, 2004a; Bern et al., 2008).

This mainly dichotomous classification of leishmaniosis as of either cutaneous (benign) or systemic (severe) involvement is also applied to leishmanioses in other mammals. *Leishmania* sp. are also responsible for cutaneous disease in animals, such as *L. braziliensis* infection in horses (Vedovello Filho et al., 2008), *L. infantum* in cats (Navarro et al., 2010), or *L. major* in sand rats (Fichet-Calvet et al., 2003). On the visceral "front", *L. infantum* infection in dogs can lead to severe systemic disease (CaL), similar to HuVL (Baneth & Solano-Gallego, 2012). However, CaL presents a more complex nosology, and it is generally referred to as viscerocutaneous: although strictly cutaneous disease occurs, characteristic CaL presents with severe systemic involvement, commonly accompanied by dermatological lesions (Baneth & Solano-Gallego, 2012). Infections in laboratory animals and other experimental models with *Leishmania* sp also have been able to replicate patterns of disease akin to HuCL and HuVL (Garg & Dube, 2006).

Endemic leishmaniosis in Southern Europe belongs to a *nosodemiological unit* in which *Leishmania infantum* causes HuCL (or, more infrequently, HuVL) in children and aged adults (Ready, 2010). Several *Phlebotomus* sand fly species are implicated as vectors, transmitting the parasite from dogs, the main reservoir host (Ready, 2010; Baneth & Solano-Gallego, 2012). HuL in these areas is a relatively minor public health issue in healthy persons, disease is a rare occurrence and outbreaks warrant ad hoc reports in scientific literature (Noguerol Álvarez et al., 2012).

However, HuVL in Southern Europe emerged as an important complication in HIV-infected patients at the end of the twentieth century, and is among the more important complications of HIV infection (Alvar et al., 2008). By contrast, in South America HuVL by *L. infantum* (syn. *L. chagasi* [Maurício et al., 2000]), is a major public health concern. CaL there is therefore relevant as the reservoir of human disease, and is targeted by public health programs in Brazil and other South American countries in an attempt to control HuL (Alvar et al., 2004).

Unlike HuL, CaL by *L. infantum* in the ecoregions around the Meditarranean basin is a highly prevalent disease of domestic dogs, commanding much veterinary attention. Thus, dogs are not a "proper" reservoir host (Haydon et al., 2002) of *L. infantum*, as they themselves are prey to disease

(Baneth & Solano-Gallego, 2012). Leishmaniosis by *L. infantum* in these ecoregions is therefore characterized by a population of hosts which are highly susceptible to disease (domestic dogs), and which are considered the reservoir of the parasite for HuL. Involvement of other hosts, such as rats, is considered minor or incidental and may be dead-end hosts (Ashford, 1996).

Prevention, diagnosis and therapy of CaL therefore warrant much interest in pet dog medicine. In depth understanding of how infection progresses in dogs over time, from inoculation to disease and infectivity to vector sandflies is the necessary basis for proper clinical and epidemiological management of CaL. Although the body of scientific literature available on these subjects is vast, much remains to be understood before we have a sound and coherent understanding of the complex processes and human relations involved in CaL.

Acquired immune response to *Leishmania*

Host immunity is a key determinant of establishment and progression of infection by *Leishmania* sp. in mammals. The host immune responses to *Leishmania* have be-en extensively researched in the standard experimental model for HuL, *L. major* infection of inbred laboratory mouse strains. Through methods of varying refinement, research has successfully emulated clinical presentations of leishmaniosis ranging from rampaging multisystemic disease in “susceptible” mouse strains such as BALB/c, to mild self healing skin lesions in “resistant” strains such as C57BL/6 infected with *L. major* (Sacks & Noben-Trauth, 2002; McMahon-Pratt & Alexander, 2004). Highly refined implementations of this model have exposed exquisite details on the processes following parasite inoculation by sandflies, and throughout the course and outcome of infection (Belkaid et al., 2000; van Zandbergen et al., 2006).

T-helper 1 and T-helper 2 responses

In the mouse *L. major* experimental model, distinct forms of acquired immune response were shown to underlie the cutaneous vs. visceral pattern of disease, either keeping infection in check or failing to do so. Following inoculation into the mammalian host, *Leishmania* promastigotes are eventually phagocytosed by macrophages, wherein they transform into amastigotes. Whereas the host innate immunity plays a key role in the initial phase of infection leading up to phagocytosis, subsequent survival and ability to replicate and propagate within the macrophagic cell line is determined by the acquired (specific) immune response (Solbach & Laskay, 2000). Two distinct

subsets of T helper lymphocytes were shown to drive the specific immune response in inbred mice, towards either an effective response which controls infection and restricts lesions to the skin (Th1 lymphocytes), or an ineffective response which fails to curtail parasite replication and with ensuant systemic dissemination (Th2 lymphocytes) (SACKS & NOBEN-TRAUTH, 2002). In fact, this finding is the basis of the identification of dichotomic T helper lymphocyte responses and the subsequent development of the Th1/Th2 paradigm of mammalian acquired host immune response in the late nineteen eighties (MCMAHON-PRATT & ALEXANDER, 2004).

The downstream paths initiated in laboratory mice by distinct T-helper lymphocyte subpopulations 1 and 2 are largely independent and mutually exclusive, as different cytokines, intermediary molecules, and effector cell populations are involved in either response and can cross-down regulate (SOLBACH & LASKAY, 2000; SACKS & NOBEN-TRAUTH, 2002). Ultimately, a Th1-dominated immune response leads to abrogated parasite replication and clinical cure, whereas prevalence of Th2 responses leads to unchecked parasite replication, tendency to visceral involvement and, eventually, death.

Identification of Th1/Th2 responses in mice spurred many efforts to demonstrate distinct Th1 and Th2 responses to *Leishmania* in other mammalian hosts, albeit only with partial success. Unsurprisingly, the immune response in natural mammalian hosts is not as polarized as in inbred laboratory mice (KRAMER et al., 2006; TRIPATHI et al., 2007). Despite most of the molecules and pathways (or their counterparts) identified in murine Th1 and Th2 responses have also been identified in multiple mammal species, studies have failed to show such clear-cut patterns in these species. Indeed, the Th1/Th2 paradigm does not apply directly even in other murine leishmanioses. For instance, ongoing research has shown dissimilar roles and importances of signalling and effector molecules in different instances of *Leishmania* infections in mice (MCMAHON-PRATT & ALEXANDER, 2004). In fact, even the a priori simple model of *L. major* infection in inbred laboratory mice strains has shown nuances in which different molecules and mechanisms interact in more complex ways than initially thought (ALEXANDER & BRYSON, 2005).

Acquired immunity to Leishmania in the field: humoral and cellular responses

Paradoxically, the old and looser classification of immune response components as either cellular or humoral (WORLD HEALTH ORGANIZATION, 1969) still holds, to a point, and applies coarsely to observed responses to *Leishmania* infection by mammals, regardless of the underlying molecular

mechanisms. Predominance of either component of acquired immunity leads to opposing outcomes; control of infection and clinical cure, or unchecked parasite replication and runaway clinical symptoms respectively (Solbach & Laskay, 2000; Alvar et al., 2004; Murray et al., 2005). Cellular immunity can be grossly likened to Th1-type responses, in which macrophages are rendered competent to control *Leishmania* replication, and is considered protective. Likewise, Th2-like pathways underly humoral responses, ineffective in controlling *Leishmania* infections and which may even contribute to pathogeny, as happens in dogs (Alvar et al., 2004; Baneth & Solano-Gallego, 2012).

Long before the discovery of Th1/Th2 responses, it was recognized that HuCL and HuVL are linked to different patterns in the acquired host immune response to *Leishmania* (World Health Organization, 1969; Herwaldt, 1999). In HuCL, patients develop potent cell-mediated immunity (cellular immunity, CMI) which controls infection and prevents relapse. Practical, if unwitting, use of this fact predates modern medicine: to prevent visible scarring secondary to HuCL, infants were inoculated with *Leishmania* in the arm (Davies et al., 2003) , a procedure termed leishmanization. By contrast, in HuVL and disseminated and mucosal leishmanioses (both infrequent, but severe, forms of disease), specific humoral immunity is more prominent, and CMI weak or lacking (Murray et al., 2005).

Acquired immunity to Leishmania *in domestic dogs*

Like humans, dogs develop specific humoral and CMI responses to *L. infantum* infection. Humoral immunity is particularly relevant in CaL, both pathologically and diagnostically. Dogs suffering disease by *L. infantum* develop exuberant antibody levels which eventually result in a hallmark (practically pathognomonic) polyclonal gammapathy (Baneth & Solano-Gallego, 2012). Subsequent generation of circulating immune complexes cause life-threatening glomerulonephritis, and eventually result in renal failure, a major cause of death of dogs with leishmaniosis (Baneth & Solano-Gallego, 2012).

Exacerbated production of specific antibodies during disease has also historically been the basis for diagnosis of CaL (Slappendel & Greene, 1990). Even without using specific assays for *Leishmania*, elevated serum gammaglobulin levels in dogs from endemic areas are highly suggestive of CaL. Consequently, numerous serological assays have been developed and implemented to exploit this characteristic of CaL, ranging from crude agglutination tests to ELISAs against recombinant

antigens (Maia & Campino, 2008). Despite the development of more sophisticated tools such as PCR, serological assays remain the standard method for diagnosing and evaluating CaL (Maia & Campino, 2008; Solano Gallego et al., 2009; Baneth & Solano-Gallego, 2012).

Whereas the humoral component of the immune response in CaL has been researched over decades, mainly for its diagnostic potential, up to the 1990's there was very little interest in the canine CMI to *Leishmania*. In fact, at one point, dogs were actually considered anergic to *L. infantum* infection (Slappendel & Greene, 1990). Investigations into canine CMI to *Leishmania* infection only began in the 1990's following seminal studies of cell-mediated responses which uncovered an unexpected prevalence of infection by *Leishmania* in otherwise healthy dogs (Cabral et al., 1992; Pinelli et al., 1994). *L. infantum* infection in domestic dogs in ecoregions around the Mediterranean basin has since been shown to be highly prevalent, much more so than disease or even seropositivity (Baneth et al., 2008). Detection of parasite DNA in dogs in different studies demonstrated prevalences of infection greater than 50% which contrasted with much lower seroprevalence and prevalence of disease (Berrahal et al., 1996; Solano-Gallego et al., 2001; Leontides et al., 2002), and confirmed that the observed prevalences of CMI were due to current infection, with parasite presence (as opposed to “memory” responses). A significant about-turn in our understanding of CaL and *Leishmania* infections in mammals in general, these findings effectively shifted the emphasis in our understanding of CaL from infection to host immune response (Ferrer, 2002), vindicating the determinant role of specific host immunity in the course and outcome of *L.infantum* infection in dogs.

Acquired immunity to Leishmania *in other mammals*

Knowledge on the immune response elicited by *Leishmania* infections in other mammalian hosts is fragmentary, mostly deriving from empirical attempts at host or reservoir identification, as well as at clinical diagnosis. Serological studies of *Leishmania* infection are by far the more common form of immunological evaluation of putative hosts such as domestic ruminants or wild canids (Mukhtar et al., 2000; Alam et al., 2011; Millán et al., 2011; Jusi et al., 2011). However, in the natural and experimental host species where this has been researched, *Leishmania* has been shown to elicit both humoral and cellular responses (Dube et al., 1999; Garg & Dube, 2006). Although the paucity of data precludes conclusions on the prevalence of CMI in these species, it is valid to conclude that dual CMIr and humoral responses are universal in mammalian hosts of *Leishmania*.

Evaluating Cell Mediated Immunity

Immunological assays of leishmanioses target both cellular and humoral acquired responses (Murray et al., 2005; Maia & Campino, 2008). Humoral immunity is gauged by measuring circulating specific antibody levels, for which a vast number of assays have been advanced. Variety in these assays largely hinges on the *Leishmania* epitopes targeted (i.e. from whole cell homogenates to selected epitopes in recombinant proteins) and on how these antibodies are detected (i.e. latex agglutination to IgG isotype-specific conjugated secondary antibodies) (Gomes et al., 2006). A lot of effort has been put into cross-validating these assays for sensitivity and specifity, in search of a gold standard (Maia & Campino, 2008; Rodríguez-Cortés et al., 2010).

By contrast, evaluation of CMI presents more difficulties. Most assays of CMI are either in vivo tests, or must be performed shortly upon obtention of samples. Furthermore, they tend to be more complex than antibody assays, require more infrastructure and have lower throughput. These drawbacks may justify the lower number of studies which attempt to evaluate CMI. They are also a major obstacle in the furthering of our knowledge on the host-parasite interactions in leishmaniosis.

Assays of CMI against *Leishmania* sp. in mammalian hosts include a delayed-type hypersensitivity skin test (LST), assays evaluating ex vivo lymphocyte proliferation (LPA), and bioassays for in vivo and ex vivo detection of CMI-related molecules, chiefly Interferon-ɣ (Maia & Campino, 2008). These assays have been partially been made obsolete by the development of molecular assays such as reverse-transcriptase PCR of cytokine mRNA, and cytokine identification with species-specific antibodies. However, crude assays such as LST or LPA still afford some advantages; LST gauges in vivo responses and potentially yields comprehensive information on CMI, whereas LPA affords a gross evaluation of in vitro CMI as compared to, perhaps more fickle, expression patterns of molecules.

Leishmanin skin test

Developed by analogy with the Mantoux test, the leishmanin, or Montenegro, skin test (LST), was the first assay of specific cellular immunity used in leishmaniosis research (Montenegro, 1926). Like the Mantoux test in tuberculosis, a specific amount of inactivated antigen (leishmanin) is injected intradermally and the response measured as of induration at 72 hours (Cardoso et al., 1998). Possibly due to misunderstanding of the immune competence of dogs against *Leishmania*, (Slappendel & Greene, 1990), LST was only tardily applied, at the end of the 20th century, in dogs

in several studies and resulted in uncovering elevated prevalences of specific CMI against *Leishmania* in healthy dogs from endemic areas (Martínez-Moreno et al., 1995; Reithinger & Davies, 1999; Solano-Gallego et al., 2000). Although simple and requiring a minimal logistical framework in field studies, the need for revisiting the study subject at a precise time and variability inherent to in vivo assays hamper its applicability in broad field studies. Attempts at fine tuning the assay in order to enhance its prognostic value using selected antigens have only been partly successful (Todolí et al., 2010).

Lymphocyte proliferation assays

Together with LST, circulating lymphocyte proliferation assays (LPA) were the first CMI assays applied to research in CaL (Cabral et al., 1992; Molina et al., 1994; Pinelli et al., 1994). In LPA, circulating lymphocytes are stimulated in vitro with a pertinent *Leishmania* antigen and the proliferation of sensitized lymphocytes is measured following an adequate incubation period. Although the thimidine (H^3+) incorporation is considered the best method for measurement of proliferation, alternative, non-radioactive methods have been developed, of which bromo-deoxyuridine (BrdU) incorporation yields the best results (Wagner et al., 1999). Although more complex than LST, non-radioactive LPA presents a viable alternative for CMI evaluation in modern laboratory settings, without requiring revisiting the subject to make readings..

Cytokine bioassays

Evaluation of molecules involved in the processes underlying the phenotype of cellular or humoral immune responses has become central in mammalian immunology. Prior to the development and availability of molecular tools such as PCR and monoclonal antibodies, this was achieved chiefly through bioassays developed to (specifically) detect or measure the effect of the target molecule on a biological system (House, 1999; Meager, 2006). Although there has been a rather timid use of bioassays in CaL research, it spearheaded the advance in knowledge in canine immune response to *Leishmania* (Pinelli et al., 1994, 1995).

IFN-γ is a salient effector molecule in CaL, as it plays a key role in activating infected macrophages and renders them competent to kill phagocytosed amastigotes (Sacks & Noben-Trauth, 2002). Determination of IFN-γ activity can be achieved by measuring its antiviral effect on cultured cells (Pinelli et al., 1994; Iwata et al., 1996). Although this assay has critical drawbacks, including the need for biocontainment facilities due to the virus used, it was the only method available for

estimation of canine IFN-γ prior to the development of molecular assays.

Molecular assays

Where the above methods provide functional information on CMI, molecular assays can be designed to target specific mediator and effector molecules in acquired immunity. These assays rely either on polymerase chain reactions (PCR) or development of specific antibodies against target molecules.

Unlike humoral immunity, characterized by production of specific antibodies, the cellular component of acquired immunity lacks singular stable markers. Instead, assessment of cellular responses is dependent on in vivo or ex vivo evaluation of transient processes, and/or measurement of concentrations of short-lived molecules mentioned previously. Furthermore, antigenic targets of CMI assays tend to vary among species, requiring production of species-specific antibodies used to detect them. Because of this, CMI assays tend to be more sophisticated than serological assays, have lower throughput and are more subject to repeatability constraints. Standardization across CMI assays is also severely hampered, at times rendering interpretation of results more an art than science. All this also difficults their widespread use, particularly under field conditions. To complicate matters further, different tests of cellular immunity in fact target different processes involved in CMI, and an effective gold standard is lacking. Even further variability is due to the lack of standardized, easily obtainable antigens and antigen preparations.

Because of the difficulties involved, particularly with non-molecular assays, CMI is normally evaluated using a single assay. Very little effort has been advanced to standardize results across assays and to establish a consensual interpretation of CMI assays.

Infection in healthy dogs

Where CMI was disregarded or ignored as an integral part of the immune response to *Leishmania* in dogs, it led to gross misinterpretations of the host-parasite relationship. Strict application of Koch's postulates of infectious disease to leishmanioses implies disease is a necessary consequence of infection (Inglis, 2007). Under this classical model of disease, infected hosts either belong to a “reservoir species” (Haydon et al., 2002), and do not develop disease, or to a “susceptible species”

which upon infection inevitably develops disease following an incubation period which is described as lasting weeks to years for *Leishmania* (Slappendel & Greene, 1990; Alvar et al., 2004). However, the greatest breakthrough in leishmanioses came from the demonstration of infection in healthy dogs two decades ago, which could not be conceivably be written off as “incubating disease”. Contrary to domestic dog's perceived condition as anergic to *L. infantum* (Slappendel & Greene, 1990), studies in the early 1990's showed not every dog experimentally or naturally infected with *Leishmania* developed disease (Cabral et al., 1992; Pinelli et al., 1994; Cardoso et al., 2007). Furthermore, complete elimination of the parasite was shown to not be necessary for clinical healing (Pinelli et al., 1994; Killick-Kendrick et al., 1994).

Recognition of non-pathogenic infection by *Leishmania* in the 1990's coincided with application of PCR, and other exquisitely sensitive molecular biology assays. Together with data from reinterpretation and reimplementation of immunological assays, studies showed infection by *Leishmania* was shown to be much more common than disease. It thus resulted that non-pathogenic infection was not unique to “pure reservoir” host species (Ashford, 2000). Highly sensitive DNA detection assays such as PCR have even shown instances of prevalence of infection of up to 100% (Berrahal et al., 1996). Subsequent studies showed high prevalences of infection are the norm in exposed dogs and humans (Solano-Gallego et al., 2001; Riera et al., 2008).

The implications of such high prevalences of unapparent infection on our understanding of *Leishmania* epidemiology and pathogeny are profound. Some authors even adopted Thomas Khun's postulates (Kuhn, 2012) and recognized this as a paradigm shift (Ferrer, 2002). This new understanding has a direct bearing not only on clinical management of leishmanioses, but much more importantly, it completely subverts many of the undepinnnings of previous veterinary and public health programs targeting leishmanioses (Quinnell & Courtenay, 2009; Costa, 2011).

The effect of generalized non-pathogenig infection by Leishmania in susceptible hosts is highly relevant. Large populations of healthy infected hosts can have a massive effect on epidemiology of leishmanioses (Quinnell & Courtenay, 2009). How infectious these hosts are for vector sandflies can significantly affect overall rates of parasite transmission. Although healthy infected hosts have been shown to be hardly infective to sandflies, if at all (Costa et al., 2000; Courtenay et al., 2002), this does not necessarily rule out their participation in *Leishmania* epidemiology. Indeed, the lower infectivity of healthy infected dogs to sandflies may be countered by their high numbers, and they may, for instance, conceivably suffice to maintain the epidemiological cycle of *Leishmania*. For

example, where the failure of dog-culling programs to control *L. infantum* in Brazil was hitherto ascribed to “silent” phases of disease, the role of healthy infected hosts warrants research (Quinnell & Courtenay, 2009; Costa, 2011).

Infectivity of healthy hosts to sandflies depends on how accessible *Leishmania* are to feeding sandflies. Some attention has already been given to distribution of the parasite within these healthy hosts (Solano-Gallego et al., 2001; Travi et al., 2001). The role of “reservoir tissues or organs” in the transmission of disease thus emerges, ; if *Leishmania* is harbored in the skin, as opposed to spleen or other internal organs (Solano-Gallego et al., 2001), it can be picked up by sandflies, and thus be transmitted to other hosts.

Epidemiological repercussions aside, infection in healthy hosts warrants in depth reconsideration of the link between *Leishmania* infection and leishmanioses. Following Koch's postulates, infection in healthy hosts classically was ascribed to a prolonged “incubation period” over which infection evolved to eventually induce disease. However, this explanation is no longer tenable in view of the contrast between prevalence of infection and incidence of disease. Non-pathogenic infection thus arises as the most plausible explanation. This in turn makes the pathogen (*Leishmania*) a necessary, but not sufficient, cause of disease (it is interesting to note that this situation is not restricted to *Leishmania*, but also affects other pathogen-disease systems (Inglis, 2007).

Furthermore, it is not unreasonable to envision infection in healthy hosts not as a static condition, but rather a dynamic equilibrium. It is would therefore be the determinants of this equilibrium what ultimately lead to the different courses and outcomes of infection. The identification of non-pathogenic infection as a relevant entity therefore also warrants further research into the putative concomitant determinants of health and disease in *Leishmania*-infected hosts, and the evolution of the host-parasite interactions (Solano-Gallego et al., 2004).

The role of other hosts

Despite a plethora of *nosodemiological units* involving a wide range of mammals, description of natural disease by *Leishmania* sp is largely circumscribed to human and canine hosts (Herwaldt, 1999; Baneth & Solano-Gallego, 2012). Descriptions of natural disease in other hosts (Maia & Campino, 2011) are fewer and have been less documented.

Sandfly vectors of *Leishmania* sp. usually feed on more than one vertebrate species, and they show

varying preferences for the different host species on which they feed (KILLICK-KENDRICK, 1999; GÁLLEGO, 2004) This host preference in turn has a direct bearing on the pressure of infection by *Leishmania* on these hosts. Like their sandfly vectors, *Leishmania* sp. lack host-species specificity and almost all *Leishmania* species are known to infect more than one mammalian host (ASHFORD, 2000). Such lack of host specificity results in a wide range of mammalian species for which *Leishmania* infection has been reported. Indeed, natural infection by *Leishmania* has been observed in terrestrial mammals ranging from domestic dogs and wild carnivores (CURI et al., 2006; SOBRINO et al., 2008), to bats (DE LIMA et al., 2008) to kangaroos (DOUGALL et al., 2009), and more recently even artyodactils (BHATTARAI et al., 2010; LOBSIGER et al., 2010). Experimental infections have also been succesfully performed in many species such as hamsters and laboratory-bred primates (GARG & DUBE, 2006).

Description of *Leishmania* infections in non-human mammalian hosts is almost invariably from a human health perspective, as reservoirs (proven or else) of human disease (ASHFORD, 2000). In fact, instances of anthroponotic leishmanioses, such as urban *L. tropica* in Afghanistan or *L. infantum* in India (BERN et al., 2008), where other mammalian hosts do not partake in the epidemiology of leishmanioses are a rare exception. Indeed, humans are generally considered marginal, or even dead-end hosts in epidemiological cycles that usually involve several mammalian hosts, one or two of which act as main reservoirs. Rural cycles of leishmanioses can involve domestic and peridomestic animals, such as dogs and rats (*L. infantum* in South America and Mediterranean ecoregions) or equines (*L. donovani* in Sudan). *L. major* in North Africa also has a rural cycle, but with a wild reservoir host, a gerbil-like rat (*Psammomys obesus*) (ASHFORD, 2000). Other instances of human involvement are restricted to sporadic infection from strictly sylvatic epidemiological cycles of *Leishmania,* such as *L. panamensis*, maintained by sloths, in South America (BERN et al., 2008).

Despite extensive research into epidemiological involvement of different mammalian species in *Leishmania* epidemiology, reports on disease in hosts other than humans or dogs are rare, and there is little information available on the pathological consequences of *Leishmania* sp. infection in sylvatic or peridomestic mammalian hosts. Although experimental infections have been performed in some natural hosts in search for a cogent model of human disease, they shed little information on disease following natural infection in these species (WHITE et al., 1989). Furthermore, reservoir hosts are normally infected, and even infectious, in apparent absence of disease or lesions

(Brandão-Filho et al., 2003; Svobodová et al., 2006; Sobrino et al., 2008; Molina et al., 2012). Nonetheless, benign lesions associated to *Leishmania* sp. infection have been observed in some wild mammals (Fichet-Calvet et al., 2003; Rose et al., 2004), and clinical manifestations of infection may occur, perhaps infrequently, in most mammalian hosts.

The domestic cat is perhaps one such host. Although a rare condition, feline leishmaniosis by L. infantum has been reported sporadically in Europe. Clinical presentations are primarily cutaneous, although some cases of visceral involvement have been reported (Mancianti, 2004), and FIV seropositive cats have been found to be PCR positive in lymph node samples (Vita et al., 2005). As reports of feline leishmaniosis have accumulated, interest in the possible participation of cats in *L. infantum* epidemiology has increased (Mancianti, 2004; Solano-Gallego et al., 2007; Maia et al., 2008; Diakou et al., 2009). However, feline leishmaniosis remains a minor veterinary concern (Maia & Campino, 2011), as could be expected based on resistance to experimental infection observed in cats (Kirkpatrick et al., 1984), and is possibly the freak result of massive pressure of infection on domestic cats living in endemic areas. Infection of cats by other *Leishmania* sp. has also been reported, although they are likewise of little clinical relevance (Solano-Gallego & Baneth, 2012).

Besides dogs, horses and donkeys (*Equus* sp.)are the only domestic species for which a significant body of studies on *Leishmania* infection has been reported. Reports on detection of lesions by *L. braziliensis* in domestic equines in South America in the course of investigations into epidemic foci of HuCL became common in the 1980's (Grimaldi & Tesh, 1993). Although the parasite species affecting equine hosts was not investigated in all cases (Ramos-Vara et al., 1996), whenever it was, *L. braziliensis* was consistently identified (Ashford, 1996; Brandão-Filho et al., 2003). Where investigated, exposed equines were shown to develop humoral responses against *Leishmania*. Clinically, most cases of equine leishmaniosis by *L. braziliensis* present as highly prevalent benign single or multiple large nodular, sometimes ulcerated, skin lesions in horses, donkeys and mules (Aguilar et al., 1984, 1986). The benign nature of lesions by *L. braziliensis* in equines is in accordance with the absence of disease in horses in established endemic foci, in the face of elevated prevalences of infection detected in serological and parasitological surveys in healthy equines (Follador et al., 1999; Brandão-Filho et al., 2003; de Castro et al., 2005; Vedovello Filho et al., 2008). The frequent involvement in *L. braziliensis* foci has led to suspect domestic equines as reservoirs of human disease, although this remains unconfirmed (Vedovello Filho et al., 2008).

Equine involvement in Palearctic leishmanioses has been seldom reported. Despite domestic *Equus*

sp. are present throughout the rural areas endemic for leishmaniosis in Asia, Africa and Europe, they have received scant attention. However, equine infection cannot be ruled out, and indeed has been reported in some cases (MUKHTAR et al., 2000).

EXPERIMENTAL DESIGN

In the following chapters we describe five studies which attempt to improve our knowledge on the interactions between naturally infected mammalian hosts and *L. infantum* in NE Spain as previously described in the aims for this thesis.

The first is a methodological study in which we evaluate three assays of specific CMI to *L. infantum* in as indicators of CMI. Two studies on the host-parasite interactions between *L. infantum* and naturally infected dogs in Mallorca follow. The first study characterizes infection of grossly normal skin, a potentially critical point for transmission of *Leishmania* to sandflies, in healthy and sick dogs. How CMI and other immunological and parasitological parameters of *L. infantum* infection in dogs change after the transmission season is the subject of a subsequent study.

These investigations in CaL are complemented by two studies into a previously unknown host of *L. infantum*: domestic horses. The first report is a preliminary approximation to domestic horses as hosts for *L. infantum*, including pathological, immunological and parasitological data. This was followed up by an extended investigation into the immune responses by healthy horses to *L. infantum*, to help characterize their status as hosts of the parasite.

CHAPTERS 2–6: PUBLICATIONS

“one of the classic features of science [is that] in explaining something,
you've merely redefined the unknown.”
Robert M. Sapolsky, *Monkeyluv*

CHAPTER 2

Comparison of three assays for the evaluation of specific cellular immunity to *Leishmania infantum* in dogs

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Comparison of three assays for the evaluation of specific cellular immunity to *Leishmania infantum* in dogs

Hugo Fernández-Bellon [a], Laia Solano-Gallego [b], Alhelí Rodríguez [b], Victor PMG Rutten [c], Aad Hoek [c], Antonio Ramis [a,*], Jordi Alberola [b], Lluís Ferrer [a]

[a] *Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, Spain*
[b] *Departament de Farmacologia, Terapèutica i Toxicologia, Universitat Autònoma de Barcelona, Spain*
[c] *Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands*

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Abstract

Cellular immune response to *Leishmania* plays a key role in canine leishmaniosis. However, there are few assays to evaluate this response in the dog. Here, we evaluated and compared three assays of specific cellular immune response to *Leishmania infantum* in dogs: the leishmanin skin test (LST), lymphocyte proliferation assay (LPA) and IFN-γ cytopathic effect inhibition bioassay (IFNB). Fifty-six healthy dogs from an endemic area for leishmaniosis on the island of Mallorca were studied. In all, 37 dogs showed a positive LST and 32 a positive IFNB, 24 were positive for both assays. The 17 dogs positive for LPA also gave positive results for either LST or IFNB, or both. These findings indicate that although LST is the method of choice, IFNB is a complementary test. Therefore, both assays should be performed and analyzed jointly. In comparison with LST and IFNB, LPA is much less sensitive, and yields many false negative results.

Keywords: Dog; *Leishmania infantum*; Cellular immune response; Lymphocyte proliferation; Interferon-γ; Leishmanin skin test

1. Introduction

The term leishmaniosis includes a number of diseases with distinct clinical symptoms, transmission routes, vectors, hosts and geographical distributions caused by different species of flagellate protozoans of the genus *Leishmania*. Despite the heterogeneity of these diseases, the host's immune response to the parasite is always crucial for the outcome of infection. In inbred mice strains, the predominance of a T-helper

Abbreviations: IFNB, IFN-γ cytopathic effect inhibition bioassay; LPA, lymphocyte proliferation assay; LSA, *Leishmania infantum* soluble antigen; LST, leishmanin skin test

* Corresponding author. Present address: Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, E-08193, Campus UAB, Spain. Tel.: +34 935813144; fax: +34 935813142.
E-mail address: antonio.ramis@uab.es (A. Ramis).

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control. An induration diameter of >5 mm after 72 h was considered positive.

2.2.1. Extraction and stimulation of peripheral blood mononuclear cells (PBMC)

PBMC extraction and stimulation were performed for both LPA and IFNB. Ten millilitres of heparinized blood was collected by jugular venopuncture from each dog and shipped at room temperature to the laboratory. On arrival, the samples were diluted in 10 ml sterile PBS, and PBMC were collected after centrifugation on Histopaque 1077 (Sigma–Aldrich Chemie Gmbh, Germany) density gradient at 400 g for 30 min. After one wash in PBS, the cell pellet was resuspended for 2 min in an erythrocyte lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2 EDTA and pH 7.4) and washed again in PBS. Trypan blue exclusion was used to count viable PBMC, after which the cells were resuspended at 10^6 cells/ml in culture medium (RPMI supplemented with 10% FCS, 2 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, penicillin (100 U/ml) and streptomycin (100 μg/ml)).

PBMC were stimulated with either *Leishmania infantum* soluble antigen (LSA) (kindly provided by Dr. Montserrat Gallego, Departament de Parasitologia, Universitat de Barcelona) at 10 μg/ml or phytohemaglutinin (PHA) (Sigma–Aldrich Chemie Gmbh, Germany) at 5 μg/ml. Unstimulated cells were incubated as negative controls in every case.

2.2.2. Lymphocyte proliferation assay

Proliferation of circulating lymphocytes to LSA and PHA was detected by measuring the incorporation of BrdU by replicating cells into newly synthesized DNA. Stimulated PBMC were incubated in triplicate in flat-bottomed 96-well polypropylene microtiter plates (2×10^5 cells/well, 200 μl total volume) for 3 days (PHA) or 6 days (LSA). Wells with 200 μl of complete medium only were included in each plate to measure background. Twenty-four hours prior to the end of incubation, BrdU (10 μM) was added to each well. At the end of incubation, plates were centrifuged ($300 \times g$, 10 min), the supernatants were then discarded, and the plates were warm-air dried and stored at 4 °C. BrdU incorporation was detected using a commercial ELISA kit (Cell Proliferation ELISA, BrdU, Roche Diagnostics GmbH, Germany) within 7 days. Cell proliferation was expressed as a stimulation index (SI): OD stimulated cells-OD background/OD unstimulated cells-OD background: values >2 were considered positive.

2.2.3. Interferon-γ cytopathic effect inhibition bioassay

A modified viral cytopathic effect inhibition assay (Pinelli et al., 1994b) was used to detect IFN-γ produced by circulating lymphocytes in cultured supernatants. Briefly, stimulated and unstimulated PBMC were incubated in 12-well polypropylene plates (10^6 cells/ml, total volume = 1 ml) for 72 h, at which time the culture medium was removed and centrifuged and the supernatants were then stored at −80 °C. Two-fold dilutions of supernatant were incubated for 16 h with 10^5 Madin-Darby canine kidney cells (MDCK2) in flat-bottom 96-well polypropylene microtiter plates in RPMI-1640 medium (total volume 100 μl) at 37 °C and 5% CO_2. Cultured supernatants were then removed, and adherent cells were infected with 50 μl of vesicular stomatitis virus at 10^5 pfu/ml. After further incubation for 24 h, supernatants were again discarded and surviving cells were stained with 50 μl 0.75% crystal violet in 0.9% formaldehyde for 15 min. Production of IFN-γ was expressed as the ratio of the reciprocal of the maximum dilution that protected 50% of the cell monolayer (as estimated visually) of stimulated versus unstimulated cells: values >2 were considered positive.

3. Results and discussion

Specific cellular immune response to *Leishmania* as detected by LPA, IFNB and LST was measured in 56 healthy dogs in an area with endemic *Leishmania infantum* on the island of Mallorca. Twenty-eight of these dogs were Ibizan hounds, the predominant breed in the region (Table 1).

PBMC stimulation with PHA was used as a positive control for the LPA and IFNB assays. Briefly, all but three dogs were positive either by proliferation or IFN-γ production upon stimulation with PHA, 44 out of 56 were positive for both IFNB and LPA. None of the PHA negative dogs were positive for LSA by the same assay. However, some dogs presented an in vivo

Table 1
Results of cellular immunity assays in 56 dogs

Dog	Breed	LPA–PHA	IFNB–PHA	LST	LPA–LSA	IFNB–LSA
1	Ibizan hound	+	+	+	−	+
2	Ibizan hound	+	+	+	−	+
3	Ibizan hound	+	+	+	−	−
4	Ibizan hound	+	+	+	−	+
5	Ibizan hound	+	+	−	−	−
6	Ibizan hound	+	+	+	−	−
7	Saint bernard	+	+	+	+	+
8	Ibizan hound	+	+	+	+	+
9	Ibizan hound	+	+	+	+	+
10	Ibizan hound	+	+	+	+	+
11	Ibizan hound	+	+	+	−	+
12	Ibizan hound	+	+	+	−	+
13	Border collie	+	+	+	+	+
14	Border collie	+	+	+	+	+
15	Crossbred	+	+	+	−	+
16	Border collie	+	+	+	−	+
17	Crossbred	+	+	−	+	+
18	Crossbred	+	+	−	−	−
19	Crossbred	+	+	−	−	+
20	Ibizan hound	+	+	+	−	+
21	Ibizan hound	+	+	+	−	+
22	Ibizan hound	+	+	+	−	+
23	Ibizan hound	+	+	+	+	+
24	Ibizan hound	+	+	−	−	+
25	Ibizan hound	+	−	−	−	−
26	Ibizan hound	+	+	−	−	−
27	Ibizan hound	+	+	+	−	−
28	Ibizan hound	+	+	+	+	+
29	Ibizan hound	+	+	+	−	−
30	Ibizan hound	+	+	+	+	+
31	Ibizan hound	+	+	+	+	+
32	Ibizan hound	+	+	+	−	+
33	Ibizan hound	+	+	+	−	+
34	Ibizan hound	+	−	−	−	−
35[a]	Ibizan hound	+	−	+	−	−
36	Ibizan hound	+	+	−	−	+
37	Bulldog	+	−	−	−	−
38	Pug	+	+	+	−	−
39	Pug	+	+	+	+	−
40	Dachshund	+	+	+	−	−
41[a]	Epagneul breton	+	−	+	−	−
42[a,b]	Pointer	−	−	+	−	−
43	Portuguese warren hound	+	−	−	−	−
44	Crossbred	+	−	−	−	−
45	Crossbred	−	−	−	−	−
46	Crossbred	−	−	−	−	−
47[b]	Dachshund	−	+	+	−	−
48	Dachshund	+	+	+	+	+
49	Epagneul breton	+	+	−	+	+
50	Bull mastiff	+	+	+	−	−
51	Pug	+	+	+	+	+
52	Crossbred	+	+	−	−	+
53	German short haired pointer	+	+	−	+	+

Table 1 (*Continued*)

Dog	Breed	LPA–PHA	IFNB–PHA	LST	LPA–LSA	IFNB–LSA
54[a]	Crossbred	+	−	+	−	−
55	Old Spanish pointer	+	+	−	−	−
56	Epagneul breton	+	+	−	+	+

Results of the cellular immunity assays of the 56 dogs included in this study. LST, leishmanin skin test (positive result: induration >5 mm); LPA, lymphocyte proliferation assay to phytohemaglutinin (LPA–PHA) or *Leishmania infantum* soluble antigen (LPA–LSA), positive result, stimulation index >2; IFNB, IFN-γ production after stimulation with phytohemaglutinin (IFNB–PHA) or *Leishmania infantum* soluble antigen (IFNB–LSA), positive result, stimulation index >2.

[a] LST positive dogs with undetectable IFN-γ production after PHA stimulation.

[b] LST positive dogs with undetectable lymphocyte proliferation after PHA stimulation.

specific response to the *Leishmania* antigen by LST, even in the absence of a detectable response to PHA by one or both in vitro assays of the 11 dogs with undetectable IFN-γ production after PHA stimulation, 4 were LST positive, and of the 4 dogs with undetectable lymphocyte proliferation to PHA and 2 were LST positive (Table 1).

Regarding the *Leishmania infantum*-specific cellular immunity, the LST yielded the highest number of positives, with 37 dogs (66%). The IFNB gave similar results, with 32 dogs (57%) positive. In contrast, only 17 (30%) dogs were positive for the LPA. Overall, most of the dogs presented specific cellular immune response to *Leishmania* and only 11 (20%) gave negative results for all three tests (Table 1).

The groups of dogs positive for the three tests only partially overlapped (Fig. 1). Twenty-nine of the 45 dogs with a detectable cellular immune response to *Leishmania* were positive for more than one assay. Most of these were positive either for all three assays (12 dogs) or for LST and IFNB alone (12 more dogs). On the other hand, 16 dogs were positive only for one assay: 12 were positive only for LST and 4 positive only for IFNB. No dogs were positive for LPA only.

The accurate determination of specific cellular immune response to *Leishmania* in dogs is a crucial indicator of a Th1-like phenotype associated with an effective control of host infection and survival. A practical and standardized assay for cellular immune response evaluation would be applicable in clinical settings both to monitor the evolution of leishmaniosis and response to treatment and to help establish prognosis. More importantly, current efforts to develop vaccines for canine leishmaniosis depend greatly on the accurate and reproducible assessment of specific cellular immunity induced by candidate vaccines. Here, we examined three such assays under field conditions to test their applicability and significance.

Predictably, many of the dogs studied (80%) presented specific cellular immune response to *Leishmania*. This figure is not epidemiologically significant: the dogs lived outdoors in an area with endemic *Leishmania*; they were healthy (as opposed to leishmaniotic dogs, with an ineffective cellular immunity to the parasite), and the predominant breed was the Ibizan hound, which shows a high prevalence of cellular immunity to *Leishmania* (Solano-Gallego et al., 2000).

The LST yielded the highest number of positive results, closely followed by IFNB, whereas LPA was the least sensitive assay. In addition to the differences

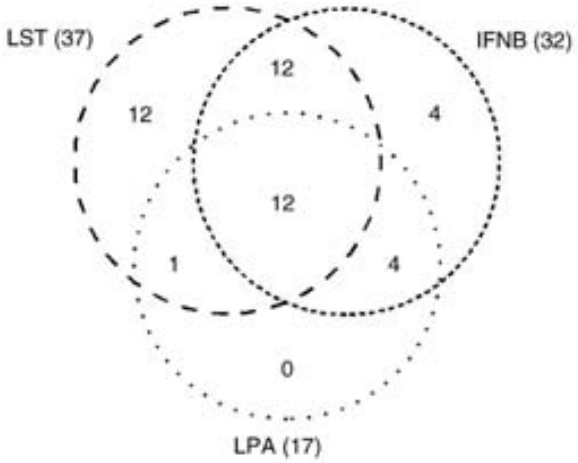

Fig. 1. Summary of the results of three assays for specific cellular immunity to *Leishmania infantum* for the 45 dogs that were positive for at least one assay. The animals positive for each assay are included in the respective circle, animals positive for more than one assay are in the areas where the circles overlap. In parentheses, total number of positive results for each assay. LST, leishmanin skin test; IFNB, IFN-γ cytopathic effect inhibition bioassay (stimulation with *Leishmania infantum* soluble antigen (LSA)) and LPA, lymphocyte proliferation assay (stimulation with LSA).

in prevalence, the three assays presented incongruous results. Thus, the selection of LST as the "golden standard" is not supported by the results because eight dogs were positive for IFNB but negative for LST, and, in turn, 13 of the dogs were positive for LST but negative to IFNB. The discrepancies between the results of the three assays are further reflected by the observation that anergy to PHA, as detected by one or both in vitro assays, did not preclude a positive *Leishmania*-specific response with another assay (Table 1). These results indicate that although the three assays assess the cellular component of the immune response to *Leishmania*, they measure distinct aspects of this response. These assays are therefore complementary.

Our results also indicate that LST and IFNB can be used for the evaluation of specific cellular immune response to *Leishmania* in dogs. However, in the case of IFNB the sensitivity is not as high as desired (the test was negative in 11 dogs after stimulation with PHA), possibly as a consequence of the bioassay used in the present study, which probably is less sensible than other methods of measuring IFN-γ as the ELISA. The weak anti-viral activity of IFN-γ could be part of the explanation of the low sensitivity of this bioassay.

In all, and despite its simplicity and having been described over 75 years ago, LST is still the method of choice, particularly when the sophisticated laboratory equipment required for the in vitro assays is not readily available. IFN-γ production by stimulated PBMC, although less sensitive than LST in this study, warrants further research. The recently marketed assays using specific anti-canine IFN-γ mAbs (ELISA, flow cytometry) will probably increase the sensitivity of IFN-γ detection, while eliminating the need to work with vesicular stomatitis virus, an OIE list A pathogen. A slight increase in sensitivity would probably make IFN-γ quantification the method of choice for the evaluation of specific cellular immune response in dogs.

LPA showed a lower sensitivity, which might be partially due to the use of a non-radioactive method instead of [^{3}H+], although the method used in our research has been validated for canine PBMC (Wagner et al., 1999). It is also plausible that lymphocyte proliferation per se is not as sensitive as LST or IFNB as an indicator of specific cellular immunity to *Leishmania*.

In summary, on the basis of our results, we conclude that LST and IFNB are the most sensitive assays for the detection of specific cellular immunity to *Leishmania* in dogs. However, since the positive results of these two assays partially overlap dog populations, they must be analyzed jointly.

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CHAPTER 3

Histological and immunohistochemical study of clinically normal skin of *Leishmania infantum*-infected dogs

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Histological and Immunohistochemical Study of Clinically Normal Skin of *Leishmania infantum*-infected Dogs

L. Solano-Gallego, H. Fernández-Bellon*, P. Morell†, D. Fondevila*, J. Alberola, A. Ramis* and L. Ferrer*

*Departament de Farmacologia, Terapèutica i Toxicologia and *Departament de Medicina i Cirurgia Animal, Facultat de Veterinària, Universitat Autònoma de Barcelona 08193, Bellaterra, Barcelona, and †Centre Sanitari Municipal, Palma de Mallorca, Spain*

Summary

Skin lesions are the most usual manifestation of canine leishmaniosis. The aim of this study was to investigate the histological pattern and parasite load in clinically normal skin of *Leishmania*-infected dogs. Two groups of *Leishmania*-infected dogs were studied. Group A consisted of 15 symptomless animals which, although seronegative or only mildly seropositive, gave a positive polymerase chain reaction (PCR) for *Leishmania* in the skin. Group B consisted of 20 clinically affected dogs which were highly seropositive and PCR-positive. Biopsies of normal skin from all dogs were processed for routine histology and *Leishmania* immunohistochemistry. The study demonstrated microscopical lesions and the presence of parasites in the skin from dogs of group B, but not group A. The results cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniosis. In contrast, however, the clinically normal skin of sick dogs harbours the parasite and probably plays a role in the transmission of leishmaniosis.

Keywords: dog; *Leishmania infantum*; leishmanial transmission; parasitic infection

Introduction

Canine leishmaniosis is endemic in the Mediterranean basin and, in most cases, is caused by the parasite *Leishmania infantum*. The main clinical findings are skin lesions, local or generalized lymphadenopathy, loss of body weight, glomerulopathy, ocular lesions, epistaxis and lameness. Non-pruritic skin lesions are the usual manifestation, and several forms have been described, such as exfoliative dermatitis and alopecia, and ulcerative, nodular and pustular dermatitis. The histopathological picture of such skin lesions commonly consists of a diffuse granulomatous inflammatory reaction with variable numbers of plasma cells and parasites in the dermis (Slappendel and Ferrer, 1998). Recently, several reports described a high prevalence of infection by *Leishmania* in dogs throughout the Mediterranean basin, as demonstrated by a variety of methods such as the determination of specific humoral and cellular immunity and the presence of leishmanial DNA. These investigations demonstrated that in an endemic region there is a large population of *Leishmania*-infected but clinically healthy dogs and a smaller proportion of dogs with clinically patent leishmaniosis (Berrahal *et al.*, 1996; Cabral *et al.*, 1998; Solano-Gallego *et al.*, 2000; Solano-Gallego *et al.*, 2001). Many different research groups are currently investigating the role played by these infected but symptomless dogs in the epidemiology

Correspondence to: A. Ramis.

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of leishmaniosis. The skin, which is considered an important tissue reservoir of parasites in healthy and sick *Leishmania*-infected dogs (Abranches *et al.*, 1991; Solano-Gallego *et al.*, 2001), is clearly of epidemiological importance. Despite this, however, the parasitic load and the microscopical changes in clinically normal skin of *Leishmania*-infected dogs have not been investigated. The aim of the present study was to make good this deficiency.

Materials and Methods

Animals

The study was carried on the island of Mallorca, an endemic area of canine leishmaniosis. The subjects were 35 dogs of various breeds and ages which were to be humanely destroyed at the animal pound of Palma de Mallorca for reasons relating to the city sanitation policy. The samples from these animals were selected from a pool of samples collected in Mallorca by our research group in recent years. The skin of all 35 dogs studied was polymerase chain reaction (PCR)-positive for *Leishmania*. The animals were, however, divided into two groups, A (symptomless) and B (clinically affected). Group A consisted of 15 clinically healthy dogs, with "background", low or medium concentrations of anti-*Leishmania* IgG antibodies; and group B consisted of 20 dogs with patent leishmaniosis and high concentrations of anti-*Leishmania* IgG antibodies. Before sampling and euthanasia, all dogs were examined for clinical signs compatible with leishmaniosis (skin lesions, local or generalized lymphadenopathy, loss of body weight). They were then premedicated with acepromazine maleate and anaesthetized intravenously with sodium thiopental. After the collection of samples (see below) the animals were killed by an overdose of sodium thiopental.

Collection and Examination of Serum Samples

Blood was collected by venipuncture to supply serum samples; these were stored at −20 °C for examination later by an ELISA (with *Leishmania infantum* antigen and Protein A peroxidase conjugate; Riera *et al.*, 1999) to detect and quantify leishmanial specific antibodies.

Collection and Examination of Skin Samples

Duplicate cutaneous samples were collected by 5-mm punch biopsy from the upper part of the muzzle, as this is the area where sandflies preferentially feed (Killick-Kendrick and Killick-Kendrick, 1999). The skin of the area from which the biopsies were taken was always macroscopically normal. One of each pair of skin samples was fixed in 10% neutral buffered formalin for routine histological and immunohistochemical examination (Ferrer *et al.*, 1988), and the other was stored at −20 °C for DNA extraction and PCR (Solano–Gallego *et al.*, 2001), with primers originally described by Rodgers *et al.* (1990).

The dermal inflammatory pattern and the cell population were evaluated histologically on haematoxylin and eosin (HE)-stained sections. The inflammatory infiltrate was graded as follows; −, no inflammatory infiltrate; +, isolated foci of inflammatory cells; ++, isolated to coalescing areas of inflammatory infiltrate; +++, diffuse areas of inflammatory infiltrate.

The parasite load was determined in immunolabelled sections as the average number of micro-organisms counted in five × 400 fields of areas with inflammatory infiltrate (Ridley and Ridley, 1983): −, no micro-organisms; +, 1–10; ++, 11–30; +++, >30.

Results

The skin biopsies from the symptomless group A animals had negligible if any lesions, and immunohistochemical examination failed to reveal *Leishmania* amastigotes. By contrast, 17 out of 20 clinically affected group B dogs had significant microscopical lesions, and 14 of these animals were shown immunohistochemically to have *Leishmania* amastigotes in the skin (Table 1).

In the 17 group B cases with microscopical lesions, the most common histopathological pattern was perifollicular dermatitis, mainly around the isthmus (Fig. 1). The inflammatory infiltrate generally extended to encompass the sebaceous glands, even obliterating them in some cases. A perivascular to diffuse dermal infiltrate usually accompanied the perifollicular inflammation. The intensity of the inflammatory infiltrate is shown in Table 1. Macrophages were predominant, with scattered lymphocytes and plasma cells. Isolated neutrophils could also be seen in some sections (Fig. 2). In three cases, the dermatitis extended to affect the whole hair follicle. Sebaceous adenitis was seen in six cases (data not shown in Table 1). The parasite load within the lesions, evaluated on immunolabelled sections, was extremely variable, ranging from 1 or 2 to over 100 amastigotes per high power field (Table 1, Fig. 3). Both the microscopical lesions and the parasite load were

Table 1
Inflammatory infiltrate (graded histologically) and parasite load (graded immunohistochemically) in apparently normal skin from 20 dogs with clinical signs of leishmaniosis (group B)

Dog	*Inflammatory infiltrate**	*Parasite load†*
1	−	−
2	+++	++
3	−	−
4	++	++
5	++	++
6	+	−
7	+	+
8	+	+
9	+	−
10	+	+++
11	++	+++
12	++	+
13	++	+++
14	+	++
15	+	++
16	+	++
17	+	−
18	+++	+
19	+	+
20	−	−

*For method of grading, see Materials and Methods.
†For method of grading, see Materials and Methods.

more severe in the mid-dermis than in the superficial or deep dermis.

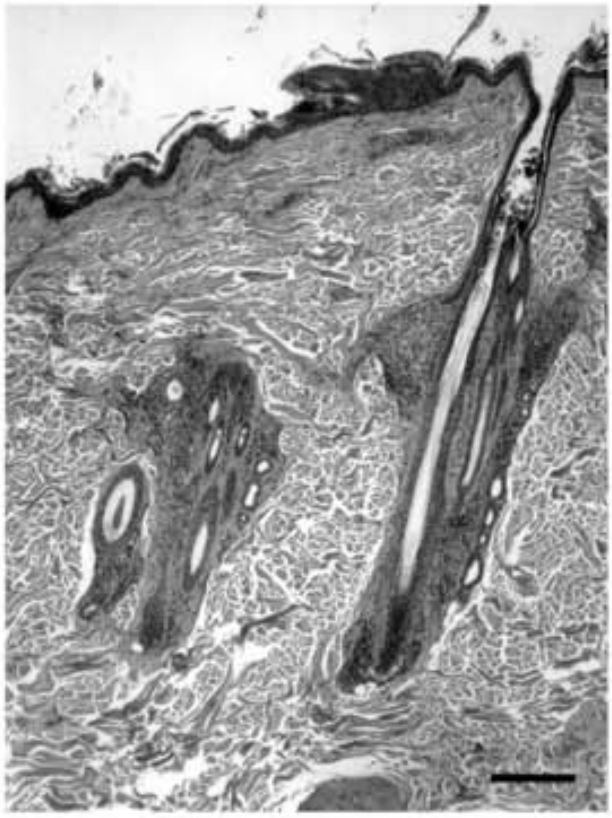

Fig. 1. Perifollicular dermatitis centred around the isthmus affecting the sebaceous gland. A mild superficial perivascular dermatitis is also present. HE. Bar, 400 μm.

Discussion

Apparently normal areas of skin in the majority of clinically affected dogs showed microscopical lesions and large numbers of amastigotes. The most severe lesions and the greatest parasite loads were located around the hair follicles, mainly around the isthmus, associated with the middle vascular plexus of the dermis. This finding was suggestive of haematogenous dissemination of the parasite and tropism for the skin.

Muzzle skin of symptomless dogs had no demonstrable microscopical lesions or amastigotes. This, together with the positive PCR results for *Leishmania*, indicates that the number of parasitic elements in the skin samples from the muzzle must have been very low. Little is known of the pathogenesis of natural infection by *Leishmania* in the dog. However, studies of experimental *Leishmania major* infection in mice have re-examined the basic relationship between parasite growth, persistence, dissemination, lesion formation and immunity (Belkaid *et al.*, 2000; Kamhawi *et al.*, 2000; Nicolas *et al.*, 2000). According to these studies, two possible scenarios might account for the positive PCR results in the apparently healthy skin of symptomless dogs. The first scenario is based on the notion that parasites detected by the PCR were the result of recent inoculation by sandflies. This would correspond to the silent phase of infection described by Belkaid *et al.* (2000) in experimental *L. major* infections in mice; in this silent phase, which lasts 4–6 weeks, parasites multiply in the dermis without the formation of macroscopical or microscopical lesions. The second scenario is based on the notion that the skin is a reservoir of amastigotes, when the host is immunocompetent, in a host–parasite equilibrium similar to that of the final phase of infection in the *L. major* mouse model. In the mouse model, the final phase is characterized by the persistence of 100–10 000 parasites, primarily within macrophages, at the cutaneous site of inoculation for up to one year following resolution of the skin lesions (Belkaid *et al.*, 2000).

In this second scenario, the persistence of such a parasite load might account for the constant stimulation of memory T cells, thus providing protection from the reinfection that can occur

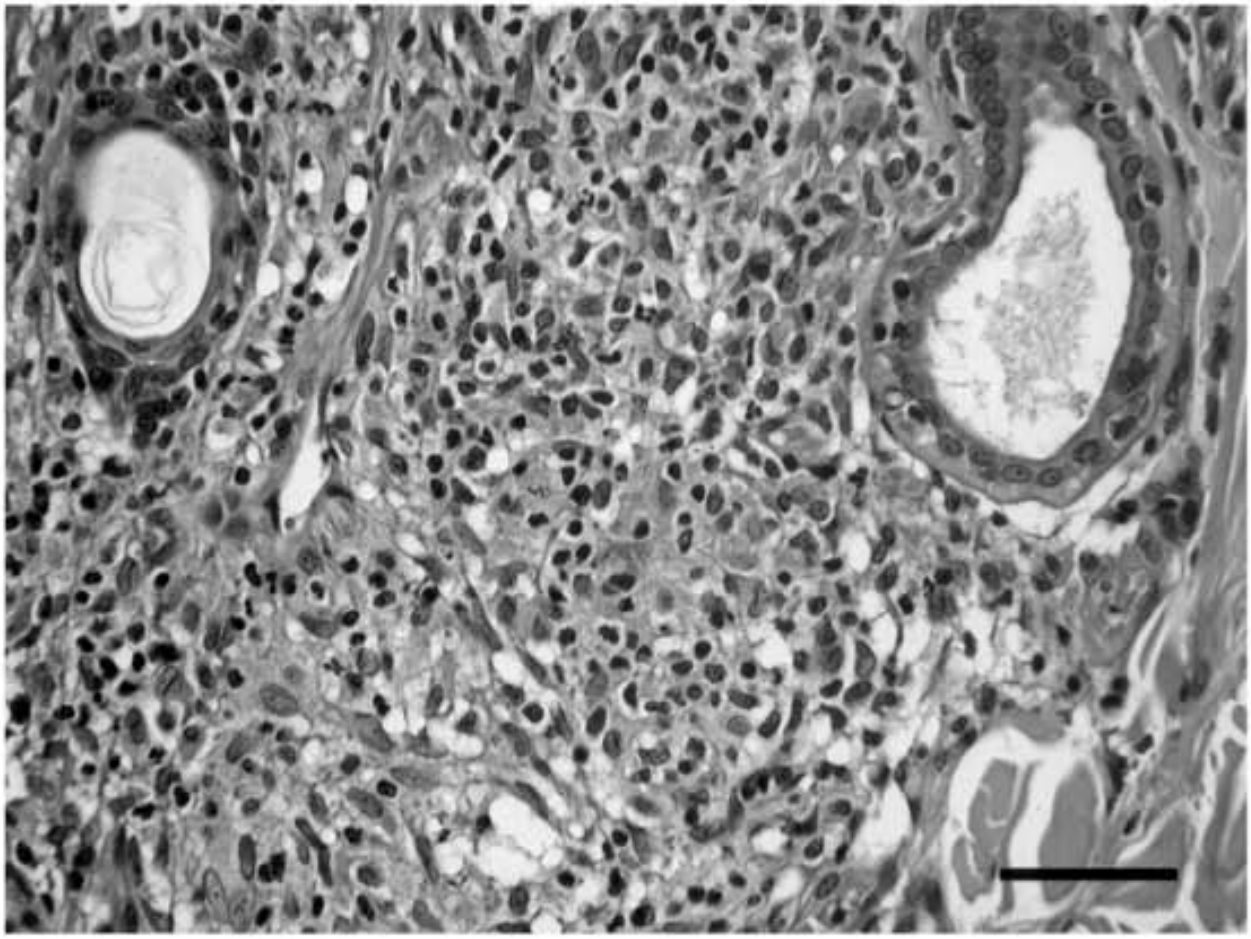

Fig. 2. Periadnexal inflammatory infiltrate composed of macrophages with scattered lymphocytes, plasma cells and neutrophils. HE. Bar, 50 µm.

yearly in dogs living in endemic areas (Aebischer *et al.*, 1993). T-cell memory would be short-lived in the absence of antigen stimulation (Gray and Matzinger, 1991). This would be a situation similar to that seen in human beings (Schubach *et al.*, 1998a,b) and mice (Aebischer *et al.*, 1993; Belkaid *et al.*, 2000; de Rossell *et al.*, 1992; Nicolas *et al.*, 2000), which remain infected but clinically healthy for extended periods of time. In any case, the possible infectivity for sandflies of symptomless but PCR-positive dogs is a question of major epidemiological relevance, to which histological or immunohistochemical techniques cannot give a definitive answer. Attempts should be made to resolve this issue by xenodiagnosis, i.e., by evaluating the infectiveness of such dogs for phlebotomine vectors feeding on them (Molina *et al.*, 1994).

Our hypothesis is that symptomless dogs found positive to *Leishmania* by the PCR technique do not

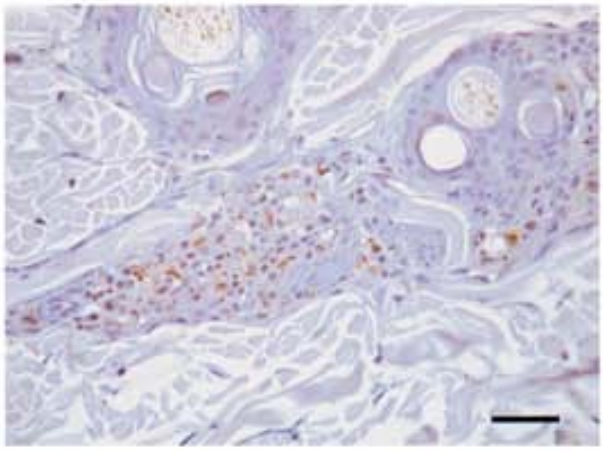

Fig. 3. Immunohistochemical labelling (brown) of numerous amastigotes in the inflammatory infiltrate. Avidin–biotin complex immunoperoxidase technique, haematoxylin counterstain. Bar, 50 µm.

play a significant role in infection of phlebotomine sandflies. Several studies on *Leishmania*-infected dogs support this hypothesis. Thus, Molina (1997) reported that the proportion of infected sandflies increased with the appearance and severity of the clinical signs. Moreover, a recent study (Courtenay *et al.*, 2002) in Brazilian dogs indicated that the best predictor of infectiveness was antibody titre and clinical disease, no dogs having been found to be infective before the detection of anti-*Leishmania* IgG antibodies.

In conclusion, the results of the present study cast doubt on the relevance of infected but symptomless dogs in the epidemiology of canine leishmaniosis. In contrast, however, they demonstrate that most of the skin of sick dogs—not merely the skin with macroscopically patent lesions—harbours parasites and probably plays a role in the transmission of infection.

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CHAPTER 4

Little evidence of seasonal variation of natural infection by *Leishmania infantum* in dogs in Spain

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Little evidence of seasonal variation of natural infection by *Leishmania infantum* in dogs in Spain

H. Fernández-Bellon [a], L. Solano-Gallego [b,f], A. Rodríguez-Cortés [b], L. Ferrer [c], M. Gallego [e], J. Alberola [b], A. Ramis [a,d,*]

[a] *Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[b] *Departament de Farmacologia, de Terapèutica i de Toxicologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[c] *Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[d] *Centre de Recerca en Sanitat Animal (CreSA) UAB-IRTA, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[e] *Departament de Parasitologia, Universitat de Barcelona, Avinguda Joan XXIII, 08028, Barcelona, Spain*
[f] *Department of Pathology and Infectious Diseases, Royal Veterinary College, London, UK*

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Abstract

Leishmania infantum, the etiological agent of canine leishmaniosis in the Mediterranean region, is vectored by *Phlebotomus* spp sandflies, which are active during the warmer months of the year. In order to determine whether seasonality in transmission induces seasonal changes in the prevalence of infection by *L. infantum* and of parasite-specific immune response, two groups of dogs, one in February ($n = 37$) and another in October ($n = 42$), were studied. Clinical signs compatible with leishmaniosis, as well as presence of microscopic skin lesions in the muzzle were recorded for all dogs. Assays were also performed for detection of *L. infantum* parasites in muzzle skin samples (PCR, immunohistochemistry and culture), specific serum antibodies (ELISA), and specific lymphocyte proliferation and interferon-γ production. Although prevalence of non-specific clinical signs increased significantly after the sandfly season, this was not the case for *Leishmania*-specific markers: positivity by PCR (24% vs. 21%) or immunohistochemistry (3% vs. 2%) of muzzle skin samples, as well as lymphocyte proliferation (59% vs. 50%) or interferon-γ production (21% vs. 27%) were similar in February and in October. Only prevalence of positive specific antibody titers increased noticeably in October (8% vs. 20%), although this was not statistically significant. Overall, the sandfly season did not have a marked impact on the prevalence *L. infantum* infection or parasite-specific immune responses analyzed in this study.

Keywords: Dog; Epidemiology; Immune response; *Leishmania infantum*; Seasonal dynamics; Spain

1. Introduction

Canine leishmaniosis (CaL) in the Mediterranean basin is a severe chronic disease caused by *Leishmania infantum* (Gállego, 2004). In Spain, CaL is vectored by two sandflies species belonging to the subgenus *Larroussius*, *Phlebotomus ariasi* and *P. perniciosus* (Guilvard et al., 1996), which are active during the warmer months of the year, generally from May to October (Lucientes-Curdi et al., 1991). Dogs exposed to sandfly bites can therefore become infected only over a discrete number of months every year. Canine leishmaniosis should therefore conceivably present a seasonal pattern, with onset of clinical signs and

* Corresponding author at: Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel.: +34 9358 13144; fax: +34 9358 13142.
E-mail address: antonio.ramis@uab.cat (A. Ramis).

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development of disease taking place towards late summer and early autumn. However, to the authors' knowledge there is consensus among clinicians that seasonality in the presentation of clinical CaL is not observed in field settings, where case presentation is scattered throughout the year. Despite abundant scientific literature on various aspects of epidemiology of canine leishmaniosis worldwide, there is little information on the phenology of infection and immune response, and its putative seasonality. This study explores the possible seasonal differences in the prevalence of infection by *L. infantum* and in immune response against the parasite in dogs.

2. Materials and methods

2.1. Dogs and samples

Seventy-nine dogs euthanized at the dog pound in Mallorca (NW Mediterranean Sea), an area endemic for CaL, were included in this study. Upon capture, the dogs were housed for 2 weeks at the pound, after which they were euthanized following city sanitary policy. Physical examination was performed on all dogs, which were classified as symptomatic (presenting at least one sign compatible with CaL) (Baneth, 2006) or non-symptomatic. All dogs were premedicated 1–2 h prior to euthanasia with acepromazine maleate (0.5 mg/kg estimated body weight). Ten mL whole blood was collected by jugular venepuncture before euthanasia was performed by intravenous thiopental overdose (200 mg/kg estimated body weight). Blood was stored in lithium heparin sterile tubes (8 mL) and serum tubes (2 mL) at room temperature, and shipped overnight to the laboratory. In addition, three 6 mm diameter skin punch biopsies were obtained from the muzzle area of each dog: one was preserved in 10% neutral buffered formalin, another in sterile saline solution 0.9% with penicillin overnight at +4 °C, and the third frozen at −20 °C.

Sampling was performed before the sandfly season (late February, $n = 37$) and at the end of October ($n = 42$). The age (estimated), sex and breed distributions were similar in both groups. Males were predominant (67% in both samples), as were 10–20 kg crossbreds (estimated body weight).

2.2. L. infantum serology

An enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Solano-Gallego et al., 2005) was used to detect antibodies to *L. infantum* in the serum samples. Briefly, polystyrene microtiter plates, sensitized with sonicated *L. infantum* (MHOM/ FR78/LEM 75), were incubated with the sample sera and then with Protein A horseradish peroxidase conjugate (Sigma–Aldrich Chemie, Germany), and developed with *o*-phenylenediamine. Optical densities were read in an automated ELISA reader, and corrected against a reference sample (included in each plate). The cutoff was established using sera from negative dogs from a non-endemic area.

2.3. Peripheral blood mononuclear cells (PBMC) extraction and lymphocyte stimulation assays

Extraction and stimulation of PBMC, as well as subsequent lymphocyte proliferation assay (LPA) and interferon-γ detection (IFN) were performed as described elsewhere (Fernández-Bellon et al., 2005). Briefly, heparinized blood samples were subjected to Histopaque 1077 (Sigma–Aldrich Chemie, Germany) density gradient extraction to obtain PBMC upon arrival at the laboratory. PBMC were washed down, counted and resuspended in an enriched culture medium at 10^6 cells/mL. PBMC were then stimulated with *L. infantum* soluble antigen (LSA) at 10 mg/mL, and cultured in parallel with unstimulated cells (negative controls). PBMC yield varied between dogs, and in some cases not enough cells were obtained to perform all the lymphocyte stimulation tests.

Lymphocyte proliferation was measured with a commercial kit (Cell Proliferation ELISA, BrdU, Roche Diagnostics GmbH, Germany). Stimulated PBMC were incubated for 6 days (LSA), at the end of which BrdU incorporation was measured following manufacturer's instructions. Cell proliferation was expressed as a stimulation index (SI), values >2 were considered positive.

Interferon-γ production was measured in PBMC supernatants after 72 h of stimulation using a viral cytopathic effect inhibition assay. Briefly, MDCK-2 adherent cells were incubated with the supernatants and then infected with vesicular stomatitis virus. Results were expressed as the ratio of the reciprocal of the maximum dilution that protected 50% of the cell monolayer (as estimated visually) of stimulated versus unstimulated cells, values >2 were considered positive.

2.4. Histological and immunohistochemical (IHC) evaluation of skin biopsies

Skin biopsies fixed in 10% neutral buffered formalin were subjected to routine histological and IHC

Table 1
Test results before and at the end of the sandfly season

Test	February	October	*p*-Value*
Clinical signs	8% (3/37)	29% (12/42)	0.024
Microscopic skin lesions	5% (2/37)	10% (4/42)	0.679
L. infantum skin IHC	3% (1/37)	2% (1/42)	1
L. infantum skin PCR	24% (9/37)	21% (9/41)	1
Leishmania skin culture	0% (0/37)	0% (0/42)	1
IFN	21% (7/34)	27% (10/37)	0.586
LPA	59% (22/37)	50% (20/40)	0.494
Detectable anti-*Leishmania* Ab	8% (3/37)	20% (8/41)	0.199
Positive by at least one *Leishmania*-specific assay	73% (27/37)	64% (27/42)	0.472

Results of tests performed on two groups of dogs before (February) and at the end of the sandfly season (October). Data in parenthesis are number of dogs positive/*n*. IHC: *L. infantum* immunohistochemical labeling; IFN: interferon-γ bioassay; LPA: lymphocyte proliferation assay.
* Fisher's Exact Test for Count Data (two sided, confidence interval = 95%).

examination. The dermal inflammatory pattern and the cell population were evaluated histologically on hematoxylin and eosin-stained sections, and classified based on the presence or absence of dermal granulomatous or pyogranulomatous inflammatory infiltrates. Presence of *L. infantum* amastigotes was detected by immunohistochemical labeling (Ferrer et al., 1988).

2.5. Culture of Leishmania strains

Skin samples were cultured in Schneider's insect medium supplemented with 20% heat-inactivated fetal calf serum and gentamicine (25 μg/mL) and/ or in Novy-McNeal-Nicolle's medium. Cultures were incubated at 24–26 °C, examined twice a week and subcultured and maintained for 2–6 months before considered negative (Gállego et al., 2002). Liquid phases of both culture media were supplemented with 1% sterile human urine as described elsewhere (Rioux et al., 1990).

2.6. Detection of L. infantum DNA in skin samples

Frozen skin biopsies were digested in 1 mL TE buffer (50 mM Tris [pH 8.0], 20 mM EDTA) with sodium dodecyl sulfate (2%) and proteinase K (0.2 mg/mL) overnight at 56 °C. DNA was then obtained from the lysates by phenol–chloroform extraction and resuspended in 100 μL TE buffer. PCR was performed using primers for a 120-bp fragment of *Leishmania* kinetoplast minicircle DNA as described elsewhere (Solano-Gallego et al., 2001). Amplified fragments were analyzed by electrophoresis in a 1% high resolution agarose gel containing ethidium bromide (0.5 mg/mL).

2.7. Statistical analysis

Seasonal differences in prevalence of positivity to the assays were tested with Fisher's Exact Test for Count Data (two sided, confidence interval = 95%) using R statistical software 2.4.1 (R Development Core Team, 2006). A p-value <0.05 was considered statistically significant.

3. Results

The results of the clinical examination and of the assays performed on each group of dogs, as well as the statistical significance of the differences are displayed on Table 1. Although differences between both groups were observed in several cases (clinical signs, microscopic skin lesions, serology), these were statistically significant only for clinical signs, and not for any *Leishmania*-specific immunologic assays or markers of infection.

Physical examination revealed non-specific clinical signs compatible with leishmaniosis, such as exfoliative dermatitis, lymphadenopathy, skeletal muscle atrophy, and papular dermatitis of the ears, in 8% dogs studied before the sandfly season, rising up to 29% dogs after the sandfly season ($p = 0.024$). Although prevalence of microscopic skin lesions compatible with leishmaniosis in muzzle skin biopsies was lower than that of clinical signs in both groups, they were again more frequent after the sandfly season (5% dogs and 10% dogs, respectively), but this difference was not significant ($p = 0.679$).

Demonstration of the parasite, either by immunohistochemistry or PCR, yielded similar prevalences before and after the sandfly season. Only one dog in each group (3% and 2%, respectively) was positive by

immunohistochemistry, and parasite DNA was detected by PCR in skin of the muzzle in 24% dogs and 21% dogs in either group, respectively. *Leishmania* culture of muzzle skin samples from all dogs was negative.

As with parasite detection, specific cellular immune response to *L. infantum* was observed in similar proportions of dogs in both seasons. Prevalence of positivity by IFN remained at around one fourth of the population in either season (21% and 27%), whereas that of LPA was about one half of the population in either season (59% and 50%). By contrast, prevalence of detectable anti-*Leishmania* antibody titers rose from 8% before the sandfly season to 20% after the sandfly season, although this difference was not statistically significant ($p = 0.199$).

Finally, over two thirds of dogs in either group (73% before the sandfly season and 64% after it) were positive for at least one *Leishmania*-specific assay (PCR, IHC, IFN, LPA or serology).

4. Discussion

The results of this study show no evidence of strong seasonal changes in parasitological (parasite detection in skin samples) and immunological (humoral and cellular responses) markers of infection by *L. infantum* in dogs, remaining grossly stable throughout the year. While clinical manifestations, mainly gross skin lesions, compatible with leishmaniosis detected during physical examination were significantly more frequent in those dogs studied in October, microscopic skin lesions suggestive of leishmaniosis were scarce in both groups of dogs. In the absence of a significant increase in the number of dogs positive for *Leishmania*-specific assays, the higher prevalence in non-specific signs might be due to other etiological agents, infectious or otherwise, to which dogs are also more exposed during the warmer months of the year.

Levels of specific cellular and humoral immune responses to *Leishmania* were not statistically different between both seasons. However, whereas prevalence of specific cellular immune response was high and similar for both seasons, humoral immune response was observed in much fewer dogs, and increased more than two-fold after the sandfly season. Although not significant statistically, the increase in antibody titers observed in early October is in accordance with reports of similar trends (Acedo-Sánchez et al., 1998; Paradies et al., 2006). An increase in frequency of positive antibody levels in face of a stable, highly prevalent, cellular immune response is suggestive of a higher sensitivity of the humoral immune response to re-exposure to *Leishmania*. Absence of a similar trend in cellular immune response might be due to a greater inertia of this component of immunity. Another possible explanation is that the subjects of this study make up a largely resistant population of infected dogs, which mount a sustained protective cellular immune response (only one dog negative for both IFN and LPA had positive antibody levels, data not shown), in which antibody response is scarce, and is elicited only after massive re-exposure to *Leishmania* through infected sandfly bites. Negative skin culture, particularly in the two dogs showing amastigotes by IHQ, might be due to zymodemes present in Majorca which are difficult to culture, such as MON-24 and MON-108 (Chicharro et al., 2002).

Although it might be expected that dogs exposed to infection or superinfection by *L. infantum* will show increased prevalence of infection and/or immune response to the parasite after the sandfly season, the results of this study show little evidence of this. Direct impact of the sandfly season on parameters of CaL is probably undermined by prolonged course of infection and immune response, which can span up to several years (Baneth, 2006). Furthermore, incidence of primo-infection during individual sandfly seasons should be relatively low compared to superinfection, due to the high prevalence of asymptomatic *L. infantum* infection in dogs living in endemic areas, as observed in this study and elsewhere (Cabral et al., 1998; Quinnell et al., 2001; Solano-Gallego et al., 2001; Leontides et al., 2002). Thus, the mild seasonal variations observed in this study would be more likely due to superinfection rather than to primo-infection by *L. infantum*.

The sensitivity of this study for changes in the parameters evaluated can be improved by following a closed set of subjects, as well as including further sample points throughout the year and including more than one transmission season. Nonetheless, the data presented herein suggest that the prevalences of the immunological and parasitological parameters studied remain largely stable throughout the year. This inertia against exposure to *L. infantum* infection through sandfly bites is probably a consequence of elevated prevalence of chronic infection by *L. infantum* in healthy dogs, together with a prolonged incubation period of CaL. Whether or not the non-significant difference in humoral immunity observed in this study is indicative of an actual increase after the sandfly season would require much broader studies, as well as longitudinal follow up studies of dogs through one or more sandfly seasons.

5. Conclusion

Infection or superinfection by *L. infantum* during the sandfly season does not seem to have a strong direct impact on the different epidemiological or immunological markers of infection by *L. infantum* in dogs. We have identified a putative transient mild increase in antibody levels in dogs presenting cellular immunity which warrants further research. Lastly, our results suggest that the time of year in which epidemiological studies on CaL are performed does not significantly impact their results.

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CHAPTER 5

Cutaneous leishmaniosis in three horses in Spain

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Case Reports

Cutaneous leishmaniosis in three horses in Spain

L. SOLANO-GALLEGO, H. FERNÁNDEZ-BELLON†, P. SERRA†, M. GÁLLEGO‡, A. RAMIS†, D. FONDEVILA† and L. FERRER*†

Departments of Pharmacology, Therapeutics and Toxicology and †Animal Medicine and Surgery, Faculty of Veterinary, Autonomous University of Barcelona, 08193 Bellaterra and ‡Department of Microbiology and Sanitary Parasitology, Faculty of Pharmacy, University of Barcelona, Avenue, Joan XXIII, 08028, Barcelona, Spain.

Keywords: horse; *Leishmania infantum*; cutaneous leishmaniosis; Spain

Introduction

Leishmaniosis is a disease caused by protozoan parasites of the genus *Leishmania*. Their life cycle includes the presence of sandflies (*Diptera, Phlebotomidae*), that act as vectors transmitting the parasites to different species of mammals suffering the disease (Anon 1990). Canids, rodents and man are the main species affected (Lainson and Shaw 1987), although the infection has been described in many other animals including cats (Ozon *et al.* 1998), sheep (van der Lugt *et al.* 1992), goats (Williams *et al.* 1991) and horses (Aguilar *et al.* 1986). Mazza (1927) described the first reported case of cutaneous lesions in a horse, in Argentina, caused by *Leishmania* parasites. Since then, cutaneous leishmaniosis in horses, mules and donkeys has been diagnosed in both South America (Aguilar *et al.* 1987; Falqueto *et al.* 1987; Barbosa-Santos *et al.* 1994) and Central America (Ramos-Vara *et al.* 1996). In those cases where biochemical characterisation of the parasite was performed, *Leishmania braziliensis* was always the species identified (Falqueto *et al.* 1987; Oliveira-Neto *et al.* 1988; Barbosa-Santos *et al.* 1994). In southern European countries, leishmaniosis is caused by *Leishmania infantum*, where dogs and man are the most affected hosts (Anon 1990). In dogs, leishmaniosis is a severe systemic disease where nonpruritic skin lesions such as exfoliative dermatitis and ulcerations, lymphadenopathy and loss of weight are the main clinical signs (Slappendel and Ferrer 1998). A review of the literature failed to identify a confirmed case of equine leishmaniosis in the Mediterranean basin (Gállego *et al.* 2001). Here, we report the first cases of equine cutaneous leishmaniosis described in Europe.

Case details

History and clinical findings

Horse 1: A 3-year-old male Andalusian horse in Cerdanyola del Vallès (41°30'N 2°09'E, Catalonia, Spain) with a history of facial nodules was examined by a veterinary practitioner in 1996. The horse presented several cutaneous papulonodular lesions on the head that measured 5–10 mm diameter. A thorough physical examination revealed no other clinical signs, apart from the skin lesions mentioned above. The lesions healed spontaneously after 5 months.

Horse 2: A 10-year-old female mixed breed horse in Pamplona (42°49'N 1°38'O, Navarra, Spain) with nodules over a 2 month period on the inguinal region was examined by a veterinary practitioner in 1999. The horse presented with several cutaneous nodules on the inguinal area measuring 10–20 mm in diameter. A thorough physical examination revealed no other clinical signs, other than the skin lesions mentioned above. The lesions healed spontaneously after 4 months.

Horse 3: A 3.5-year-old female Andalusian horse in Vilanova i la Geltrú (41°13'N 1°43'E, Catalonia, Spain) with a history of papules and nodules on facial, axillary and inguinal regions was examined by a veterinary practitioner in 2000. The horse presented papules and ulcerated nodules on the head, axillae and groin over a 2 month period. The papulonodular lesions measured 5–20 mm diameter and some of them had alopecic and crusted surfaces (Fig 1). The horse did not respond to anti-inflammatory therapy with a single dose (30 mg i.m.) dexamethasone sodium phosphate. A thorough physical examination revealed no other clinical signs, apart from the skin lesions mentioned above. The lesions resolved after 3 months without the need of antimicrobial drugs.

Histopathology

Incisional biopsies of the lesions were taken from the 3 horses. Samples of the lesions were fixed in 10% (v/v) neutral-buffered formalin and embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin (H&E). Immunohistochemical detection of *Leishmania* was accomplished using a standard immunohistochemical protocol (Ferrer *et al.* 1988). Histopathology in all horses revealed a nodular to diffuse granulomatous dermatitis with some multinucleated giant cells scattered between the cellular infiltrates (Figs 2, 3). An intense infiltrate of lymphocytes was evident, especially in the periphery of the granulomatous reaction. Most macrophages presented amastigotes in their cytoplasm as revealed by immunohistochemical staining for *Leishmania*.

*Author to whom correspondence should be addressed.
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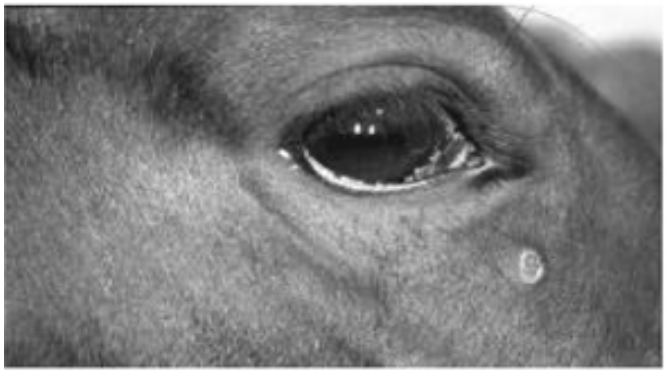

Fig 1: Cutaneous leishmaniosis in Horse 3. *Nodule with an alopecic and crusted surface on the facial region.*

Immunology

ELISA: In *Horse 3*, an ELISA to detect specific antibodies for *Leishmania* was performed as described previously (Riera *et al.* 1999). The working dilutions of horse sera and Protein A (Prot A) conjugated to horseradish peroxidase[1] were 1:50 and 1:1000, respectively. Sera from 16 healthy horses living in a region where leishmaniosis is nonendemic were tested to set a cut-off for the ELISA. The cut-off absorbance was established as the mean plus 3 s.d, resulting in a optical density (OD) of 0.307 for Prot A (mean ± s.d. 0.127 ± 0.06). All determinations included the serum from a sick dog with confirmed infection as a positive control. *Horse 3* was considered serologically positive, with low levels of anti-*Leishmania* antibodies (OD at 490 nm: 0.325).

Lymphocyte proliferation assay (LPA): An LPA was also performed in *Horse 3*. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood samples of the horse by standard ficoll-hypaque (Histopaque 1.077)[1] density gradient centrifugation, as described (Böyum 1968). PBMC were cultured in a flat-bottomed 96-well-microtiter plate, at a density of 1 x 10^5 cells per well in supplemented RPMI. The cells were incubated (37°C, 5% CO_2) with either 5 µg/ml phytohaemagglutinin (PHA) for 3 days, or 10 µg/ml leishmanial soluble antigen (LSA) for 5 days and pulsed during the last 24 h with 10 µmol/l 5-bromo-deoxyuridine (BrdU). Unstimulated PBMC were used as controls for both PHA and LSA cultures. Cell proliferation was determined using a nonradioactive ELISA technique according to the manufacturer's instructions (Cell proliferation ELISA, BrdU [Colorimetric])[2]. The stimulation index (SI) was determined as the ratio of the mean of OD (450 nm) of triplicate cultures stimulated with LSA or PHA to the mean of OD of cultures with RPMI alone. A stimulation index greater than 2 was indicative of a positive response. *Horse 3* had a strong lymphocyte proliferative response to phytohaemagluttinin (SI: 20) and to leishmanial soluble antigen (SI: 15).

Parasite culture and biochemical characterisation

Culture and identification of the parasite was attempted in *Horse 3*. The skin biopsy was maintained 24 h at 4°C in a 9% (w/v) saline solution containing 25,000 iu penicillin/ml. Subsequently, the biopsy was cultured at 25°C in Schneider medium[1] supplemented with 20% (v/v) fetal calf serum (FCS)[3] and 1% (v/v) sterile human urine. The parasite growth was obtained after 5 days of sample inoculation in culture and the strain named as MEQU/ES/2001/BCN-304, according to the WHO recommendations (Anon 1990). Mass rearing culture, to obtain sufficient parasites for the biochemical study, was done in Schneider medium (10% FCS). The biochemical characterisation of the strain was carried out by isoenzyme electrophoretic analysis in starch gel using 15 loci: malate dehydrogenase (MDH: E.C. 1.1.1.37), malic enzyme (ME: E.C. 1.1.1.40), isocitrate dehydrogenase (NADP+) (ICD: E.C. 1.1.1.42), 6-phosphogluconate dehydrogenase (decarboxylating) (6PGD: E.C. 1.1.1.44), glucose-6-phosphate 1-dehydrogenase (G6PD: E.C. 1.1.1.49), glutamate dehydrogenase [NAD(P)+] (GLUD: E.C. 1.4.1.3), diaphorase nicotamide adenine dinucleotide (DIA: E.C. 1.6.2.2), nucleoside purine phosphorylase 1 (NP_1: E.C. 2.4.2.1) and 2 (NP_2: E.C. 2.4.2.*), glutamate-oxalacetate transaminase 1 and 2 (GOT_1 and GOT_2: E.C. 2.6.1.1), phospho-glucomutase (PGM: E.C. 5.4.2.2), fumarate hydratase (FH: E.C. 4.2.1.2), mannose-6-phosphate isomerase (MPI: E.C. 5.3.1.8), glucose-6-phosphate isomerase (GPI: E.C. 5.3.1.9 (Rioux *et al.* 1990). Finally, the strain was identified as *Leishmania infantum* zymodeme MON-1.

Discussion

The prevalence of *Leishmania* infection in dogs is high, but varies with geographical area and type of diagnostic test performed. In Spain, seroprevalence was 10–30% (Fisa *et al.* 1999) but with other techniques i.e. leishmanin skin test and serology, rates of exposure to the parasite were as high as 80% (Solano-Gallego *et al.* 2000). To the authors' knowledge, there are few studies describing the epidemiology of *Leishmania* infection in other domestic animals (Ozon *et al.* 1998). In this report, we document the first cases of equine leishmaniosis caused by *L. infantum* in a European country.

Clinical and histopathological features of our cases of equine cutaneous leishmaniosis in Spain are the same as described by other authors in horses in South America (Barbosa-Santos *et al.* 1994) and Central America (Ramos-Vara *et al.* 1996) and, to an extent, are similar to those described in human cutaneous leishmaniosis in the Mediterranean basin (Giudice *et al.* 1998).

The disease in horses has remained undiagnosed in southern European countries, where leishmaniosis in man and dogs is endemic, probably due to the mild clinical picture and benign evolution of the lesions, even without treatment. Nevertheless, in the future, leishmaniosis should be included in the differential diagnosis of papulonodular dermatoses of horses, together with other infectious diseases (botryomycosis, sporotrichosis, pythiosis, histoplasmosis, phaeohypomycosis, habronemiasis and hypodermiasis), sterile non-neoplastic nodular dermatoses (insect bites hypersensitivity, eosinophilic nodular disorders), oozing papular urticaria, and neoplasia (nodular sarcoid, lymphosarcoma, cutaneous mastocytoma) (Fadok 1995; Pascoe and Knottenbelt 1999).

The immunoprofile of *Horse 3* was similar to that found in human cutaneous leishmaniosis; low anti-*Leishmania* antibody levels and strong specific-*Leishmania* cellular immunity (Giudice *et al.* 1998). As occurs in man (Giudice *et al.* 1998), cutaneous leishmaniosis in horses appear to heal spontaneously after a short period, probably due to a strong specific-*Leishmania* cellular immunity. Furthermore, it could be common that horses have also subclinical infections as it has been described in man (Marty *et al.* 1994) and dogs (Cabral *et al.* 1998).

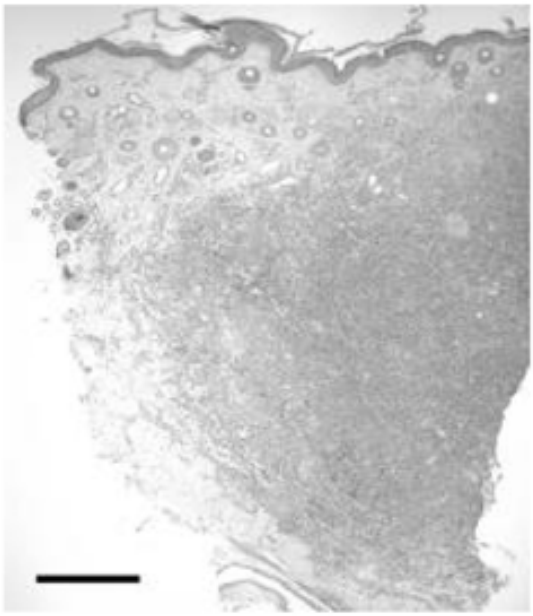

Fig 2: Nodular to diffuse dermatitis (H/E). Scale bar 500 µm.

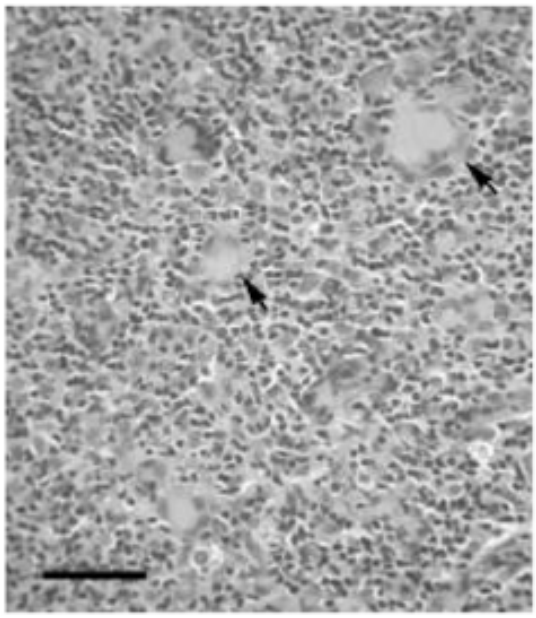

Fig 3: Granulomatous infiltrate with giant cells (arrows), macrophages, lymphocytes and plasma cells (H/E). Scale bar 50 µm.

In our cases, the lesions resolved without any antimicrobial therapy. However, if treatment were needed, we would have recommended a regime dose of conventional chemotherapy for leishmaniosis, such as pentavalent antimony as described previously in the treatment of horses affected by leishmaniosis (Barbosa-Santos *et al.* 1994; Ramos-Vara *et al.* 1996). In dogs, the combination of pentavalent antimony and allopurinol seems to be more efficacious than either drug alone (Denerolle and Bourdoiseau 1999) even though both drugs alone are capable of reducing clinical signs (Riera *et al.* 1999; Koutinas *et al.* 2001). However, there are no studies about the efficacy of allopurinol treatment in equine leishmaniosis.

In southern European countries, the main species affected by *L. infantum* are dogs and man, although the infection has been described on a smaller scale in other animals including cats (Ozon *et al.* 1998), rats (Morillas Márquez *et al.* 1985) and foxes (Marín Iniesta *et al.* 1982). The identification of *L. infantum* in *Horse 3* in Catalonia is the first finding of this species affecting the horse, as *L. braziliensis* is the species isolated from horses in South America (Aguilar *et al.* 1987; Falqueto *et al.* 1987; Barbosa-Santos *et al.* 1994) and Central America (Ramos-Vara *et al.* 1996). The finding of 2 species of *Leishmania* parasitising the same mammalian species has been described for *L. infantum* and *L. braziliensis* in dogs (Anon 1990) and, now, in horses. The first species is present in both the Old and New World while the second species is present only in the New World (Anon 1990). We suspect the species infecting *Horses 1* and *2* would have been *L. infantum*, the only species of *Leishmania* found in Spain, even though cultures from lesional skin were not performed and the diagnosis of leishmaniosis was assessed only by immunohistochemistry.

The biochemical characterisation of the strain was carried out by isoenzyme electrophoretic analysis. The use of isoenzyme electrophoretic typing is an extremely laborious and slow method of characterisation of organisms. However, at the moment, this is currently the standard method to identify strains of *Leishmania* and therefore extensively used in taxonomic and epidemiological studies on this protozoa (Gállego *et al.* 2002). This technique has revealed a considerable variation within the majority of *Leishmania* species, expressed by the stable and relatively specific electromorphs leading to the differentiation between *Leishmania* species (Pratlong and Lanotte 1999). Polymorphic DNA sequences from *Leishmania* have been amplified using different PCR-based techniques and used for species identification, strain discrimination with the exception of *L. infantum* and population genetic studies. Cultivation of the parasites is, however, mandatory (Schonian *et al.* 2001). For this reason, the isoenzyme electrophoretic analysis is the only method that permits to identify strains of *L. infantum* from mammalian populations (Schonian *et al.* 2001). *L. infantum* MON-1 was identified from a lesional skin sample of *Horse 3*. This zymodeme is the most commonly found in the Old World, where it has been identified in 66.5% of the cases between 38 zymodemes, mostly in dogs and man (Gállego *et al.* 2001).

The prevention of acquisition of the disease for horses travelling or living in endemic areas is difficult and currently depends mainly on control of the insect vector, because of the lack of effective prophylactic drugs and vaccines (Gradoni 2001). Measures to protect the individual mammalian host include keeping the animal indoors from 1 h before sunset to 1 h after dawn during the vector season and the use of repellents and insecticides for sand flies (Slappendel and Ferrer 1998). Deltamethrin collars are used to protect dogs from bites of sand flies (Killick-Kendrick *et al.* 1997) and decrease the incidence of seropositive dogs in endemic areas (Maroli *et al.* 2001). Deltamethrin is an insect repellent and antiparasitic drug commonly used in large animals (Parashar *et al.* 1991; Mestres and Mestres 1992). Therefore, it could be helpful to topically treat horses with deltamethrin to prevent infection. However, there are no studies of the protection against sandflies by using deltamethrin collars in horses.

The data obtained suggest that horses living in areas of leishmaniosis endemicity in the Mediterranean basin can be infected by *L. infantum*, develop clinical signs of leishmaniosis and, possibly, play a role in the epidemiological cycle of this species of *Leishmania*. In southern European countries, dogs and man are the main source of infection for the female sand fly vector. Nevertheless, *Phlebotomus perniciosus*, one of the two vector species of *Leishmania* in Spain, has shown an opportunistic behaviour, feeding on those animals that are available, including horses (Colmenares *et al.* 1995). According to these findings, horses could be involved in the transmission of *Leishmania* infection. Extensive epidemiological and immunological studies should be undertaken to investigate *Leishmania* infection in horses living in endemic areas in Europe.

Manufacturers'addresses

[1]Sigma, St Louis, Missouri, USA.
[2]Roche Diagnostics Corporation, Basel, Switzerland.
[3]Cansera, Etobicoke, Ontario, Canada.

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CHAPTER 6

Immune response to *Leishmania infantum* in healthy horses in Spain

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Short communication

Immune response to *Leishmania infantum* in healthy horses in Spain

Hugo Fernández-Bellon [a], Laia Solano-Gallego [b], Mar Bardagí [a], Jordi Alberola [b], Antonio Ramis [a,*], Lluis Ferrer [a]

[a] *Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain*
[b] *Departament de Farmacologia, de Terapèutica i de Toxicologia, Universtitat Autònoma de Barcelona, Spain*

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Abstract

Leishmania infantum infection has recently been described in horses in Europe. We report the results of a study on the immune response to *L. infantum* in horses living in an area endemic for leishmaniosis in NE Spain. Two ELISAs using protein A and anti-horse IgG conjugates were adapted to measure specific antibodies to *L. infantum* in horse sera. A lymphocyte proliferation assay (LPA) of peripheral blood mononuclear cells to *L. infantum* antigen was also performed to detect specific cellular immune response to *Leishmania*. Anti-*L. infantum* antibodies were detected in the serum of 16 of the horses studied ($n = 112$) using the protein A assay but not in the assay using the anti-horse IgG conjugate. Specific lymphocyte proliferation was observed in 20 out of 55 horses. This study shows that horses in the area studied mount specific immune responses to *L. infantum*, and must therefore be considered among the species exposed to the parasite in this region. The infrequency of leishmaniosis in horses suggests that the immune response in this species is effective in controlling the infection.

Keywords: *Leishmania infantum*; Horse; Lymphocyte proliferation assay; ELISA; Immune response

1. Introduction

In the Mediterranean basin, human leishmaniosis due to *Leishmania infantum* is infrequent among nonimmunocompromised subjects (Ashford, 2000). In contrast, the dog, the main reservoir of *L. infantum*, is highly susceptible of developing the disease (Slappendel and Ferrer, 1998). Interestingly, in both species there is a significant proportion of healthy individuals from endemic areas which are parasitemic, but remain free of clinical signs. In fact, it has been shown that the prevalence of infection is much greater than the prevalence of overt leishmaniosis in humans and dogs living in endemic areas (Solano-Gallego et al., 2001; Riera et al., 2004). This raises the possibility that in other mammal species in which the disease is rarely

* Corresponding author. Tel.: +34 93 581 3144; fax: +34 93 581 3142.
E-mail address: antonio.ramis@uab.es (A. Ramis).

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manifested there may also be a percentage of healthy infected individuals. However, although animals other than dogs have for some time been identified as hosts of *L. infantum* (European fox, black rat, cat), no evidence has been found to indicate that any of these is a putative reservoir of the disease (Abranches et al., 1982; Gradoni et al., 1983; Fisa et al., 1999; Mancianti et al., 1994; Poli et al., 2002).

Recently, several cases of equine cutaneous leishmaniosis caused by *L. infantum* have been described in Europe (Koehler et al., 2002; Solano-Gallego et al., 2003; Rolão et al., 2005). Previous case descriptions of equine leishmaniosis had been restricted to America, with only *L. braziliensis* having been identified as the etiologic agent. Indeed, clinical equine leishmaniosis is so abundant in South America that the horse has been proposed as a reservoir of human cutaneous leishmaniosis caused by *L. braziliensis* (Tolezano, 1994). However, no studies to assess the exposure of healthy horses to *L. braziliensis* have been reported. The only evidence of exposure to *Leishmania* in healthy equines was reported by Mukhtar et al. (2000), who stated that 69% ($n = 96$) of the donkeys studied presented antibodies to *L. donovani*, in a serological survey covering different domestic animal species in Sudan.

Our interest was generated by the identification of equine leishmaniosis due to *L. infantum*. We proposed to determine whether otherwise healthy horses living in an endemic area develop an immune response to *L. infantum*. Here, we report the study of specific humoral and cellular immunity to *L. infantum* in horses living in an area endemic for the parasite in NE Spain.

2. Materials and methods

This study was performed on sera and whole blood samples obtained from 111 healthy Andalusian and mixed breed horses of both sexes and different ages (1–25 years) belonging to five different herds in the province of Barcelona (NE Spain), an endemic area for leishmaniosis. Serological ELISA tests were performed on all 111 horses. A lymphocyte proliferation assay (LPA) to *L. infantum* antigen was performed on 54 of these horses from which whole blood could be collected. A horse with papular dermatitis which was parasitologically confirmed as caused by *L. infantum* (Solano-Gallego et al., 2003) was also included in the study and tested for ELISA and LPA.

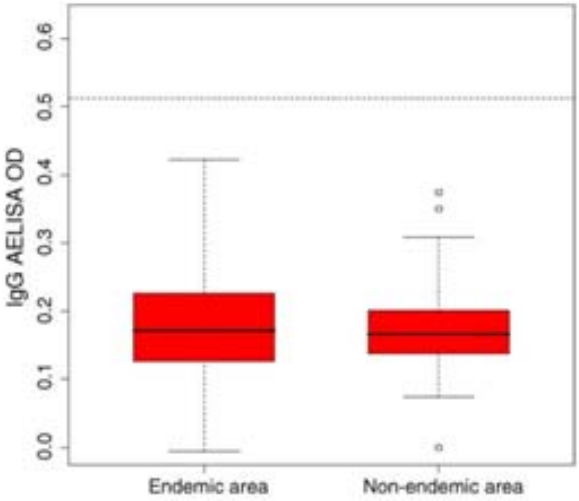
Sera were obtained by centrifugation of whole blood samples extracted from the jugular vein, and frozen until use. An enzyme-linked immunosorbent assay (ELISA) used to detect canine antibodies to *L. infantum* (Riera et al., 1999) was adapted for its use on horse samples. Briefly, polystyrene 96-well microtitre plates sensitized with sonicated *L. infantum* (MHOM/FR78/LEM 75) antigen were used. Two ELISAs were performed, using either Protein A (Sigma–Aldrich Chemie, Germany) (Protein A ELISA) or rabbit anti-horse IgG (Sigma–Aldrich Chemie, Germany) (IgG ELISA) horseradish peroxidase conjugates. Optimal concentrations of the sample sera and conjugate were established at 1:100 (serum) and 1 μg/mL (Protein A conjugate) for the Protein A ELISA; and 1:200 (serum) and 1:4000 (anti-horse IgG conjugate) for the IgG ELISA respectively. A calibrator serum was included in all ELISA plates to standardize the results against variations between assays.

Heparinized blood samples (8–10 mL) obtained by venepuncture of the jugular vein were processed within 24 h of extraction for LPA. To detect specific cellular immune response to *L. infantum*, a lymphocyte proliferation assay used in dogs (Fernández-Bellon et al., 2005) was adapted for use on horse samples. Briefly, peripheral blood mononuclear cells (PBMC) were extracted from whole blood by density-gradient centrifugation on Fycoll-Hypaque (Sigma–Aldrich Chemie, Germany). Live PBMC were then counted by Trypan Blue exclusion in a Neubauer chamber. The cells were incubated in culture medium (RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/mL penicillin and 100 μg/mL streptomycin). Stimulation was performed in 96-well flat bottomed microtitre plates at 2×10^5 cells per well (1×10^6 cells/mL) with either phytohemaglutinin (PHA) (5 μg/mL) (Sigma–Aldrich Chemie, Germany) as a stimulation control, or *L. infantum* soluble antigen MHOM/FR/78/LEM 75 (10 μg/mL). Cell proliferation was measured using a nonradioactive 5′-bromo-deoxyuridine (BrdU) incorporation assay (Roche Molecular Biochemicals, Germany). At 24 h before the end of stimulation, BrdU was added at 10 mM final concentration. On day

3 (PHA) or five (LSA) after the start of incubation, the culture plates were spun down, the supernatants removed, and the plates were air dried and stored at 4 °C. The BrdU ELISA was performed following the manufacturer's instructions to quantify the incorporation of BrdU into cells.

The results of the serological tests were expressed in optical density (OD), as the OD reading for each sample minus the background level and corrected for interplate variation using the calibrator serum (Kurstak, 1985). LPA results were expressed in OD, as the mean values of the wells with stimulated cells minus those of the unstimulated cells.

Cutoffs for the ELISA serologies and the LPA, were determined using sera (ELISA, $n = 33$) and blood samples (LPA, $n = 15$) of adult horses from an area not endemic for leishmaniosis (Utrecht, The Netherlands). Cutoffs were established at the mean value plus four standard deviations ($\mu + 4\sigma$) of the results for the nonendemic area horses. Values below $\mu + 4\sigma$ were considered negative, and those greater were considered positive. Protein A ELISA results for the 33 nonendemic area horses used as controls averaged 0.114 OD, with 0.097 OD standard deviation. This yielded a cutoff value of 0.501 OD (Graph 1). IgG ELISA results for the same 33 horses were similar, with 0.180 OD mean, and 0.084 standard deviation, setting the cutoff at 0.514 OD (Graph 2).

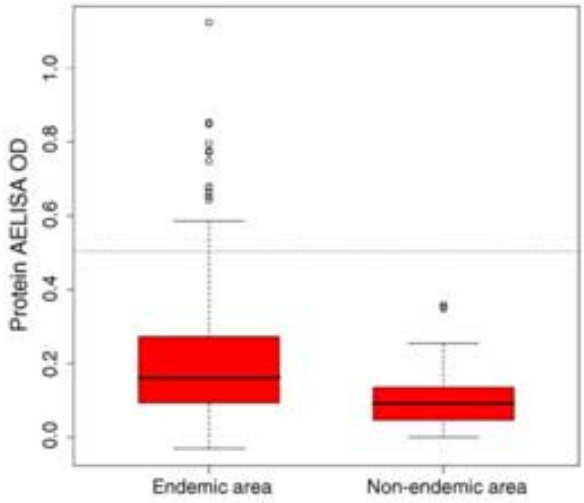


Graph 2. Boxplot of the IgG ELISA results of the endemic ($n = 112$) and nonendemic area horses ($n = 33$). Results are expressed as OD. Dotted line: cutoff.

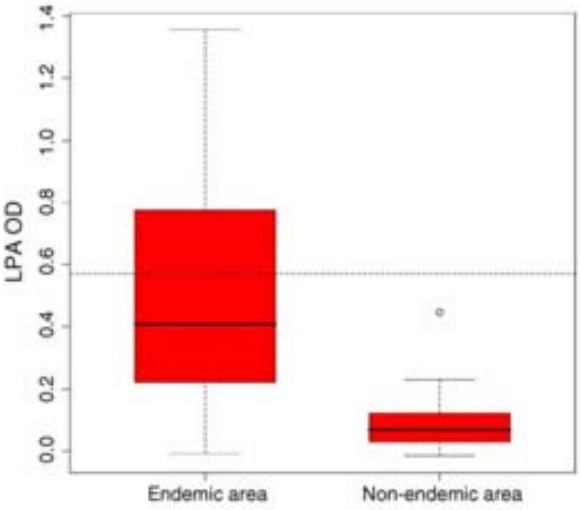
Whole blood samples from 15 of these horses were also used to set the LPA cutoff (0.571 OD): 0.103 OD mean and 0.117 OD standard deviation (Graph 3).

3. Results and discussion

One hundred and twelve horses (including one parasitologically confirmed as *L. infantum* infected)



Graph 1. Boxplot of the protein A ELISA results of the endemic ($n = 112$) and nonendemic area horses ($n = 33$). Results are expressed as OD. Dotted line: cutoff.



Graph 3. Boxplot results of the LPA of the endemic ($n = 55$) and nonendemic area horses ($n = 15$). Results are expressed as OD. Dotted line: cutoff.

Table 1
Cutoffs and results of assays for humoral immune response (protein A ELISA, IgG ELISA) and cellular immune response (LPA) to *L. infantum* in endemic area horses

Assay	*n*	Cutoff (OD)	Number of positive horses (%)
Protein A ELISA	112	0.501	16 (14.3)
IgG ELISA	112	0.514	0 (0.0)
LPA	55	0.571	20 (36.4)

living in *L. infantum* endemic areas were tested for antibodies to the parasite using two ELISA assays. Sixteen horses were positive by protein A ELISA, with OD values greater than 0.501 (Table 1, Graph 1). In contrast, no differences were observed between results for the IgG ELISA of the nonendemic and of the endemic area horses: all 112 endemic area horses had IgG ELISA results below 0.514 OD (Table 1, Graph 2). Cellular immunity to *L. infantum* was tested in 55 horses using a lymphocyte proliferation assay. Non-specific proliferation to PHA was strong (>0.5 OD) in all the horses on which LPA was performed. Twenty of these horses presented proliferation to LSA above the cutoff (Table 1, Graph 3). Of the 55 horses for which protein A ELISA and LPA were performed, 4 were positive to both assays. It is important to point out that the horse with papular leishmaniosis due to *L. infantum* as confirmed by immunohistochemistry and culture (Solano-Gallego et al., 2003) included in the study showed negative results for both ELISA tests (protein A ELISA: 0.232 OD, IgG ELISA: 0.314 OD), but was positive for LPA (0.571 OD).

Leishmanioses are caused by several species of *Leishmania* parasites in a wide range of hosts throughout the world. In all species investigated, the host's immune response plays a determinant role in the outcome of infection. For instance, in the case of canine leishmaniosis, hosts which mount an effective cellular immune response are protected from developing clinical signs, whereas the humoral immune response is ineffective in controlling infection and can be pathologic (Slappendel and Ferrer, 1998).

In the Mediterranean basin, leishmaniosis due to *L. infantum* is maintained by domestic dogs, which act as reservoir for humans and present an elevated prevalence of both infection and disease (Ashford, 2000). Disease in humans is not of concern in the non-immunocompromised population, even though prevalence of infection by the parasite has been found, as in dogs, to be over 50% (Solano-Gallego et al., 2001; Riera et al., 2004). In both species, most infected individuals mount an effective cellular immune response which controls infection and prevents disease progression (Cabral et al., 1993; Ashford, 2000; Riera et al., 2004).

Sporadic reports indicate that leishmaniosis affects domestic mammals other than dogs in the Mediterranean basin, namely cats (Poli et al., 2002) and horses. The first reports of equine leishmaniosis due to *L. infantum* in Europe have been published recently (Koehler et al., 2002; Solano-Gallego et al., 2003; Rolão et al., 2005). These cases raise the possibility that the horse is frequently exposed to infection by *L. infantum* in endemic areas. Although direct evidence of transmission of *Leishmania* by the phlebotomine vector to equine hosts is lacking, studies suggest that the vector is not species specific (Bongiorno et al., 2003). Under these conditions, dog and horse cohabitation in endemic areas will plausibly result in frequent exposure of horses to *L. infantum*. The results presented herein bear out this hypothesis; the horses in this study present both humoral and cellular immune responses to *L. infantum*, indicating that exposure and development of an immune response to the parasite occur in the absence of clinical manifestations.

Protein A ELISA yielded positive results in 16 out of 112 endemic area horses studied versus 20 out of 55 for LPA. This difference is greater taking into account that, compared to the same ELISA used for routine serological diagnosis of canine leishmaniosis at our laboratory, the protein A ELISA was performed at higher serum and conjugate concentrations (Solano-Gallego et al., 2001). The lack of positive results for the second serological assay (IgG ELISA) further backs the hypothesis that the humoral response to *L. infantum* in the horses studied is weak in comparison to that observed in the dog. In contrast, more than one third of the horses on which LPA was performed presented cellular immune response to *L. infantum*, and the assay was not significantly modified from that used in dogs (Fernández-Bellon et al., 2005). Although previous studies have already detected immune response to *L. infantum* in horses with leishmaniosis (Solano-Gallego et al., 2003; Rolão et al., 2005), this is the first time specific humoral and cellular immune responses to the parasite have been detected in healthy horses. These results resemble what is observed in many healthy dogs

and humans infected by *L. infantum*, which mount specific cellular immune responses to the parasite without developing clinical symptoms (Cabral et al., 1993; Ashford, 2000).

As a conclusion we can state that a percentage of horses living in *L. infantum* endemic areas present specific humoral and cellular immune responses, suggesting that exposure to the parasite is common. Cases of equine leishmaniosis are very rare, however, indicating that the immune response mounted by horses is generally effective in preventing the development of disease. Further studies are warranted to investigate whether, as our results suggest, horses living in endemic areas can be inapparently infected by *L. infantum*, as well as to further characterize the immune response detected in this study.

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CHAPTER 7
DISCUSSION

“all science would be superfluous if the outward appearance and the essence of things directly coincided.”

Karl Marx, *Capital, volume III*

In Chapters 2 through 6, we presented the results of investigations into natural infection by *L. infantum* in healthy mammalian hosts (dogs and horses) from endemic areas in SW Europe. Although they do not pretend to be an exhaustive study of all aspects of infection and host response, they address key outstanding issues on response to natural infection in healthy mammals, and yield insights into cross-species homologies of *L. infantum* immunology and epidemiology.

Chapter 2 is a methodological study, comparing assays of canine CMI, a key determinant of *L. infantum* pathogenesis and immunology. Chapters 3 and 4 go on to characterize *L. infantum* infection in macroscopically normal skin of infected dogs, and parasitological and immunological markers of infection in healthy dogs before and after the transmission season. Finally, Chapters 5 and 6 explore infection and immunity to *L. infantum* in horses living in an endemic area.

In this chapter, we review the results of these studies, and their implications, as well as considerations on general issues regarding *Leishmania* infections

EVALUATING THE CELLULAR IMMUNE RESPONSE TO L. INFANTUM IN DOGS

Acquired cell mediated immunity (CMI) is a hallmark of infection by *Leishmania* in mammals. Although this has been recognized for decades (WHO 1969), research into visceral *Leishmania* infections have for years relied on measuring specific antibodies and parasite culture. For lack of better assays, serology was used mainly as indicator of exposure to *L. infantum*, and secondarily as an indicator of host immune status, while demonstration of infection relied chiefly on microscopical identification or parasite culture (Slappendel & Greene, 1990; Acha & Szyfres, 2003). High anti-*Leishmania* antibody levels and parasite identification matched up nicely and appeared to fully account for infection and disease (Slappendel & Greene, 1990). Where serological assays can be simple and inexpensive, cellular immunity assays lack the convenience of serological assays, can be more costly, and perhaps for these reasons were historically shunned in leishmaniosis research, particularly in field settings. Indeed they are still ignored as relevant in some cases (Gomes et al., 2006; Miró et al., 2008). However, the first studies that evaluated specific CMI to *L. infantum* in dogs unmasked an unexpectedly large population of infected but healthy dogs (Cabral et al., 1992, 1998; Oliveira et al., 1993; Cardoso et al., 1998). These results were initially interpreted as evidence for prepatent infection (Cabral et al., 1998), or a unique “resistant” phenotype (Pinelli et al., 1994).

Despite the renewed interest in CMI since the aforementioned studies, the drawbacks to the assays for evaluating specific cellular immunity to *Leishmania* infection still remain. Unlike humoral immunity, defined by circulating specific antibody levels, CMI lacks a stable, easy to quantify indicator. In fact, most CMI assays available for research in veterinary leishmaniosis target responses of cells or tissues, either in vivo (LST) or in vitro (LPA, cytokine assays) (Pinelli et al., 1994; Solano Gallego et al., 2001). This difficulty is increased by the lack of molecular tools (mainly antibodies against cytokines) for some mammalian host species of interest in *Leishmania* research.

Delayed skin hypersensitivity to *Leishmania* antigen (LST) was the first assay of CMI available for CaL. It requires use of a standardized antigen preparation (available from WHO reference laboratories), but is otherwise easy to perform in field settings, and requires almost no infrastructure. Its main drawback is subjectivity of readings, as well as putative false positive results caused by repeated testing. Also, the test lacks proper standardization, and the thresholds used are therefore arbitrary. The assay is nonetheless perhaps the most used CMI assay in *Leishmania* infection research (Maia & Campino, 2008).

In vitro assays are the main alternative to LST for evaluation of CMI in CaL. They measure different variables in the response of circulating lymphocytes to stimulation with *Leishmania* antigens. These assays are labor intensive and require cell culture infrastructure. However, they allow better assay standardization and objectivity of the readings than LST. Also, they do not require visiting the study subject twice, but only once for drawing blood, a significant advantage in the case of field research. The simplest in vitro CMI assay, LPA, consists in measuring proliferation of circulating lymphocytes upon stimulation with *Leishmania* antigen, and has been used extensively as an assay of CMI in CaL (Pinelli et al., 1994; Martínez-Moreno et al., 1995; Cabral et al., 1998; Quinnell et al., 2001). Molecules involved in lymphocyte responses to *Leishmania* antigens, mostly cytokines, have also been studied extensively in leishmaniosis research. One such cytokine, IFN-γ is central to CMI and is routinely evaluated in experimental murine leishmaniosis (Sacks & Noben-Trauth, 2002). However, until specific antibodies against canine IFN-γ became available (R&D Systems), the choice of tests in dogs was restricted to bioassays or mRNA reverse-transcriptase PCR.

We performed a comparison of LST, LPA and IFNB on a healthy dog population in Mallorca

(Chapter 2) in an attempt to gain knowledge on the significance of the three assays of CMI available at our laboratory. Fifty-six dogs with outdoor lifestyles (mainly hunting dogs), including 28 Ibizan hounds, were tested by LST, LPA, and IFNB.

As could be expected in a study population with a high percentage of Ibizan hounds (SOLANO-GALLEGO et al., 2000), a vast majority (80%) of dogs studied presented specific CMI to *L. infantum*. However, the results of the three assays differed substantially. No one assay emerged as clearly better than the other ones, although LPA displayed the least sensitivity and was only positive for dogs also positive for another assay. LST yielded the highest number of positive results (37/56), followed by IFNB (32/56). Twenty four of these dogs were positive by both assays, including 12 which were positive for all three tests. On the other hand, there was a higher coincidence between LPA and IFNB than LPA and LST. Discrepancies among assays of CMI to *L. infantum* have been noted elsewhere (MARTÍNEZ-MORENO et al., 1995). Their significance, however, is uncertain. Analysis of circulating anti-*L. infantum* IgG in these dogs not shown in Chapter 2 (Figure 1), shows no clear relation between humoral immunity and either CMI assay. In fact, specific antibodies were detected only in dogs showing CMI, and were well spread out among the different subsets of CMI results. This homogeneity in the humoral response suggests the differences in results of CMI assays are not

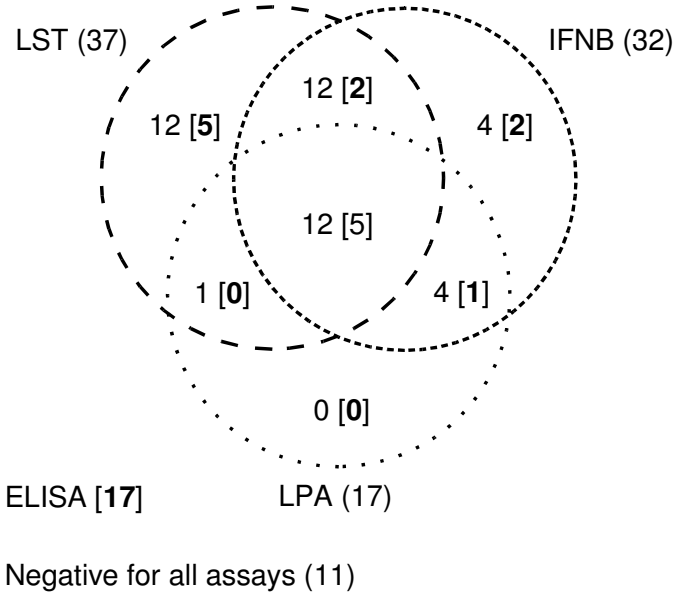

Figure 1: Venn diagram as published in Chapter 2, with serological results added: in brackets, number of dogs within each subset which were seropositive by ELISA. n =56. LST: Leishmanin Skin Test, IFNB: Interferon-γ cytopathic effect-inhibition bioassay, LPA: Lymphocyte Proliferation Assay, ELISA: protein-A conjugate assay for specific antibodies.

necessarily a reflection of different immunological status among the subjects. Evaluation of CMI (LPA, IFNB) vs humoral immunity in a population of stray dogs from the same geographical area studied in Chapter 4 shows a similar pattern (Figure 2).

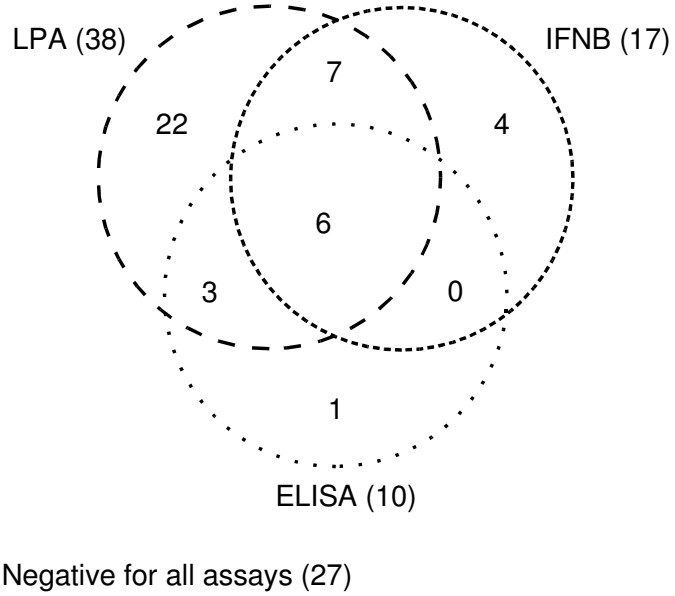

Figure 2: Venn diagram of cellular and humoral immune responses to L. infantum *in dogs tested at a dog pound in Mallorca (Chapter 4). n = 70. IFNB: Interferon-γ cytopathic effect-inhibition bioassay, LPA: Lymphocyte Proliferation Assay, ELISA: ELISA: protein-A conjugate assay for specific antibodies.*

As a whole, our results suggest that none of the CMI assays evaluated can be considered a gold standard of specific cellular immune response to *L. infantum* in dogs. On the contrary, they are complementary, each detecting CMI in partially overlapping subsets of dogs. These discrepancies are not specific of CMI to *Leishmania*. In a study comparing CMI assays for human tuberculosis (Talati et al., 2009), tuberculin skin test and two IFN-γ assays only yielded coinciding results in 3 out of 27 positive subjects. The remaining 24 subjects were each only positive by one IFN-γ assay. Other studies have found similarly incongruent results (Adetifa et al., 2007).

Putative causes of these incongruent results among CMI assays include methodological causes, as well as factors intrinsic to CMI itself. Performance of CMI assays depends on many variables, including tempo, measurement systems, molecule concentrations, and cell culture conditions. These make for extreme variability in how the assays are performed. It may be possible that the discordant

results of the CMI assays evaluated are in fact due to suboptimal assay standardization. However, arbitrary modifications of cutoff values in our studies did not increase congruence among results (data not shown), in line with other reports (Talati et al., 2009). On the other hand, the lack of a specific measurable target for evaluation of CMI, may underly discrepancies among CMI assays, as each assay targets subtly different aspects of CMI. For instance, the cell populations involved in delayed hypersensitivity tests are distinct from circulating lymphocytes assessed in in vitro assays (Vukmanovic-Stejic et al., 2006). Given this, it is possible that a “gold standard” for evaluation of CMI is intrinsically unattainable.

Finally, although this study did not include parasitological assays (culture, PCR), and thus only provides indirect evidence for infection rates, if specific CMI is taken as an indicator of current infection, i.e. with viable parasites (Sacks & Noben-Trauth, 2002), the dogs studied showed highly prevalent infection by *Leishmania* in absence of disease. This is in agreement with the current understanding that a majority of infected dogs in endemic areas mount effective CMI to *L. infantum* (Baneth & Solano-Gallego, 2012).

NATURAL L. INFANTUM INFECTION IN HEALTHY DOGS

CaL is highly prevalent in domestic dogs living in endemic areas. Disease is, however, “the tip of the iceberg” as regards infection (Baneth et al., 2008). Over the past fifteen years, many epidemiological studies have demonstrated massive prevalence of infection and specific immune responses in domestic dogs. This was partly brought about by the use of tests other than serological assays in epidemiological studies on *L. infantum* in dogs, namely CMI assays and molecular biology tools (Pinelli et al., 1994; Berrahal et al., 1996). Although these results were originally attributed to prolonged prepatence or to the fact that some dogs may be clinically cured but still infected (Berrahal et al., 1996; Cabral et al., 1998), it has become evident that disease is the anomalous outcome of infection, with unapparent infection being the norm.

This paradigm shift provides a novel perspective on the epidemiological dynamics of *Leishmania* infection. For instance, it helps account for the clamorous failure of dog culling programs to control HVL in Brazil (Quinnell & Courtenay, 2009; Costa, 2011).

Research on natural *Leishmania* infection has consequently shifted its attention away from disease and pathogeny to encompass (latent) infection and mechanisms involved in a sustained stable host-parasite equilibrium. This new scenario raises questions about the characteristics and significance of chronic infection in healthy dogs. Although much work has been performed on *Leishmania* pathogenesis, absence of disease has received comparatively little attention. The evolution of infection and host responses over time is key to an adequate model of leishmaniosis. In Chapters 3 and 4 we investigated some aspects of infection in heatlhy naturally infected dogs living in Mallorca. Chapter 3 is a pioneering study that investigates parasite loads and microscopical lesions in the grossly healthy skin of infected dogs. Chapter 4 explores the variations in markers of infection and disease before and after the sandfly season in healthy dogs.

L. infantum in grossly normal skin

Following studies by (Berrahal et al., 1996), who using PCR demonstrated widespread presence of *L. infantum* in the skin of dogs living in an endemic zone (Southern France), our research group confirmed the skin of infected dogs as a putative significant reservoir of amastigotes in healthy dogs (Solano-Gallego et al., 2001). In this study, prevalence of PCR detection of *L. infantum* DNA in the skin was three-fold that of the bone marrow, classically considered a reservoir tissue. These results

opened the possibility that healthy infected dogs may be infective to sandflies, which in turn may prove to be a determinant factor in the transmission and epidemiology of CaL.

As an initial study into the epidemiological relevance of normal dog skin (without macroscopic lesions) in CaL, we analyzed skin biopsies from healthy and diseased dogs infected by *L. infantum* (Chapter 3). We studied skin biopsies which were positive for *L. infantum* by PCR from 35 dogs from an endemic area (Mallorca), diagnosed as healthy (symptomless, seronegative) or diseased (with symptoms, strongly seropositive). Histopathological and immunohistochemical studies revealed stark differences between the skins of healthy and diseased dogs. Healthy dogs, positive by PCR of the skin, but with low or undetectable antibody levels and no clinical signs, had microscopically normal skin, which was negative for *L. infantum* immunohistochemical staining. By contrast, most diseased dogs (presenting elevated antibody levels and clinical signs) harboured large numbers of *L. infantum* amastigotes in moderate to severe microscopical lesions of macroscopically normal skin.

Dogs with CaL therefore appear to harbor large numbers of *L. infantum* amastigotes even in macroscopically normal skin. Our results were subsequently confirmed by Giunchetti et al. (2006), who linked parasite load in the skin with severity of CaL, and Saridomichelakis et al. (2007), who found similar amounts of parasites in the skin at different sites in diseased dogs. This high density of parasites, specific of dogs with overt disease explains results of xenodiagnosis, by which only sick or highly seropositive dogs were found to be infectious to vector sandflies (Molina et al., 1994; Travi et al., 2001; Courtenay et al., 2002).

Significantly, we showed that *L. infantum* amastigotes persist in very small numbers (detectable by PCR but not IHC) in the skin of healthy dogs, in absence of significant inflammatory infiltrates. These results are in accordance with findings in other instances of *Leishmania* infection. In an experimental model emulating natural infection of laboratory mouse strains with *L. major*, (Belkaid et al., 2001) found persistence of unapparent infection at the site of inoculation up to a year after infection, in a dynamic equilibrium between parasite and host immunity. Similarly, a study on clinically cured HuCL patients in Brazil, (Mendonça et al., 2004) detected *L. braziliensis* DNA in lesion scars in the absence of microscopical lesions. As mentioned previously, however, xenodiagnosis studies of healthy dogs and humans harbouring *L. infantum* (Costa et al., 2000; Travi et al., 2001; Courtenay et al., 2002), suggest that infectivity of these hosts to sandfly vectors is far lower (if any) than that of diseased hosts. Nonetheless, participation of healthy infected hosts in the

epidemiology of *L. infantum* cannot be completely ruled out. Even a minimal rate of infectivity to sandflies in such hosts may be by a large enough proportion of such hosts. Furthermore, host-parasite equilibrium may go through transient periods of enhanced infectivity in healthy hosts as a result of dynamic interactions between infection, host immune response and environmental factors.

Effect of seasonality of the sandfly vector on *L. infantum* infection in dogs

The sandfly vectors of *L. infantum* in the ecoregions around the Mediterranean basinare seasonal, with activity, and transmission, of the parasite limited to the warmer months of the year (Rossi et al., 2007). In a classical model of disease, each transmission season would be responsible for a cohort of CaL cases in the domestic dog population, albeit with variable, and in some cases protracted, incubation periods. However, widespread chronic infection by *L. infantum* in healthy dogs implies a different role and consequences of vector seasonality on the rates of infection and on the course of novel and ongoing infections.

The elevated prevalences of infection by *L. infantum* demonstrated over the past 15 years in dogs living in endemic areas (Baneth et al., 2008) imply that perhaps a majority of infected sandflies actually feed on dogs that are already infected. Therefore, *L. infantum* will in most cases be inoculated into infected dogs which have already developed an immune response to the parasite (Saridomichelakis, 2009). Instances of novel infections in unexposed hosts will be comparatively rare. Consequently, the actual impact of the sandfly season on *L. infantum* infection in dogs is therefore not easily deducible.

In order to advance knowledge on the impact of the sandfly season on infection and disease, we evaluated parasitological and immunological markers of *L. infantum* infection and disease in dogs culled at a dog pound in Mallorca before and after the sandfly season (Chapter 4).

Analysis of specific immune response to *L. infantum*, and parasite detection in the muzzle skin were performed on 37 dogs in late February, and 42 in late October. Remarkably, prevalence of almost all specific markers included in this study were roughly similar for both groups of dogs. Overall, prevalence of infection by *L. infantum* was greater than 60%, in agreement with a previous study on dogs at the same location (Solano-Gallego et al., 2001). Also, as was to be expected in healthy dogs, CMI, evaluated by LPA and IFNB, was the predominant form of specific immunity, being over three times more prevalent than humoral immunity, and almost fully overlapping it (Figure 2).

The only noticeable difference between results before and after the sandfly season was in prevalence of detectable serum antibodies against *L. infantum*, which were observed in twice as many dogs sampled in October vs. February. However, this difference was not statistically significant and did not correlate with an increase of clinical leishmaniosis. Such absence of subtstantial changes in prevalence of infection have also been observed by other researchers (Gramiccia et al., 2010). If real, the increase we observed in specific antibody levels may be a response of healthy chronically infected dogs to superinfection. Such reexposure may trigger a mild transient increase in memory T cell activity, more easily evidenced in humoral immunity because of its lower baseline levels (about 15%) compared to CMI (about 50%). As with the results presented in Chapter 2, this study underscores the need for comprehensive evaluation of host immunity in CaL studies, including both the humoral and cellular components of specific immunity.

The results of the studies presented in Chapters 3 and 4 contribute to the new epidemiological scenario for CaL in which cases of disease constitute a minor proportion of infected dogs (Baneth et al., 2008). Our results also point to a reformed model of dynamics of infection and disease in which host-pathogen interaction constitute a complex dynamic system, and disease is not a necessary outcome (Inglis, 2007). As previously reported (Berrahal et al., 1996; Cabral et al., 1998; Solano-Gallego et al., 2001), we have found that infection by *L. infantum* is generalized among domestic dogs living in endemic areas. However, high prevalence of infection is countered by widespread protective CMI, and maintained in a dynamic equilibrium in which infection normally remains latent and does not induce disease. Although unproven in dogs, it is probable that persistent infection is necessary for sustained specific protective immunity (Belkaid et al., 2000), in a process which should perhaps be equated to concomitant immunity in nematode immunology (Chapel, 2006).

In this setting, healthy dogs are a major epidemiological factor in the *L. infantum* cycle. Our findings (Chapter 3), together with other studies in dogs and humans (Costa et al., 2000; Travi et al., 2001; Courtenay et al., 2002; Quinnell & Courtenay, 2009), indicate healthy infected dogs do not readily transmit the parasite to sandflies. Nonetheless, the large number of healthy infected dogs (Chapter 4, (Baneth et al., 2008) may compensate their low infectivity to the point that they become a relevant reservoir of disease. Much more significantly, however, infected healthy dogs appear to be a pool from which CaL emerges. Disrregulation or shifts in the host immune status or balance

can be brought about by numerous intrinsec and extrinsec factors (nutrition, concurrent infections, environmental stress) (Serafim et al., 2010) and upset the host-*Leishmania* equilibrium leading to overt disease (Ferrer et al., 2002; Alvar et al., 2004).Disease would hence be the outcome of a complex interactions at molecular, cellular, organism and ecological level. Therefore, efforts to control CaL and zoonotic HVL must inevitably fail if they only target disease in dogs (Courtenay et al., 2002; Moreira et al., 2004; Quinnell & Courtenay, 2009; Costa, 2011). By contrast, integrative approaches, such as curtailing transmission through the widespread use of insecticides (Miró et al., 2008) or vaccines (Dantas-Torres, 2006) is a much more promising road.

Susceptibility of dogs to CaL is frequently referred to as an example of lack of coadaptation between dogs and *L. infantum*, and attributed to relatively recent infection of domestic dogs by *L. infantum* (Ashford, 2000; Acha & Szyfres, 2003). However, accumulating data suggests that in many instances, host and parasite have indeed coadapted into a largely non-pathogenic relation. Latent non-pathogenic chronic infection has been shown, in our studies and others, to be the normal outcome of infection by *L. infantum* in dogs. Furthermore, at least one dog breed (Solano-Gallego et al., 2000) (and perhaps others) living in endemic areas has developed efficient CMI responses that protects it from disease. Even the wolf, the sylvatic ancestor of domestic dogs, also seems to have adapted to *L. infantum*. A study demonstrated *L. infantum* infection in 8/39 wild Iberian wolves (*Canis lupus signatus*), as well as in other wild carnivores (Sobrino et al., 2008). It is possible that coadaptation with *L. infantum* has indeed taken place in wolves and some dog breeds, but not in others, which would have been exposed more recently to *L. infantum*. Many new dog breeds were created in recent centuries (Parker et al., 2004; Larson et al., 2012), and it is also probable that this was accompanied with arrival of genotypes which had not previously been exposed to *L. infantum* to endemic areas.

INFECTION BY, AND IMMUNE RESPONSE TO, L. INFANTUM IN OTHER SPECIES: HORSES, HUMANS, CATS

The accepted epidemiological cycle of *L. infantum* in SW Europe implicates domestic dogs as reservoir hosts, and human beings as occasionally suffering disease (Ashford, 2000; Bern et al., 2008). Human infection, now mainly affecting immunocompromised persons, has become one of the main opportunistic infections in HIV positive patients and a worldwide health concern (Alvar et al., 2008) Besides the zoonotic importance in the recent re-emergence of HuVL due to the AIDS epidemic, canine leishmaniosis is relevant in veterinary practice, as it is highly prevalent, and can pose a diagnostic and therapeutic challenge. By contrast, veterinary significance of *L. infantum* infection in other animal species is minor. Some studies have evaluated other putative reservoirs of *L. infantum* in Spain and other Mediterranean countries, but although infection has been often demonstrated in rodents and carnivores, they have been generally considered accidental hosts (Alvar et al., 2004; Gállego, 2004; Millán et al., 2011).

Horses cohabit extensively in with dogs in *L. infantum*-endemic areas, and are also fed upon by the sandfly vectors of *L. infantum* (Bongiorno et al., 2003), many of which are opportunistic in their host preferences (Gállego, 2004). Despite a high probability of inoculation of *L. infantum* by sandflies, however, horses appear to be remarkably free of disease. In fact, there are few reports of equine leishmaniosis in the Mediterranean region (Koehler et al., 2002; Rolão et al., 2005). Over a five year period, three horse skin biopsies were submitted for histopathological diagnosis to the Servei de Diagnòstic de Patologia Veterinària (UAB) and diagnosed as equine cutaneous leishmaniosis (Chaper 5). Prompted by this trickle of cases, we undertook a preliminary investigation into the immune response to *L. infantum* in healthy horses living in an endemic area (Chapter 6).

Our pathological findings (Chapter 5) were similar to those described in the bibliography. All three horses presented a similar gross lesional pattern, with multiple small nodular lesions especially evident on thinly haired parts of the body (head or inner thighs). Histologically, the three cases were showed a similar picture, consisting of an intense and diffuse histiocytic infiltrate in the dermis, with associated multinucleated cells. Immunohistochemical staining for *Leishmania* demonstrated large numbers of intracytoplasmatic amastigotes in macrophages within the granulomatous infiltrate. Also, a mild to intense lymphocytic infiltrate was observed in the periphery of the

granulomatous lesions. In all three cases, the skin lesions regressed spontaneously in a few months, and no other clinical signs were observed.

A preliminary analysis of the specific immune response to *L. infantum* performed on the most recent case demonstrated specific antibodies, as well as specific lyphoproliferative response. This prompted us to develop a more in depth survey, aimed at estimating exposure to *L. infantum* infection in horses, as well as to evaluate the humoral and cellular immune response to *L. infantum* in healthy horses living in an endemic area (Chapter 6).

Canine assays in use at our laboratoy were adapted for their use on horse samples. Cutoffs to determine positivity were set using horse samples from Utrecht, the Netherlands, considered not endemic for *L. infantum*. Specificity was prioritized over sensitivity, and cutoffs therefore set at highly stringent thresholds (Kurstak, 1985).

Specific anti-*L. infantum* antibodies were evaluated in horse sera using modified ELISAs. Several variations were tested in order to increase the sensitivity of the assays, including reagent concentrations and different conjugates. Specific antibodies were detected in 16 out of 112 horses using a protein A conjugate, using higher concentrations of serum and conjugate than those for the canine assay. Even so, the tested sera did not yield OD values beyond 1.5. Moreover, no horses were positive by the IgG assay. Although these results are hardly quantifiable, they suggest much lower circulating anti-*Leishmania* antibody levels in the horses studied than observed in dogs. This is in accordance with findings from a study in Greece (Kouam et al., 2010). By contrast, adaptation of a canine LPA for use on horse PBMC required very few adjustments. Moreover, the response signal was strong, comparable to results for Ibizan hounds at our laboratory (data not shown), and despite the stringent cutoffs, nearly half the horses tested had positive results. These results suggest generalized infection of horses by *L. infantum* in endemic areas, with a marked predominance of protective CMI.

Although equines have received scarce attention as putative *Leishmania* hosts in the Palearctic, this is not so the Neotropic ecozone, where equine cutaneous leishmaniosis by. *L. braziliensis* is a well defined entity (Ashford, 2000; Vedovello Filho et al., 2008) Equine leishmaniosis is usually reported within wider outbreaks, also involving humans and dogs. Indeed, horses and donkeys have been studied as possible reservoir host of *L. braziliensis* in peridomestic settings (Vedovello Filho et al., 2008).

Clinically, horses and donkeys with cutaneous leishmaniosis by *L. braziliensis* present single or multiple large, ulcerating nodules usually at exposed body parts, which tend to heal spontaneously. *L. braziliensis* is readily detected and cultured from such sites. Studies in horses living in areas with active HuCL foci have shown moderate prevalences of positive antibody titers, as well as infection demonstrated by PCR (Brandão-Filho et al., 2003; Vedovello Filho et al., 2008).

By contrast, equine involvement in *Leishmania* epidemiology in the Palearctic ecozonehas received little attention. Mukhtar et al.(2000) analyzed donkeys as putative *L. donovani* reservoirs in an outbreak in Sudan. They found over two thirds of the donkeys studied were positive by DAT for *L. donovani*. However, no clinical symptoms are reported. Donkeys have also been investigated as putative reservoir host for *L. infantum* in Brazil with negative results (Cerqueira et al., 2003). Other reports of equine *L. infantum* infection in Europe been published have only been published since the turn of the century (Koehler et al., 2002; Rolão et al., 2005) As with our own findings (Chapter 5), these consisted of isolated cases of self-limiting benign nodular skin lesions. In the Portuguese case, specific antibodies were also detected (Rolão et al., 2005), and the authors considered these suggestive of a visceral involvement.

The paucity of reports of equine leishmaniosis by *L. infantum* is in consonance with the mild nature of disease observed in horses (Chapter 5, Koehler et al., 2002). It is plausible that the disease is more frequent than what existing reports might suggest, but being mild and self-limiting is largely ignored and is therefore underdiagnosed. In any case, our results suggest that although infection may be commonplace in horses, the parasite does not induce a severe pathological process.

Our results suggest sandfly vectors readily feed on horses and inoculate them with *L. infantum* promastigotes. This is in accordance with entomological data, which show a marked lack of host preference by sandflies (Gállego, 2004) However, instead of being a dead end for *Leishmania*, our data suggest that infection can progress in horses, at least enough to induce and maintain strong protective cellular immunity. In this scenario, clinical symptoms in horses would not be the result of chance infection, but rather the infrequent outcome of highly prevalent infection.

The clinical presentation of equine leishmaniosis by *L. infantum* is similar to papular dermatitis by *L. infantum* at sandfly bite sites in resistant dogs. Dogs are frequently presented with benign papular lesions on the head, consisting of granulomatous inflammation, which are thought to be elicited by innoculation of *Leishmania* by sandflies at these sites. Affected dogs have been shown to mount

specific cellular immune response, and can be therefore considered resistant to disease (Ordeix et al., 2005) In the case of horses, the pattern of specific immune response against *L. infantum* we observed is also indicative of a potent CMI response which confers protection against disease. Specific antibodies were detected in only 14% of the horses studied, despite having increased reagent concentrations to maximize sensitivity. The assay for cellular immunity, LPA, showed strong responses, and was positive in over 40% of horses studied. Thus, we believe that this pattern of immune response is also similar to that of Ibizan hounds, a breed considered resistant to leishmaniosis (Solano-Gallego et al., 2000). Ibizan hounds are not the only *L. infantum* host with an immune response similar to what we observed in horses. Although *L. infantum* is classified as causing visceral leishmaniosis in humans, clinical disease in non-immunocompromised persons in South Western Europe is very rare. When it does occur, moreover, it usually is in the form of single, self-limiting skin nodules in young or aged patients (Ashford, 2000; Ready, 2010). In fact, unapparent infection by *L. infantum* in humans has also become recognized, not as a freak occurrence, but as common (Le Fichoux et al., 1999; Riera et al., 2008). Healthy humans have been shown to develop specific immune responses in the absence of disease, not surprisingly displaying predominance of cellular over humoral immunity: a study of blood donors in Eivissa (Riera et al., 2004), found a 22% prevalence of positivity to LST compated to 5% to 11% seropositivity with either ELISA or WB. More interestingly, this study identified high prevalence (22%, 27/122) of parasitemia (nested PCR performed on PBMC), which persisted in half of the subjects (9/18) that could be followed up to a year later. Subsequent studies obtained similar results regarding positivity to the different assays (Riera et al., 2008).

Another *L. infantum* host with an apparently similar immune response is the domestic cat. It was generally accepted that cats were resistant to leishmaniosis, based both on experimental data (Kirkpatrick et al., 1984), and on an empirical absence of clinical cases. However, as with horses, and perhaps also due to increased veterinary surveillance, reports of feline leishmaniosis in South West Europe have accumulated (Baneth, 2006) to the point of triggering epidemiological studies in healthy cats and reapparaisal of their role (Maroli et al., 2007; Maia & Campino, 2011). Surveys of serological evidence of infection and parasitemia (PCR) have yielded varying prevalences of infection and exposure (Solano-Gallego et al., 2007; Maia et al., 2008; Ayllon et al., 2008), although they do not observe association among infections by *L. infantum* and FeLV or FIV described in clinical cases (Maroli et al., 2007). This incongruence may suggest that feline leishmaniosis is a secondary consequence of a failure of the immune system to check infection by

L. infantum, and healthy cats tend to remain non-symptomatic. It is possible that healthy infected cats mount strong CMI to *L. infantum*, as do horses, dogs and humans.

Full understanding of *L. infantum* epidemiology warrants an integral approach, in which populations of host species are considered not as isolated entities, but rather as interplaying elements within a complex matrix of pathogeny, parasite persistence, infectivity, and vector competence, among other factors. The epidemiology of leishmaniosis by *L. infantum* can grossly be explained by a simple model in which domestic dogs are the maintainance population of the parasite, and therefore effectively the reservoir of disease in humans (Ashford, 1996). However, growing data on novel or previously disregarded hosts (Chapter 6, Mancianti, 2004; Sobrino et al., 2008; Millán et al., 2011; Molina et al., 2012), and on widespread latent infections in different host species, imply a complex epidemiological picture. Although these latent infections are not necessarily relevant to current *L. infantum* epidemiology, environmental, ecological, and social changes can conceivably change this. Improvement or degradation of living standards can, for instance, lead to increased malnourishment in pet (e.g. horse) and feral mammal (e.g. cat) populations, and effectively modify their relative infectivity to sandflies.

Regarding identification of competent maintainance host populations (Haydon et al., 2002) of *L. infantum* in SW Europe, relevant host species include domestic and peridomestic mammals (dog, horse, cat and rat), sylvatic mammals (wolf, fox, wild rodents) and human beings (Ashford, 1996, 2000; Mancianti, 2004; Sobrino et al., 2008; Millán et al., 2011). The role of these populations in the maintainance and transmission of *L. infantum* is difficult to establish. Although it is widely accepted that domestic dogs, given the population density and prevalence of infection and disease, make up a maintainance population for *L. infantum* (Ashford, 1996), the potential role of other species cannot be easily dismissed and warrants more research.

As with healthy infected dogs, healthy infected humans (Costa et al., 2000) and equines (Cerqueira et al., 2003) do not seem to readily infecte vector sandflies. However, healthy hosts such as horses or humans, although harboring much fewer parasites than diseased dogs, may partake in the transmission cycle of *L. infantum*, albeit with a comparatively negligible impact in areas where dogs transmit *L. infantum* unchecked. If, for instance, effective prophylaxis (Dantas-Torres, 2006; Miró et al., 2008) succeeds in significantly reducing incidence of CaL, the role of such hitherto minor

actors in maintaining and transmitting *L. infantum* may be amplified. It is also plausible that when a healthy (and therefore non-infective) infected host population becomes more susceptible to disease, i. e. through malnutrition (Desjeux, 2004b; Serafim et al., 2010), it may therefore become more infective to sandflies.

In summary, despite a presumably high exposure to inoculation of *L. infantum*, leishmaniosis is a rare occurrence in horses. The high levels of specific immunity, particularly CMI, we have observed in horses suggest that although infection is frequent, it seldom develops into disease. Horses can therefore be considered resistant to leishmaniosis by *L. infantum*, as is the case of two other *L. infantum* hosts: humans and cats. Although a relevant role for horses in current maintainance of *L. infantum* is highly improbable, it can not be ruled out and may become noteworthy if the traditional reservoir (domestic dog) is controlled, or where external factors modify susceptibility to disease in other hosts (Maia & Campino, 2011; Molina et al., 2012).

ON LEISHMANIA

The studies presented in this Thesis (Chapters 2 through 6) focus on specific aspects of the interaction between *L. infantum* and its mammalian hosts in SW Europe. However, they must be interpreted in a broader context, including many "nosodemiological units" (Ashford, 2000), as well as experimental models (Garg & Dube, 2006), with different parasite, vector and host species, epidemiological and pathological characteristics. Attempting to place our results within the overall knowledge on *Leishmania* infections brings forth some issues which are rarely addressed elsewhere: 1) terms commonly used in *Leishmania* research may be inadequate, 2) infection and disease by *Leishmania* fall into a relatively simple pattern despite their heterogeneity, and 3) hallmark characterisitcs of *Leishmania* infection are also found in other infectious diseases.

Do we mean what we say? Words and meanings

Amidst a significant shift in our understanding of leishmaniosis and infections by *Leishmania* in mammals, outdated concepts still underlie the terminology used in *Leishmania* literature. This hinders advancement of novel ideas and models by the use of inadequate and misleading technical terms. A trivial, but blatant, example of this is persistent use of *L. chagasi* (Serafim et al., 2010; Antinori et al., 2012; Pinedo-Cancino et al., 2013), to designate *L. infantum* in South America despite it has been proven long ago to be the same species (Maurício et al., 2000), introduced by Europeans into South America five centuries ago (Lukes et al., 2007). *L. infantum* in South America is not an established endemism, but rather probably it is still in the process of adaptation, perhaps even to *Lutzomyia*, which appears to be a less competent vector than *Phlebotomus* (Quinnell & Courtenay, 2009)

Other instances of inadequate concepts and terminology are not as clear cut, and depend heavily on the favored interpretation of leishmaniosis and *Leishmania*-host interaction. However, they pose a major stumbling block in scientific communications when attempting to advance novel and subtle nuances in the field. A brief comment on several instances where misleading terminology and meanings hampers scientific communication follows.

Leishmaniosis

Although classically used to define disease in susceptible species, leishmaniosis is currently also

used to describe non-pathogenic infection (Sobrino et al., 2008). Historically, equivalence of infection and disease allowed for the use of a single term to define both processes, although disease was the operative target. Non-pathogenic infection was only recognized in species refractory to disease, and was described accordingly (Ashford, 1996). Although attempts at establishing a differentiated nomenclature for infection and disease in parasitic diseases are not new (Kassai, 2006), this is now a necessity. We believe the most sensible approach is restricting the term leishmaniosis to the pathological process primed by *Leishmania* infection, and referring to infection (pathogenic or not) as such, as is being done with other pathogens (Corbett et al., 2003). When specifically describing infection that does not trigger disease, perhaps the best term is "latent infection", as it does not imply disease but denotes the potential for development of disease.

Infection, and reinfection, and superinfection

Elevated prevalences of infection by *L. infantum* in mammalian hosts detected in our research and elsewhere imply that many, if at times not most, infected sandfly bites on host species are in fact on an already infected host (Saridomichelakis, 2009). First-time infection is probably a comparatively rare occurrence, making immune memory both against parasite and vector antigens (Rohoušová & Volf, 2006) key in the course and outcome of infection.

Although "reinfection" has been frequently used to describe such an "infection upon infection" (Chapel, 2006), it does not imply the second infection is concomitant with the first. "Superinfection", on the other hand, implies an overlapping of infections, and is therefore more adequate to describe inoculation of *Leishmania* sp into an already infected host. We must note a drawback to this usage is that "superinfection" is frequently used to describe heterologous infections (Doudi et al., 2012).

Concomitant immunity

Concomitant immunity, or premunition, refers to acquired immunity against a pathogen derived from persistent infection by low numbers of the same pathogen (Chapel, 2006, p. 51). This process is precisely what Belkaid et al. (2000) discovered in a natural infection model of *L. major* in laboratory mice, in which IL-10 downregulates parasite elimination enough to allow persistence of low numbers of amastigotes at inoculation sites, sustained CMI, and therefore protection from superinfection. Concomitant immunity explains how leishmanization works (Sacks & Noben-Trauth, 2002), and is probably common in *Leishmania* infections (Belkaid et al., 2002; Kaye &

Scott, 2011). High prevalence of CMIas observed in our studies (Chapters 4, 6) is probably a product of persistence of small numbers of parasites, constantly priming CMI, an instance of concomitant immunity. Openly acknowledging concomitant immunity as a key process in *Leishmania* infections (Belkaid et al., 2002; Kaye & Scott, 2011) will provide an improved theoretical background for their study, inasmuch as it is highly probable that prognosis, treatment and prophylaxis of leishmaniosis will eventually rely heavily on assessing, stimulating and maintaining concomitant immunity in susceptible hosts.

Prepatence and incubation period

As explained previously, *Leishmania* infection in mammals can no longer be understood based on a linear conception of Koch's postulates of disease. In natural *Leishmania* infection, and experimental models mimicking natural infection, disease is not a necessary (or even probable) outcome. In this setting, *when* infection begins is less important than the (theoretical) moment at which the host organism (perhaps latently infected for years (Walton et al., 1973) initiates a process leading to clinical patence, called cummulative dissonance by Inglis (2007). Prepatent or incubation periods beginning at infection are therefore inadequate for description of leishmaniosis, as they may encompass two consecutive but distinct, and perhaps independent, processes (non-pathogenic latency and pathogenic cummulative dissonance leading to disease).

The *Leishmania* leit motiv

In spite of the great variability of factors and parameters implicated in *Leishmania* infections , infection across all groups of mammals leads to surprisingly few courses and outcomes of infection. Despite a regular trickle of reports of new *Leishmania* sp. - mammalian host combinations, no truly unique novel presentations have been described (Rose et al., 2004; Libert et al., 2012; Molina et al., 2012). Furthermore, these states are not mutually exclusive, and individuals of the same species may fall under more than one category, andeven a single individual may change from one state to another, (i.e. through effective chemotherapy, or disease reactivation as in post-kala-azar dermal leishmaniosis) (Shaw, 2007; Bern et al., 2008). In fact, the different states of host-pathogen interaction can be, to a point, part of a continuum, partially addressed by some authors (Murray et al., 2005; Saridomichelakis, 2009). This continuum can be expressed across multiple axis, such as predominance of humoral vs. CMI, local vs. systemic involvement, susceptible vs. resistant hosts, asymptomatic vs. symptomatic infection.

Despite involving different hosts species in different settings, the results of our studies on natural infection by *L.infantum* in dogs and horses also fall within these states in the host-parasite relation:

- *Chronic progressive disease*, potentially lethal if untreated, such as HuVL, human mucocutaneous leishmaniosis, human diffuse leishmaniosis, and CaL, characterized by inadequate immune response and massive parasite proliferation (Alvar et al., 2004; Desjeux, 2004b). The dogs in group B in Chapter 3 probably belong to this subset; an exacerbated humoral response does not check parasite replication, and is accompanied by progressing disease with high parasite loads in the skin.
- *Self-limiting mild disease*, (followed by chronic latent infection), such as HuCL (Desjeux, 2004b). Equine leishmaniosis by *L. infantum* (Chapter 5) falls into this category, with horses developing self-healing mild cutaneous lesions in absence of other symptoms.
- *Chronic latent infection* with low parasite load and sustained effective immune response, exemplified by symptomless latent infection identified in different species (Riera et al., 2004; Baneth et al., 2008; Sobrino et al., 2008). This is perhaps the most frequent form of infection, and represents most of the subjects included in our studies; all dogs studied in Chapter 2, dogs in Group A in Chapter 3, and almost all infected dogs studied in Chapter 4, as well as the horses in Chapter 6.
- *Abrogated infection* with or without specific host immune response, is the outcome of inoculation of *Leishmania* by sandflies into other, "non-competent" hosts, probably the case of artiodactyls (Anjili et al., 1998; Moraes-Silva et al., 2006).

Notably, the ability of a system to return a discrete number of outcomes irrespective of initial variability is characteristic of complex systems (Gribbin, 2004; García, 2007). Although it is well outside the scope of this thesis, research in leishmanioses would perhaps benefit significantly from an approach based on the complex systems theory (Inglis, 2007; Kaye & Scott, 2011).

Beyond *Leishmania*

As mentioned in Chapter 1, the scientific relevance of *Leishmania* infections in mammals is partly due to their use as models for research in basic immunology. Therefore, the new "concepts and insights" on CaL (Baneth et al., 2008; Miró et al., 2008) are part of a broader reevaluation of infection and disease by *Leishmania* (Tripathi et al., 2007).

Many of the novel ideas and concepts in the field of leishmaniosis have also arisen in the study of *Mycobacterium* infections (Caminero & Torres, 2004; Nicod, 2007). As with *Leishmania*, *Mycobacterium* are obligate intracellular pathogens infecting a wide range of mammalian hosts and inducing several forms of disease, from leprosy through paratuberculosis to classical pulmonary tuberculosis. The interplay of CMI vs. humoral host immune response is also a key determinant in the development of disease. For instance, as in human diffuse cutaneous leishmaniosis, lepromatous

leprosy results from immune anergy in patients that would otherwise develop non-lepromatous leprosy (or HuCL in the case of leishmaniosis) (Britton & Lockwood, 2004; Murray et al., 2005; Kaye & Scott, 2011). Also, healthy humans infected with *M. tuberculosis* appear to control infection effectively, as one third of the human population is estimated to be infected by *M. tuberculosis* but clinical disease is observed in fewer than 10 million humans (Corbett et al., 2003). As with HuVL, a direct consequence of the high prevalence of latent infection and determining role of CMI in controlling infection is that tuberculosis is a frequent complication in HIV-infected patients (World Health Organization, 2004).

These similarities help underscore the futility of studying infection by *Leishmania* in mammals, from a reductionist standpoint, limited to specific aspects of a discrete “nosodemiological unit” or experimental model. As with our results, an integrative approach, across species, models and even diseases, is warranted in order to develop a solid understanding of infection and disease by *Leishmania.*

CONCLUSIONS

1. Taken separately, the evaluated assays of CMI are insufficient to provide a proper evaluation of cellular immunity.

2. Intradermal delayed-type hypersensitivity test is more sensitive than lymphocyte proliferation or Interferon-γ bioassay for *Leishmania*-specific cellular immunity. However, as concluded above, no single test provides a full picture of CMI.

3. Healthy dogs in endemic ecoregions, e.g. Mallorca, are by and large infected with *Leishmania*, mount strong CMI and, weaker specific humoral immuity.

4. *Leishmania* specific-CMI is highly prevalent in infected healthy dogs, which have low parasite loads in the skin.

5. Leishmaniotic dogs present high parasite loads associated with microscopic lesions in grossly normal skin.

6. Seasonality in the transmission of *L. infantum* by sandflies does not substantially impact parasitological and immunological markers of infection.

7. Horses living in endemic ecoregions may infrequently develop mild self-limiting cutaneous leishmaniosis by *L. infantum*.

8. As in healthy dogs and humans, horses from endemic ecoregions mount generalized strong CMI, to *L.infantum*, but weak humoral responses.

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