Vaccine trials against canine leishmaniasis

by

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Acknowledgements

Declaration

I hereby declare that the work presented in this thesis is the result of original research carried out by the author, Connor Carson, under the supervision of Dr. Orin Courtenay, unless otherwise stated. No part of this thesis has been submitted for a degree at another University.

Data presented in this thesis has been published as detailed below:

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Summary

Zoonotic visceral leishmaniasis (ZVL) is a fatal disease caused by the sandfly-borne intracellular protozoan parasite *Leishmania infantum*, and vaccine development in the reservoir host (the domestic dog) is a current research priority. The aims of this study were (1) to conduct safety and immunogenicity trials of two candidate vaccines in dogs, and (2) to compare and demonstrate the utility of immunological and molecular tools for measurement of vaccine efficacy in naturally exposed dogs.

DNA/ modified vaccinia virus Ankara (MVA) prime/boost canine vaccines expressing the *Leishmania* proteins TRYP and LACK were safe, and elicited a type-1 cytokine response, *in vivo* delayed-type hypersensitivity and IgG2 class responses, consistent with superior protective immunogenicity of TRYP over LACK. However, inconsistent associations were found between progressive disease in infected dogs and IgG class levels, prompting caution in use of the latter as a proxy for protective immunogenicity. Specific serological responses in vaccinated dogs did not cross-react with an unrelated diagnostic antigen rK39, and responses to crude parasite antigen (CLA) were minimal, enabling serological detection of infection incidence in vaccinated dogs. Particularly in early stage infection, CLA ELISA was more sensitive than rK39 ELISA and an rK39-based rapid diagnostic test, though rK39 serology was sensitive for diagnosis of symptomatic clinical cases.

A commercially available PCR kit incorporating a rapid oligochromatographic detection step was tested for the first time in dogs, and proved highly sensitive for detection of ZVL infection in bone marrow, comparable to existing nested PCR methods. Molecular methods were investigated as proxy measures to replace labour-intensive xenodiagnosis for detection of the infectiousness of dogs to biting sand flies. Conventional and real-time PCR of tissues from naturally infected dogs were sensitive tests to identify infectiousness, but showed low to moderate specificity. Recommendations are made to improve the application of molecular methods as proxy measures of infectiousness and hence vaccine efficacy.

List of abbreviations

ALM   autoclaved *Leishmania major*
BCG   bacillus Calmette-Guerin
CL   cutaneous leishmaniasis
CLA   crude *Leishmania* antigen
ConA   concanavalin A
DC   dendritic cell
DNA   deoxyribonucleic acid
DTH   delayed type hypersensitivity
ELISA   enzyme-linked immunosorbent assay
EST   expressed sequence tag
FITC   fluorescein isothiocyanate
FML   fucose mannose ligand
HRP   horseradish peroxidase
IDT   intradermal skin test
IFAT   immunofluorescent antibody test
IFN-γ interferon gamma
IL   interleukin
iNOS   inducible nitric oxide synthase
kDNA   kinetoplast DNA
LACK   *Leishmania* homologue of the mammalian receptor for activated C kinase
mRNA   messenger ribonucleic acid
MHC   major histocompatibility complex
MVA   modified vaccinia virus Ankara
PBMC   peripheral blood mononuclear cell
rRNA   ribosomal RNA
rVV   recombinant vaccinia virus
TNF-α tumour necrosis factor alpha
TRYP   tryparedoxin peroxidase
VL   visceral leishmaniasis
WBA   whole blood assay
WHO   World Health Organization
ZVL   zoonotic visceral leishmaniasis

Chapter 1: Introduction

1.1 Background

Leishmaniasis is a disease caused by vector borne protozoa of the genus *Leishmania* (*Kinetoplastida: Trypanosomatidae*), which are obligate intracellular parasites of macrophages and dendritic cells. The disease is endemic in 88 countries worldwide, and the annual incidence is estimated as 2 million cases. Three quarters of these are patients with the disfiguring skin disease cutaneous leishmaniasis (CL), while the remaining 500,000 are cases of systemic visceral leishmaniasis (VL) that is fatal unless treated (Desjeux, 2004). The annual mortality attributable to leishmaniasis is approximately 60,000 (WHO, 2006), and the overall disease burden is calculated by WHO as 2.4 million disability adjusted life years (DALYs). Incidence of VL is rising with urban migration of people from rural areas (WHO, 2002), and increasing overlap of the distribution of VL and human immunodeficiency virus (HIV) has led to the emergence of *Leishmania*/HIV co-infection which has so far been reported from 34 countries (WHO, 2002; Desjeux, 2004; Alvar *et al.*, 2008).

There are around 20 well-recognised species of *Leishmania* which are known to cause CL or VL in humans (Desjeux, 2004). VL results from infection with members of the *L. donovani* complex: *L. donovani*, *L. infantum* and *L. chagasi*, and as a visceralizing infection VL is responsible for more severe clinical disease than CL. VL is subdivided into anthroponotic and zoonotic forms. Zoonotic Visceral Leishmaniasis (ZVL) caused by *L. infantum* (=*L. chagasi*) (Mauricio *et al.*, 2000) causes disease in humans in South America, the Mediterranean, and parts of Asia. Infection of humans occurs when *Leishmania spp.* are injected into the dermis as the metacyclic promastigote life stage following the bite of an infected female phlebotomine sandfly (*Diptera: Psychodidae: Phlebotominae*). ZVL is maintained in the environment by a reservoir host, principally the domestic dog – anthroponotic transmission of ZVL is thought not to occur, though infection may be acquired by drug users sharing needles (Cruz *et al.*, 2002). The expansion in recent years of sandfly vector populations and hence ZVL into non-endemic areas represents a threat to human health in both Old and New worlds (Enserink, 2000; Maroli *et al.*, 2008; Naucke *et al.*, 2008; Otranto *et al.*, 2009a), and the easing of European travel restrictions for UK domestic pets means that there is now a potential reservoir of infected dogs in Britain, should climatic change permit the

establishment of the vector (Shaw *et al.*, 2009). Novel methods for treatment, control and prevention of leishmaniasis are thus a current research priority. Existing drug therapy for human VL is expensive: the WHO-recommended first line treatment (pentavalent antimonial) currently costs US$150 per patient for a 30-day course of sodium stibogluconate injections, US$120 for meglumine antimoniate, and US$30 for generic sodium stibogluconate. Unresponsive cases require more toxic second-line drugs, such as amphotericin B or pentamidine, and although amphotericin B is available in a less toxic liposomal formulation (AmBisome®), the cost is approximately US$1,500 per patient. The first oral drug for VL (miltefosine), which was licensed for use in India in 2002, currently costs US$120 per patient (WorldBank, 2006). Resistance to chemotherapeutic agents is on the increase (Perez-Victoria *et al.*, 2001; Sundar *et al.*, 2001), and at present there are no vaccines available for routine use in humans.

Control of the phlebotomine vector using traditional methods of interior residual insecticidal spraying has been successful in reduction of disease transmission (Boelaert *et al.*, 2000; Kishore *et al.*, 2006), however this control method may promote insecticide resistance among the vector species, requires suitable infrastructure and trained personnel, and is expensive and poorly sustainable (Maroli and Khoury, 2006). Research is under way in the Indian subcontinent into the efficacy of insecticide treated bed nets (ITN) for reduction of sandfly densities, and hence transmission of VL (Joshi *et al.*, 2009).

Reduction of disease by culling dogs has been traditionally targeted as a means of disease control in man in Latin America. However, detailed studies in Brazil (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001b; Courtenay *et al.*, 2002b; Reithinger *et al.*, 2002; Quinnell *et al.*, 2003a) have demonstrated that culling of infected dogs is not an efficient means of disease control in areas of high transmission, and may serve to exacerbate the situation by replacing infected immune animals with susceptibles (Courtenay *et al.*, 2002b). Interruption of transmission using insecticidal dog collars has been successful (Mazloumi Gavgani *et al.*, 2002; Reithinger *et al.*, 2004) although the high price of collars and recurrent costs due to high loss rates (Foglia Manzillo *et al.*, 2006) make such measures impractical for community disease control. Chemotherapeutic drugs against canine ZVL (most frequently consisting of combination therapy with meglumine antimoniate (for 4-8 weeks) and allopurinol (for 6-12 months) can induce temporary remission of clinical symptoms (Noli and Auxilia,

2005), but are generally ineffective in achieving parasitological cure or abrogating infectiousness to sand flies (Alvar *et al.*, 1994; Solano-Gallego *et al.*, 2009).

In recent years, research efforts aimed at controlling leishmaniasis have turned towards vaccine development, following the observation that in humans spontaneous or drug-induced recovery from VL is accompanied by solid immunity against re-infection (Melby, 1991; Roberts, 2005). A successful human vaccine would solely prevent infection of susceptible individuals, whereas a dog vaccine would not only interrupt transmission from the reservoir host, but would also address a major veterinary health problem. In dogs, although data is lacking on immune responses following re-infection under natural conditions, experimental re-challenge of previously infected animals has shown that previous exposure confers a degree of resistance (Santos-Gomes *et al.*, 2003). Even if only partially effective, a canine vaccine which reduced clinical disease or tissue parasite load in infected animals could reduce disease transmission to humans, since infectiousness of dogs to sandflies appears to correlate with both parasite load, and the intensity of clinical signs (da Costa-Val *et al.*, 2007; Michalsky *et al.*, 2007; Vercosa *et al.*, 2008). A vaccination strategy targeting the reservoir host would be of particular benefit to prevent disease in humans who lack a functioning immune system (for example HIV patients) and are therefore unable to mount a response to a human vaccine, were one to become available. Development of canine vaccines for ZVL has been identified as a research priority by WHO/TDR (Dumonteil *et al.*, 2001), and mathematical models have highlighted canine vaccination as potentially the most practical and effective means of disease control in humans (Dye, 1996; Courtenay *et al.*, 2002b). To date, only one canine vaccine has been licensed for prevention of ZVL in Brazil (under the trade name Leishmune®) (Borja-Cabrera *et al.*, 2002; Dantas-Torres, 2006), and other vaccine candidates are urgently being sought. In order to measure vaccine efficacy, a variety of approaches have been employed in the growing number of canine vaccine trials in published research. However, unlike the situation in human vaccine research, in which well validated protocols exist for the conduct of Phase III trials of novel vaccines, there is no standardized method for quantification of vaccine efficacy in canine vaccine trials against leishmaniasis.

The overall aims of the present project were thus to carry out safety and immunogenicity trials of two novel vaccines against zoonotic visceral leishmaniasis in the domestic dog, and to validate diagnostic protocols suitable for detection of both

infection and infectiousness of naturally exposed dogs, enabling the measurement of vaccine efficacy in future large scale Phase III community-based trials of these vaccines.

1.2 Immunology of *Leishmania* infection in mice, humans and dogs

The development of a vaccine against ZVL in dogs depends on a detailed understanding of the canine immune response to *Leishmania* infection. Although differences exist between the murine, human and canine response to *Leishmania* infection, protective immunity in all species depends upon a functioning cellular immune response (Kemp *et al.*, 1993; Pinelli *et al.*, 1994; Reiner and Locksley, 1995; Kharazmi *et al.*, 1999). Impaired cellular immune responses are correlated with progression of disease, while in immune individuals strong T-cell lymphoproliferation is seen in peripheral blood mononuclear cells after exposure to *Leishmania* antigens (Cabral *et al.*, 1992; Pinelli *et al.*, 1994; Pinelli *et al.*, 1999). The CD4+ subpopulation of T-cells is important in the development of immunity, as demonstrated by the fact that human patients with low CD4+ cell counts due to co-existing HIV infection suffer from a particularly severe form of ZVL (Russo *et al.*, 2003). In the most commonly used experimental model for leishmaniasis, *L. major* infection in mice, the immunological correlates of protective immunity appear relatively straightforward. The most important determinant of murine immunity to *L. major* appears to be the balance between two subpopulations of CD4+ cells, designated Th1 and Th2. A bias towards the differentiation of Th1 cells in response to infection is associated with protection from clinical disease, while expression of a Th2-dominated response results in susceptibility (Reiner and Locksley, 1992; Mosmann and Sad, 1996). Studies in human CL support the critical role of the ratio Th1/Th2 response as a determinant of immunity or infection (Gaafar *et al.*, 1995; Kemp, 1997; Kemp *et al.*, 1998).

In the course of natural infection, *Leishmania* at the site of inoculation by the sandfly vector survive uptake by neutrophils, by trafficking to non-lytic ER-like compartments, and their carrier neutrophil is then engulfed by their target cell (macrophages) in which parasite replication occurs (Gueirard *et al.*, 2008). In immune individuals, Th1 cells play a pivotal role in a series of events that lead to the destruction of intracellular *Leishmania* parasites by activation of parasitized macrophages. The main mechanism of parasite killing involves fusion of the phagosome (the intracellular vacuole within the macrophage which contains the

engulfed parasite) with lysosomes to form a phagolysosome. This is followed by nitric oxide (NO) production driven by inducible nitric oxide synthase (iNOS), via an L-arginine dependent intracellular pathway. Other proposed mechanisms include destruction of infected macrophages by cytotoxic (CD8+) T lymphocytes (Muller *et al.*, 1991; Belkaid *et al.*, 2002b) and FasL mediated macrophage apoptosis (Huang *et al.*, 1998). However, in all species so far studied, interferon gamma (IFN-γ) produced mainly by Th1-type CD4+ T cells seems to be essential for the activation of macrophages in order to kill the parasite. When an armed effector Th1 cell contacts an infected macrophage, it produces IFN-γ in association with cell surface expression of CD40 ligand, which together are responsible for the stimulation of highly microbicidal activated macrophages. The importance of IFN-γ is shown by the failure of IFN-γ knockout mice to clear *Leishmania* infection (Wang *et al.*, 1994; Swihart *et al.*, 1995). A second important Th1 cytokine, tumor necrosis factor alpha (TNF-α) is believed to synergize with IFN-γ in mediating parasite killing: in mice it has been demonstrated that disease development after experimental infection with *L. major* was significantly reduced by intra-lesional injection of recombinant IFN-γ and TNF-α together, but not by either cytokine alone. Furthermore, it has been demonstrated that mouse peritoneal macrophages incubated with TNF-α were effective in killing *L. major* parasites *in vitro* (Liew *et al.*, 1990b), and that experimentally infected mice treated with anti-TNF-α antibody had higher *L donovani* parasite burdens than control mice (Liew *et al.*, 1990a). C57BL/6 mice, which are resistant to leishmaniasis, become extremely susceptible to fatal *L. major* infection after deletion of the TNF gene by homologous recombination, and show reduced formation of dendritic cells (DC's) at the site of infection. An improvement of survival time was observed after cutaneous administration of TNF at the time of infection (Ritter *et al.*, 2008). In marked contrast to the cytokine profile described above which correlates with resistance to infection, in BALB/c mice that are susceptible to *L. major* infection, the development of Th2 CD4+ T-cells which secrete IL-4, IL-13 and IL-5 (Janeway *et al.*, 2001), and possibly IL-10 (Roberts, 2005), has been found to antagonize the Th1 response and allow persistence of infection. Secretion of IL-10 by a third population of CD4+ cells, termed CD4+ CD25+ T regulatory cells (T$_{reg}$), was also found to be pivotal in persistence of *L. major* infection in mice (Belkaid *et al.*, 2002a), and in the failure of an experimental *Leishmania* vaccine (Stober *et al.*, 2005).

In dogs, clinical outcomes of ZVL infection comprise a whole spectrum of responses from asymptomatic, through oligosymptomatic (with one or more mild signs), to fully symptomatic disease. Clinical signs are related to granulomatous inflammatory tissue reactions, associated with the presence of parasitized macrophages, and may include generalized muscular atrophy, enlargement of lymph nodes and spleen, exfoliative/ ulcerative dermatitis, excessive nail growth, eye disease and pyrexia (Baneth *et al.*, 2008). Severe symptomatic disease frequently progresses to death of the animal due to glomerulonephritis and subsequent renal failure. Symptomatic infection is associated with the absence of delayed type hypersensitivity (DTH) in response to intra-dermal inoculation of *Leishmania* antigen (Pinelli *et al.*, 1994; Cardoso *et al.*, 1998; Solano-Gallego *et al.*, 2000), and decreased CD4+ and CD8+ T cell numbers in peripheral blood (Pinelli *et al.*, 1994; Martinez *et al.*, 1995; de Luna *et al.*, 1999; Reis *et al.*, 2006a). Other features associated with clinical disease are absence of IFN-γ production by peripheral blood mononuclear cells (PBMC) *in vitro* (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995; Santos-Gomes *et al.*, 2002; Brandonisio *et al.*, 2004) and a high titre of non-protective anti-*Leishmania* IgG antibody (Abranches *et al.*, 1991; Pinelli *et al.*, 1994; Martinez *et al.*, 1995; Pinelli *et al.*, 1995), which is partly responsible for immune-complex mediated glomerulonephritis referred to above. Resistance to disease is associated with activation of Th1 CD4+ cells producing IFN-γ, IL-2, and TNF-α (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995). In dogs, the significance of Th2 CD4+ cells in antagonising the protective Th1 response is currently under investigation. Recent research summarized below has suggested that the failure to generate a Th1 response, rather than the predominance of a Th2 response *per se*, allows progression of ZVL infection in the dog. In contrast to humans and murine infection, the role of IL-10 secreted by CD25+ CD4+ regulatory T_{reg} cells in canine infection outcome is unclear following contradictory evidence (Santos-Gomes *et al.*, 2002; Brandonisio *et al.*, 2004; Chamizo *et al.*, 2005; Pinheiro *et al.*, 2005; Rafati *et al.*, 2005). Previously published work exists showing little correlation between Th2-type cytokine response and progressive disease, for example, in bone marrow aspirates from infected Brazilian dogs, it was shown that IL-4, IL-13 and IL-10 mRNA levels were not elevated relative to uninfected controls, although infected dogs with detectable IL-4 mRNA had more severe clinical symptoms compared to those in which IL-4 was absent, which implies some exacerbatory effect of this Th2 cytokine (Quinnell *et al.*,

2001b). *In vitro* antigen-driven memory cell responses were not measured. Manna *et al.* found that, although IL-10 mRNA was expressed in naturally infected Italian dogs that progressed to symptomatic infection, IL-10 was also expressed in dogs that remained free of clinical signs of disease, suggesting that IL-10 expression could not be used as a correlate of active disease caused by *L. infantum* (Manna *et al.*, 2006). Similarly, in a Brazilian study, high IL-10 levels detected in liver and spleen of symptomatic dogs were not significantly different from levels in asymptomatic animals (Correa *et al.*, 2007). Conversely, in another study of Brazilian dogs, the expression of IL-10 mRNA was high in symptomatic animals in association with high parasite burden, whilst (consistent with the hypothesised role of Th-1 responses in protection against disease) lymph node IFN-γ and TNF-α were high in asymptomatic dogs in association with low parasite burden (Alves *et al.*, 2009). Other studies report a mixed Th1/Th2 response in association with symptomatic disease in dogs, with no particular polarization of the cytokine response. For example, in naturally infected Brazilian dogs, levels of both IL-10 and IFN-γ mRNA expression in spleen cells were positively associated with splenic parasite burdens and severity of clinical signs in (Lage *et al.*, 2007). In a study of experimental infection in laboratory beagles, mixed Th1 and Th2 responses were found after antigen stimulation of canine PBMC, with expression of mRNA for IFN-γ, IL-2 and IL-10. Though IL-2 and IFN-γ predominated in asymptomatic animals in that study, development of symptoms could not be related to IL-10 expression (Santos-Gomes *et al.*, 2002). Mixed Th1 and Th2 responses were also detected by measurement of mRNA in spleens of both experimentally and naturally infected dogs, with an increase in expression of IFN-γ mRNA implicating a Th1-type immune response. However IL-4 levels also increased one month after experimental infection while the expression levels of IL-10 and TGF-β, did not change during the 7 month follow-up period (Strauss-Ayali *et al.*, 2007).

Interpretation of data from canine vaccine trials is further complicated by controversy surrounding other proxy measures of canine cellular immune response against *Leishmania*. In experimental murine models of leishmaniasis, for example, the profile of IgG class titres against crude *Leishmania* antigen (CLA) has been well validated as a proxy measure of T-cell activation. In particular, a high ratio of specific IgG2a:IgG1 has been associated with a Th1-type pro-inflammatory cytokine response (production of IFN-γ by CD4+ helper T lymphocytes), which in turn promotes resistance to

disease caused by *Leishmania major* infection. Conversely, a comparatively low IgG2a:IgG1 ratio results from preferential activation of a non-protective Th2 response involving production of IL-4 and progression to clinical disease (Snapper and Paul, 1987; Heinzel *et al.*, 1989; Coffman *et al.*, 1993; Germann *et al.*, 1995).

As stated above, humoral immunity is not thought to contribute to resistance against leishmaniasis, since IgG antibody titres produced in response to infection with *Leishmania* are non-protective (Pinelli *et al.*, 1994), although recent claims for the existence of a functional role for anti-parasite IgG in contributing to protective immunity (Bourdoiseau *et al.*, 2009) require further investigation. However, measurement of IgG class expression as a proxy for Th1/Th2 bias, as in the murine model, is highly attractive as an additional measure of vaccine immunogenicity in dogs, due to technical simplicity of the procedure and low cost in comparison with cytokine assays. Unfortunately, the interpretation of IgG class profiles in relation to clinical outcomes in dogs is currently unclear: some studies show an association between asymptomatic and symptomatic infection, and elevated IgG2 and IgG1 levels, respectively (Deplazes *et al.*, 1995; Nieto *et al.*, 1999; Iniesta *et al.*, 2005; Carrillo *et al.*, 2007; Ramos *et al.*, 2008), whilst others show the opposite associations (Cordeiro-da-Silva *et al.*, 2003; Reis *et al.*, 2006b; Cardoso *et al.*, 2007; Iniesta *et al.*, 2007). The lack of consistent associations from analysis of IgG1:IgG2 levels in infected animals may result from lack of specificity of commercially available polyclonal conjugates used in the majority of canine studies (Day, 2007), a potential problem which has been largely ignored in published research to date.

In summary, it is clear that in all species studied, the main effector mechanism in resistance to leishmaniasis is macrophage activation by IFN-γ and TNF-α to kill intracellular parasites via the L-arginine dependent nitric oxide pathway, and in addition CD8+ cytotoxic T lymphocytes may be involved in direct lysis of infected macrophages (Pinelli *et al.*, 1995; Ramiro *et al.*, 2003; Rafati *et al.*, 2005). However, in dogs the importance of the Th1/Th2/T$_{reg}$ paradigm in developing/suppressing this end pathway is still open to debate, and in particular the current controversy regarding immunological correlates of protection in the dog (for example, specific IL-10 response and IgG class expression) requires further clarification.

Previous research has shown the importance of genetic factors in disease susceptibility in mice and humans (Blackwell, 1996; Karplus *et al.*, 2002; Jeronimo *et al.*, 2007).

Differential breed susceptibility to canine VL has also been observed in the Ibizan hound (Solano-Gallego *et al.*, 2000) which is more resistant to disease than other breeds, and this has prompted interest in other genetic factors influencing canine resistance and susceptibility to *Leishmania*, such as polymorphism at gene loci DLA-DRB1 (Quinnell *et al.*, 2003b), and NRAMP1 (Altet *et al.*, 2002). This underlines the need for a greater understanding of canine immunology and response to *Leishmania* infection in genetically diverse populations of dogs, and suggests that the pure bred beagle dogs most commonly used in experimental canine leishmaniasis studies may not be broadly representative of immune responses in natural populations of dogs in endemic areas.

1.3 Experimental vaccines against zoonotic visceral leishmaniasis

An effective vaccine against an infectious pathogen should confer long lasting protection against disease resulting from exposure to the live organism, and should be safe, biologically stable, cost-effective and easy to administer.

A variety of vaccination strategies against *Leishmania* have been attempted, in the mouse model as well as in human and canine trials. An important feature of the leishmaniases, which has been exploited in vaccine research, is that protection against one species of *Leishmania* has the potential to induce cross-protection to all species (Coler and Reed, 2005). For example, vaccination of Indian langur monkeys with *L. major* confers protection against experimental challenge with *L. donovani* (Misra *et al.*, 2001).

However, despite the use of many different vaccine candidates, delivered by different inoculation routes and protocols, no human vaccine yet exists for routine use, and only one prophylactic vaccine has been licensed (in Brazil) for administration to dogs.

Leishmania vaccines fall into three main categories:

1) Live whole parasite vaccines

2) First generation vaccines: whole killed parasites or fractions of parasite preparations.

3) Second generation vaccines: defined vaccines such as recombinant protein antigens, naked and virally vectored DNA vaccines, and genetically engineered attenuated or gene knockout parasites.

Routes for inoculation include subcutaneous, intradermal and intra-peritoneal administration (the latter more commonly used in experimental mice). Vaccines are

normally inoculated by injection using a needle and syringe, though needle-free methods have been developed, such as propulsion of small particles of vaccine-coated inert metal through intact epidermis (Liu, 2007). In addition to the vaccine itself, other pro-inflammatory chemical components known as adjuvants may be co-administered to augment the cellular immune response, and recent research has highlighted the potential of sandfly salivary proteins in this role to promote a protective vaccine response (Collin *et al.*, 2009).

1.3.1 Live whole parasite vaccines and first generation vaccines

Early attempts at vaccine formulation were based on whole live, attenuated or killed parasites. The earliest of these methods, known as "leishmanization", was practised in the former Soviet Union from the 1930's onwards for immunization of humans against cutaneous leishmaniasis, and was also used in Israel in the 1970's and in the Islamic Republic of Iran in the 1980's (Nadim *et al.*, 1983). The process involves deliberate intradermal inoculation of cultured viable *L. major* promastigotes, in a preferred part of the body where the resulting ulcerative lesion will cause least inconvenience for the patient, usually the deltoid area of the arm. A solid immunity to natural infection is usually developed, which lasts for the lifetime of the patient. This process is still used in some former soviet countries such as Uzbekistan (Modabber, 2000). However, difficulties with standardization of the dose and quality control, as well as safety concerns regarding human (or canine) inoculation with live unattenuated parasites are obstacles to widespread adoption of this method. Inoculation of live *Leishmania* is also inadvisable in areas where HIV infection is significantly prevalent in the human population.

Vaccination strategies have been developed using crude preparations of killed *Leishmania* parasites for prevention of both CL and VL (Genaro *et al.*, 1996). Trials to investigate the safety, immunogenicity and efficacy of these vaccines in humans have been conducted in South America and the Middle East, using preparations of killed whole promastigotes with or without bacillus Calmette-Guerin (BCG) adjuvant. In general these trials have been disappointing, demonstrating only partial protection in some subgroups of vaccinated patients. In Brazil a multi-strain combination of 5 autoclaved *Leishmania* strains was developed (Mayrink *et al.*, 1979) which was partially protective against CL. A simplified version of this vaccine containing only one strain (*L. amazonensis*) was tested in Columbia (Velez *et al.*, 2000) and Equador

(Armijos *et al.*, 2004), with mixed results, and has been registered in Brazil, for adjunctive use in humans alongside chemotherapy but not for prophylactic use (Khamesipour *et al.*, 2006). Other first generation experimental vaccines for humans include a combination of autoclaved *L. major* (ALM) promastigotes and BCG which has been tested in Iran and Sudan. In the Sudanese trial, ALM + BCG induced significantly higher rates of Leishmanin skin test (LST) conversion in those vaccinated (30%, vs. 7% by BCG alone), and there was a 43% lower incidence of visceral leishmaniasis in skin test responders as compared to non-responders. However, there was no significant difference overall in disease incidence between vaccinees and controls (Khalil *et al.*, 2000). In Iran, similar results were obtained with this vaccine formulation (Sharifi *et al.*, 1998; Momeni *et al.*, 1999). Adsorption of ALM onto alum (aluminium hydroxide) adjuvant has shown more promise as a vaccine candidate. In Sudan, this vaccine induced a high rate of LST conversion in those vaccinated, which was maintained for at least 90 days post vaccination (Kamil *et al.*, 2003).

In dogs, experimental trials of crude *Leishmania* vaccines have also been undertaken, with mixed results (see table in Appendix 1 for details of all published canine vaccine trials against *Leishmania* to date). In Iran, vaccination of dogs with autoclaved *L. infantum* or *L. major* plus BCG adjuvant was protective against experimental infection with *L. infantum* promastigotes delivered intraperitoneally. Field trials of this vaccine were not promising (Mohebali *et al.*, 1998), so to enhance immunogenicity the vaccine was adsorbed onto alum, as described above. A single dose of alum/ALM/BCG given to naturally exposed dogs in a double blind field trial showed 69% efficacy for prevention of seroconversion (Mohebali *et al.*, 2004a). Cell-mediated immunity and parasite persistence were not investigated.

As a refinement of crude vaccines, vaccination of dogs with *Leishmania* fractions, derived from cultured parasites by procedures such as Mendonca-Previato aqueous extraction (Mendonca-Previato *et al.*, 1983) have also been investigated. One of the first canine vaccines, a partly purified preparation of lyophilized *L. infantum* (LiF2), was administered to animals in a holoendemic area together with muramyl dipeptide adjuvant. Paradoxically, the incidence in vaccinated animals was significantly higher than controls in the first year post vaccination (Dunan *et al.*, 1989) (though these differences disappeared in the second year), despite the fact that in previous tests, this vaccine was protective against CL in experimental murine models (Frommel *et al.*, 1988; Monjour *et al.*, 1988). The authors of those studies speculated that failure to

protect dogs against natural exposure may reflect differences between experimental and natural hosts. Other research has suggested that some immunization regimes based on killed parasites may result in disease exacerbation (Titus *et al.*, 1984; Liew *et al.*, 1985). Conversely, *L. infantum* antigens (LiESAp) excreted/secreted in culture (consisting mainly of a 54kDa protein) were shown to be 100% protective against development of clinical disease and detectable infection in bone marrow culture from experimentally infected dogs after 14 months (Lemesre *et al.*, 2005). Subsequently, in field trials of several hundred dogs that were naturally exposed to infection for 24 months (two transmission seasons), the infection rate of bone marrow detected by PCR was significantly lower in the vaccinated animals, and vaccine efficacy was calculated as 92% (Lemesre *et al.*, 2007). However, the low overall number of infections (12/175 in the placebo group in comparison with 1/165 in the vaccine group) suggest that this vaccine should in future be trialled in an area of higher incidence. The *Li*ESAp vaccine is currently undergoing trials as a second generation defined peptidic formulation with QA21 adjuvant, dubbed "Cani Leish" (Papierok and Lemesre, 2009).

The most promising of the purified fraction vaccines is the fucose mannose ligand (FML) vaccine based on a glyoproteic complex purified from *L. donovani* promastigotes, which was shown to be safe, immunogenic and protective against experimental challenge in rodent models (Palatnik-de-Sousa *et al.*, 1994; Santos *et al.*, 1999; Paraguai de Souza *et al.*, 2001). In dogs, the FML vaccine with saponin adjuvant elicited enhanced levels of IFN-γ, NO and anti-*Leishmania chagasi* IgG2 (Araujo *et al.*, 2009), and in Phase III field trials in Brazil demonstrated 95% protection against the development of clinical disease with an efficacy of 80% after 41 months follow-up (Borja-Cabrera *et al.*, 2002). The vaccine was shown to block transmission by eliminating canine *Leishmania* parasite loads (hence availability to sandflies), as determined by PCR carried out on peripheral blood and lymph node aspirates (Nogueira *et al.*, 2005). The FML vaccine, formulated with saponin adjuvant (Riedel de Hahn saponin mixture) was subsequently licensed in Brazil as Leishmune[®] (Fort Dodge, Brazil) being the first licensed canine anti-*Leishmania* vaccine worldwide. In these studies (Santos *et al.*, 2002) as in other vaccine studies which employed saponin adjuvants (Schetters *et al.*, 1994), short-term side effects attributable to saponin were documented (localised pain at the site of inoculation; listlessness and anorexia for a few days; development of a swelling at the injection site

in approximately 15% of dogs) (Parra *et al.*, 2007; Santos *et al.*, 2007). These side effects may be of concern regarding potential loss of compliance among dog owners and vets, and difficulties in licensing of the Leishmune® vaccine in developed endemic countries including Europe.

1.3.2 Second generation vaccines

1.3.2.1 Recombinant antigens and peptides

Peptides are the most commonly selected defined vaccine candidates, since they engage T-cells and elicit the full spectrum of adaptive immune responses. Defined recombinant *Leishmania* antigens are an attractive target for development as vaccines because, in comparison with crude killed or purified parasite preparations, they are easily standardized to conform to Good Manufacturing guidelines, and not dependent on maintaining cultures of live parasites. The development of recombinant peptides as vaccines has been aided by genetic studies of *Leishmania* (Herrera-Najera *et al.*, 2009), in particular the sequencing of the complete genome of *L. major* (Ivens and Blackwell, 1996) and the recent addition of the complete genomes for *L. infantum* and *L. braziliensis* (Peacock *et al.*, 2007). Recombinant peptides are generated by cloning of *Leishmania* genes into bacterial plasmids, and subsequent expression of their corresponding proteins in bacterial cultures. Cloning DNA which encodes more than one protein into an expression vector permits the expression of recombinant polyproteins. The concept of multi-antigen vaccines has been suggested as a means of overcoming MHC-restricted antigen recognition which arises due to genetic differences between individuals. This method has been used to create multivalent recombinant protein vaccines presenting a broader range of protective epitopes, which are less likely to suffer from MHC related unresponsiveness in a genetically heterogeneous population, or between vaccinated individuals from different ethnic backgrounds. In general, despite some success in trials summarized below, subunit vaccines based on proteins or peptides have shown variable to poor protection against challenge. Reasons for this may include failure of *E. coli* produced proteins to achieve their native conformation with respect to folding, appropriate glycosylation and other post translational modifications (Handman, 2001).

Experimental testing of recombinant proteins *in vivo* has been conducted mainly in preclinical trials in the murine model of infection, although some work has been

conducted with primates and in dogs. So far, *Leishmania* antigens which have been trialled as protein/peptide vaccines include glycoprotein 63 (gp63) (Jardim *et al.*, 1990), *Leishmania* homologue of the mammalian receptor for activated protein C kinase (p36/LACK) (Mougneau *et al.*, 1995), membrane glycoprotein 46 (gp46) (Champsi and McMahon-Pratt, 1988), recombinant hydrophilic acylated surface protein B1 (rHASPB1) (Stager *et al.*, 2000), *L. major* promastigote surface antigen (PSA-2) (Handman *et al.*, 1995), kinetoplastid membrane protein (KMP-11) (Tolson *et al.*, 1994; Trujillo *et al.*, 1999), the *L. chagasi* antigen LCR1 (Wilson *et al.*, 1995), and histone (H1) antigen (Masina *et al.*, 2003). Recombinant proteins based on antigens which are mainly expressed in the amastigote form of the parasite include type I cysteine proteases a and b (CPa/CPb) (Rafati *et al.*, 2002; Zadeh-Vakili *et al.*, 2004), and *L. donovani* amastigote-specific antigen (A2) (Ghosh *et al.*, 2001). Recombinant protein Q, formed from genetic fusion of five *L. infantum* ribosomal and histone proteins, is protective against challenge in dogs when administered intraperitoneally together with BCG adjuvant (Molano *et al.*, 2003), and resulted in both reduced parasite load and clinical signs of disease when administered alone (Carcelen *et al.*, 2009). However, protein Q was not protective against challenge when administered with several different adjuvants in another canine trial (Poot *et al.*, 2009). *Leishmania* recombinant proteins derived from 3 immunodominant antigens, *L. major* stress-inducible protein 1 (LmSTI1) *Leishmania* elongation initiation factor (LeIF), and thiol-specific-antioxidant, presented in a variety of combinations, have conferred good protection against *L. major* in murine (Campos-Neto *et al.*, 2001) and nonhuman primate models (G. Grimaldi & S. Reed, unpublished observation). A tri-fusion vaccine has been developed (Skeiky *et al.*, 2002) in which the three antigens listed above were incorporated into one recombinant polyprotein known variously as Leish 111-f or polyprotein MML, which conferred protection to BALB/c mice against *L. major*. This vaccine is currently undergoing immunogenicity and safety trials in humans (Reed *et al.*, 2003). In dogs, although this vaccine elicited high IgG2:IgG1 antibody ratios thought to indicate a predominantly Th1-type immune response, it failed to protect against natural *Leishmania* infection or disease progression (Gradoni *et al.*, 2005). In other canine trials of recombinant protein vaccines, it was found that immunization with *L. infantum* rHASPB1 or *L. donovani* H1 proteins adjuvanted with Montanide™ ISA 720 were partially protective against development of clinical

symptoms in dogs experimentally inoculated with a high dose of *L. infantum* (Moreno *et al.*, 2007).

1.3.2.2 DNA vaccines

A common drawback of recombinant protein vaccines is failure to induce long lasting memory T-cell responses (Gurunathan *et al.*, 1998). This problem has led to the development of alternative approaches to delivery of vaccine antigens. The discovery in the 1990's that plasmid DNA injected intramuscularly could transfect muscle cells and lead to the expression of encoded protein antigens by myocytes, led to the realization that in this way recombinant nucleic acids could be used to deliver vaccine antigens, a process termed DNA or genetic vaccination (Wolff *et al.*, 1990). This vaccination approach has been trialled with success in species such as mice, hamsters, dogs and humans. Circular plasmids encoding the gene for a *Leishmania* antigen are introduced into the host by injection where they are taken up by host cells in muscle and other tissues. Under the control of a host promoter, intracellular expression and polyadenylation of the antigen are followed by expression of the molecule on the cell's surface, where it is exposed to the immune system. Peptide products of DNA vaccines interact with both MHC class I (Doe *et al.*, 1996) and II (Encke *et al.*, 1999) receptor mediated pathways, and therefore induce both CD4+ and cytotoxic CD8+ T cells. The prolonged antigen production which results from DNA vaccination is also advantageous for the development of immunological memory (Mendez *et al.*, 2001) in which CD8+ T-cells play an important role.

DNA vaccines are generally less costly to produce than peptide or protein vaccines, can be produced on a large scale with high purity, and maintain stability under a wide variety of environmental conditions (Gurunathan *et al.*, 2000). Extensive testing of DNA plasmid vaccines showed that plasmid DNA remained at the site of injection where it was slowly cleared by the host, and that genomic integration was highly unlikely (Sheets *et al.*, 2006b). Treatment related toxicities were limited to the inoculation site and no adverse systemic effects of vaccination were recorded at doses of up to 8mg DNA plasmid per inoculation (Sheets *et al.*, 2006a). In human trials (Wang *et al.*, 1998) DNA vaccines were safe, evoked specific immunity, and produced no side effects even in immunocompromised individuals (MacGregor *et al.*, 1998). DNA vaccination has been shown to be effective against a variety of pathogens in

rodents and nonhuman primates, including murine *Trypanasoma cruzi* and *Leishmania* infection (Gurunathan *et al.*, 1997; Sepulveda *et al.*, 2000; Rosati *et al.*, 2009).

Some DNA vaccine candidates against leishmaniasis were initially investigated for their potential as recombinant protein antigens in studies discussed in the previous section. Plasmid DNA expressing Gp63 was the first DNA vaccine against *Leishmania*, which conferred partial protection in mice to challenge with *L. major* (Xu and Liew, 1994). Other DNA vaccines previously tested as recombinant antigens include KMP-11 (Bhaumik *et al.*, 2009) (Basu *et al.*, 2005), and *L. donovani*-specific A2 proteins (Ghosh *et al.*, 2001). Cysteine proteinases, previously used singly as recombinant protein antigens, also show potential for development as DNA vaccine candidates in mice as a cocktail of CPa and CPb plasmids (Rafati *et al.*, 2001). In dogs, a study in Iran examined the effect of a vaccination regime involving a cocktail of CPa and CPb DNA followed by recombinant CPa and CPb protein antigens prior to experimental infection with *L. infantum* promastigotes (Rafati *et al.*, 2005). Vaccinated dogs remained free of infection in bone marrow and showed elevated lymphoproliferative responses to rCPa and rCPb protein antigens.

A highly promising DNA vaccine which was initially investigated as a recombinant protein antigen vaccine against murine *L. major* infection is a homologue of eukaryote thiol-specific-antioxidant (TSA) (Levick *et al.*, 1998; Campos-Neto *et al.*, 2002), a 22.1 kDa protein identified by screening an expression library with murine immune sera (Webb *et al.*, 1998). This molecule has been cloned, sequenced and characterized as tryparedoxin peroxidase (TRYP) during expressed sequence tag (EST) analysis of *L. major* cDNA libraries in the search for vaccine candidates (Levick *et al.*, 1996; Levick *et al.*, 1998), and a possible functional role has been identified for TRYP in resistance to antimonial drugs against *Leishmania* (Wyllie *et al.*, 2008). Vaccination of mice with a cocktail of plasmid DNA's encoding TSA (TRYP), LACK and LMSTI1 was protective against low dose intradermal challenge infection at 2 weeks and at 12 weeks post vaccination (Mendez *et al.*, 2001).

It is known that the method of delivery of DNA has an important influence on the resultant immune response (Pertmer *et al.*, 1996; Feltquate, 1998). Naked plasmid DNA may be used, however heterologous so-called "prime/boost" strategies which involve priming with DNA and boosting either with protein expressing the same antigen, or the same DNA plasmid incorporated in an alternative delivery system such as bacteria or a recombinant viral vector, may be more effective in stimulating long-

lived immune responses. DNA/ recombinant vaccinia virus (rVV) heterologous prime/boost vaccine protocols are now known to be superior to homologous challenge with DNA, since they stimulate more robust so-called 'synergistic' cellular immune responses (Gilbert *et al.*, 2006). For example, it has been shown in mice that two immunizations with naked DNA plasmids expressing TRYP elicit a similar protective effect to prime/boost vaccination with DNA plasmids, followed by rVV expressing TRYP, when infectious challenge was done in the effector phase of the immune response (2 weeks post vaccination). However, the superior longevity of the memory response induced by prime/boost DNA/rVV TRYP was confirmed by *in vitro* restimulation of draining lymph node cells at 16 weeks post vaccination: the IFN-γ responses and Th1 cytokine bias in mice vaccinated with the DNA/rVV prime-boost protocol were similar at 16 weeks to those observed at 2 weeks post vaccination, and significantly this memory response was not seen in mice vaccinated twice with DNA TRYP alone (Stober *et al.*, 2007). To date, the DNA/rVV prime/boost TRYP vaccine described here has not been tested in dogs.

The p36/LACK antigen (*Leishmania* homologue for receptors for activated C kinase) (Mougneau *et al.*, 1995) has also been studied as a target for DNA vaccination strategies against *Leishmania*. This highly conserved 36kDa protein is involved in stimulation of an early expansion of IL-4 secreting cells (Julia and Glaichenhaus, 1999). In mice, vaccination with LACK DNA has been shown to be protective against challenge with *L. major* (Mougneau *et al.*, 1995; Gurunathan *et al.*, 1997). However, DNA-LACK vaccines were not protective against other *Leishmania spp.* such as *L. donovani* and *L. mexicana* (Aguilar-Torrentera and Carlier, 2001; Melby *et al.*, 2001). Vaccination with DNA-LACK showed equivalent protective efficacy to LACK recombinant protein antigen plus IL-12, in challenge experiments conducted in mice with *L. major* (Gurunathan *et al.*, 1997). However, vaccination with LACK DNA together with DNA encoding Th1-type cytokines IL-12 or IL-18, followed by a booster of LACK DNA expressed in rVV, led to greater cellular immune responses in mice than were observed with the same protocol in the absence of cytokine DNA (Tapia *et al.*, 2003). In other work, prime/boost immunization carried out in BALB/c mice with LACK protein followed by a booster with rVV co-expressing p36/LACK and IL12 induced 52% reduction in lesion size and a reduction in parasite load (Gonzalo *et al.*, 2001).

A critical factor influencing the efficacy of recombinant vaccinia virus vectors for delivery of vaccine antigens is the strain of vaccinia virus selected. A prime/boost vaccination with LACK DNA followed by the highly attenuated MVA expressing LACK (MVA-LACK) elicited higher levels of IFN-γ and TNF-α secreting CD8+ T cells, and conferred greater protection against CL in mice, than the same protocol with a replication competent Western Reserve strain rVV (Perez-Jimenez *et al.*, 2006). In contrast to Western Reserve strain rVV, MVA is a non-replicating vector with six major genomic deletions in comparison with wild type vaccinia virus Ankara (approximately 15% of the genome), and for this reason the probability of reversion to virulence is extremely low. MVA is the current rVV strain of choice for clinical investigations having been used in immunosuppressed macaques (Stittelaar *et al.*, 2001) and in over 120,000 human patients (Mayr and Danner, 1978) without documented adverse side effects, even in immunocompromised humans (Meyer *et al.*, 2005). The DNA/MVA system has also been used for delivery of malaria vaccine in humans without adverse side effects (Moorthy *et al.*, 2003), and in dogs, a rabies vaccine which employed the MVA vector has also been tested without signs of ill effects (Weyer *et al.*, 2007). The DNA/MVA approach is also currently being applied in human vaccine development against HIV (Amara *et al.*, 2002), tuberculosis (McShane *et al.*, 2001) and tumours (Palmowski *et al.*, 2002).

In mice, a direct comparison of the protective efficacy of TRYP and LACK vaccines delivered by DNA/DNA and DNA/MVA prime/boost protocols has been carried out, which showed superior protective efficacy of DNA/MVA TRYP over LACK (Stober *et al.*, 2005). A higher ratio of antigen specific IgG1:IgG2a, and higher *ex vivo* IL-4 mRNA levels, in LACK compared to TRYP vaccinated mice suggested a bias towards a Th2 response in the former. However, both antigens induced high IFN-γ and low IL-4 following restimulation of draining lymph node cells with antigen *in vitro*. An important predictor of the outcome of experimental infection was CD4+ T cell-derived IL-10. In vaccinated mice, the ratio of post-vaccination, pre-challenge IFN-γ:IL-10 in draining lymph node cells was a clear indicator of vaccine success (Stober *et al.*, 2005), in that high IL-10 expression prior to infection predicted failure of the LACK protocol, whereas low IL-10 predicted protection using TRYP. Challenge infection caused further polarization to high IL-10/low IFN-γ with LACK and low IL-10/high IFN-γ with TRYP. *In vitro* depletion experiments and *ex vivo* quantitative reverse

transcriptase PCR demonstrated that antigen-driven CD4$^+$CD25$^+$Foxp3$^+$ T$_{reg}$1-like cells were the primary source of IL-10 in LACK-vaccinated mice. Anti-IL-10 treatment *in vivo* demonstrated that IL-10 was functional in determining vaccine failure, rendering LACK protective in the presence of high IFN-γ and low IL-4 / IL-5 responses.

In dogs, however, Ramiro *et al.* (Ramiro *et al.*, 2003) demonstrated good protective efficacy of vaccines incorporating LACK. In that study, DNA/DNA vaccination using the LACK antigen was effective in reducing organ parasite loads in dogs challenged with *L. infantum*, and a heterologous prime/boost strategy using DNA followed by recombinant rVV (wild-type Western Reserve strain) expressing LACK was even more efficient at reducing clinical symptoms/score. The heterologous prime/boost vaccination strategy gave 60% protection against infection in vaccinees tested over 17 months post experimental challenge with a high intravenous dose (1x10^8) of *L. infantum* promastigotes, and 4/5 vaccinated dogs remained free of clinical disease compared to 0/5 controls. In subsequent canine trials, comparing both DNA/Western reserve rVV, and DNA/MVA heterologous prime/boost vaccines expressing LACK it was demonstrated that fewer dogs showed clinical disease symptoms in the DNA/MVA than DNA/Western reserve groups. Splenocytes from DNA/MVA dogs showed a higher degree of antigen specific CD4+ and CD8+ T cell proliferation than Western Reserve strain vaccinated dogs (Ramos *et al.*, 2008). A further refinement of the LACK vaccine has been expression of DNA in the antibiotic resistance gene free pORT-LACK plasmid, to allay concerns regarding the spread of bacterial resistance in the environment (Ramos *et al.*, 2009). To date, there are no studies in dogs comparing immunogenicity or efficacy of DNA/rVV LACK and TRYP vaccines.

1.3.2.3 Attenuated / genetically engineered live *Leishmania* vaccines

An alternative method for delivery of recombinant antigens is by cloning into a suitable bacterial vector which is then inoculated (live) into the host. In the murine model, gp63 delivered orally by an experimental vaccine strain of *Salmonella typhimurium* has been shown to induce a significant degree of resistance against *L. major* infection (Yang *et al.*, 1990) Also in mice, the expression of the *L. chagasi* antigen LCR1 by live intracellular bacteria (BCG strain of *Mycobacterium bovis*) enhances the IFN-γ response to LCR1 and confers partial protection against challenge with *L. chagasi* (Streit *et al.*, 1996; Streit *et al.*, 2000). A live vector such as BCG

provides the advantage of prolonged exposure to the target antigen, increasing the longevity of the immune response.

Genetic manipulation of *Leishmania* parasites to attenuate virulence or increase their susceptibility to chemotherapy provides another avenue for live vaccine development. A modified *L. major* with knock-out of the dihydrofolate reductase-thymidilate synthetase (DHFR/TS) gene has been trialled in mice (Cruz *et al.*, 1991). This construct was able to survive for up to 2 months *in vivo* without causing pathology, and intravenous or subcutaneous inoculation conferred short term protection against challenge with *L. major* (Titus *et al.*, 1995) and partial protection against *L. amazonensis* (Veras *et al.*, 1999). However, although this mutant parasite generated a Th1-type response in human PBMC *in vitro*, further trials in primates were disappointing.

Introduction of genes (so-called "suicidal cassettes") into the *Leishmania* genome which confer drug sensitivity to chemotherapeutics that are harmless to the human or animal host, permits the establishment of a short-term infection that confers immunity to the host before termination of the parasite with appropriate drug therapy. Infection of BALB/c mice with a double drug sensitive strain of *L. major* induced progressively growing lesions which resolved on treatment with either drug. No recurrence was observed after 4 months (Davoudi *et al.*, 2005) and high levels of IFN-γ correlated with protection against experimental challenge were observed.

A major obstacle to commercialization of any such vaccine that incorporates a live attenuated organism is the potential for reversion to virulence, which together with negative public perceptions of genetically modified organisms may be reasons why genetically manipulated bacterial and live attenuated *Leishmania* vaccines have not progressed beyond the experimental stages described above. Vaccines containing the highly attenuated recombinant MVA are likely to be the only current exception to this, given the extensive clinical trials of this vaccine vehicle which have been undertaken, and the historical precedents described above.

1.4 Development of novel vaccines against zoonotic visceral leishmaniasis

The ability of DNA vaccines to induce long-lived CD4+ and CD8+ responses without the absolute requirement for adjuvants, together with their high stability and lower cost in comparison with recombinant antigens, makes this one of the most appropriate avenues for the development of new canine vaccines against ZVL. Existing research

summarized above suggests that prime/boost DNA/MVA LACK shows significant potential for investigation as a vaccine candidate against canine *Leishmania*, and that DNA/MVA TRYP is also highly promising, but as yet untested in dogs.

1.4.1 Safety and immunogenicity

In order to carry out trials of novel canine *Leishmania* vaccines as an initial step towards commercialization and widespread use in both developing and developed world endemic settings, it is necessary to demonstrate safety and immunogenicity of the vaccine in the target species, as described in European Union legislative frameworks (European Medicines Agency (1994; 1995)). Safety of vaccine formulations at varying dose rates must be verified by monitoring physiological parameters pre- and post-vaccination, to characterize the nature of any undesirable local or systemic side effects, with defined clinical end points. This is important since even relatively minor side effects may reduce the compliance of dog owners and veterinary surgeons in uptake of a vaccine, reducing its commercial potential. Assessment of immunogenicity requires measurement of pre-determined immunological correlates of protective immunity. In the case of canine leishmaniasis, research summarized above shows a fairly consistent association between protection against progressive disease and specific Type-1 cytokine response, particularly IFN-γ. *In vivo* delayed type hypersensitivity, measured by intradermal reaction to crude or defined parasite antigens, is also well validated as a correlate of protective cellular immunity. In addition to these measures, other Th1- and Th2-type cytokines and serological proxy measures of Th1/Th2 bias, as described, contribute to the evidence base required to validate vaccine immunogenicity, despite some controversy over the significance of Th2/T_{reg} cytokine profiles and IgG subtype expression in dogs. It is necessary initially to assess immunogenicity in dogs that are unexposed to infection, since memory responses to vaccine alone cannot be assessed satisfactorily in animals which have been boosted by challenge infection with the parasite.

1.4.2 Efficacy

Measuring the efficacy of vaccines against canine leishmaniasis is complicated by a number of factors: some of these are inherent to the mechanism of transmission of this vector-borne disease, while other issues concern the limitations of available diagnostic methods for confirming infection in dogs. Traditionally, efficacy trials require

experimental inoculation of the disease agent into vaccinated and control unvaccinated (or placebo inoculated) animals, with assessment of efficacy based on the progression of disease symptoms or evolution of parasite burdens in target tissues. In the case of leishmaniasis, this method has questionable application to the measurement of vaccine efficacy. There is increasing evidence to show that experimental intravenous injection of high numbers (10^6-10^8 cultured promastigotes), as has been done in a majority of vaccine trials to date (see Appendix I) is poorly representative of the intradermal inoculation of lower numbers of the parasite by the sandfly vector (typically 100-1000 metacyclic promastigotes, in the presence of a large assortment of immunomodulatory and vasoactive salivary compounds and parasite derived glycoconjugates) (Rogers *et al.*, 2004; Rogers *et al.*, 2006; Peters *et al.*, 2009). In theory, challenging vaccinated dogs with infected sand flies could be done under laboratory conditions. In practice, however, there are significant technical obstacles to maintaining and infecting large insectary populations of the vector, and scaling-up this procedure to enable challenge of sufficient numbers of dogs to enable observation of significant differences between vaccine and control group incidence rates. Ultimately, large scale community-based vaccine trials in dogs exposed to natural field infection will be required to validate efficacy in target populations of domestic dogs. Assessment of vaccine efficacy in field trials will be dependent on measurement of infection incidence in vaccinated *vs* control animals, however there is no widely accepted 'gold' standard method for the diagnosis of canine leishmaniasis. Clinical signs of disease are non-specific (Blavier *et al.*, 2001), and a significant proportion of infected dogs are asymptomatic (Baneth *et al.*, 2008), thus diagnosis must be confirmed with laboratory techniques. Many serological methods are available, including indirect immunofluorescence (IFAT), direct agglutination test (DAT), latex agglutination, immunochromatography, as well as a number of ELISA procedures, each with their own advantages and disadvantages (Iniesta *et al.*, 2002; Alvar *et al.*, 2004). Parasitiological techniques include direct examination of stained tissues, impression smears by microscopy, and laboratory culture of parasites (Manson-Bahr, 1987; Spreng, 1993). Culture is insensitive and time consuming, and different strains of *Leishmania* vary in their capacity to grow *in vitro* (Iniesta *et al.*, 2002). PCR detection of *Leishmania* has improved the sensitivity of parasitological diagnosis of ZVL in dogs, however there is a lack of standardization of target DNA sequences and tissue sample types in the published research to date, which complicates objective comparisons between vaccine trials. Furthermore,

molecular techniques are technically more complex and considerably more expensive than most serological methods, and these factors become important in the design of vaccine trials involving longitudinal follow-up of large numbers of dogs in the field. Thus, before embarking on vaccine efficacy field trials there is a need to validate laboratory protocols which are sensitive and specific for the detection of canine *Leishmania* infection, able to detect infection in its early stages to provide precise estimates of vaccine efficacy, and which are cost-effective for use in potentially resource-poor field environments. Finally, when measuring vaccine efficacy in the canine reservoir of ZVL, an important additional factor of relevance to public health is the infectiousness of infected dogs to sand flies, which are the vectors for transmission of disease to humans. Even if only partially effective in preventing infection and/or disease in the dog, a vaccine which reduced the infectiousness of dogs to the sand fly vector has the potential to impact on transmission. To date, information on experimental methods to measure canine infectiousness without the use of laboratory bred sand flies in xenodiagnosis experiments is scanty, and further research is needed in this area.

1.5 Overall aims

The overall aims of this study were thus:

(1) to carry out safety and immunogenicity trials of novel vaccines against canine ZVL (prime/boost DNA/MVA TRYP and LACK) in domestic dogs, and

(2) to validate experimental protocols for detection of *Leishmania infantum* infection and infectiousness in naturally exposed dogs, as diagnostic tools to enable the measurement of vaccine efficacy in future large scale community-based trials of experimental vaccines against canine leishmaniasis.

Chapter 2: Materials & Methods

2.1 General methods

2.1.1 Cytokine assays

Measurement of IFN-γ, TNF-α, and IL-10 cytokine levels expressed by antigen stimulated lymphocytes was carried out in whole blood assays (WBA) (Shifrine *et al.*, 1978). Blood collected from study dogs by jugular or cephalic venepuncture into heparin anticoagulant was diluted 1:10 in RPMI (Sigma) supplemented with 100IU/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine (Sigma), and incubated in 96-well flat bottomed polystyrene tissue culture plates (BD Biosciences). Triplicate wells (200μl per well) were incubated for each antigen or mitogen (TRYP, LACK, CLA and Concanavalin A - all at 10μg/ml), including negative control (unstimulated) wells, for a period of 5 days at 37°C in 5% CO_2 in air. Supernatants from each of the three replicate wells were pooled and stored at -80°C until required, avoiding freeze-thawing. Measurement of cytokines expressed in culture supernatants was carried out by quantitative ELISA using commercially available reagents (R&D systems). Duoset kits were used to detect IFN-γ and TNF-α, while matched pair monoclonal capture/polyclonal detection antibodies were employed for IL-10 measurement, according to the manufacturer's recommendations. Quantification of cytokine levels was measured from optical absorbance of the coloured ELISA reaction product, with reference to a standard dilution curve constructed from a known quantity of the supplied recombinant protein standards. Background levels in unstimulated control wells were deducted from antigen-stimulated values to quantify antigen-specific cytokine production (with negative values recorded as zero).

2.1.2 Intradermal tests

Cellular immune responses *in vivo* were measured in vaccinated dogs by intradermal skin testing (Pinelli *et al.*, 1994) using 0.1μg TRYP and LACK recombinant antigen in 0.1ml sterile pyrogen-free PBS (or 0.1ml PBS alone, as a control) injected intradermally at the right inner thigh, a distance of 5cm apart. The size of the indurated area was measured at 72h after injection. Two measurements were taken at 90 degrees to each other using vernier calipers, and the mean of the two numbers was recorded. A positive reaction was considered as >5mm.

2.1.3 ELISA

Total antigen-specific IgG, and IgG1/IgG2 subtypes, were measured in canine sera by ELISA. 96-well polystyrene microtitre plates (Maxisorp; Nunc) were coated overnight at 4°C with 50µl 0.05M carbonate/bicarbonate coating buffer, pH 9.6 (Sigma-Aldrich) containing the appropriate amount of antigen (0.5µg TRYP, 0.25µg LACK, 50ng rK39, or 1.0µg crude *Leishmania* antigen (CLA), which were obtained as described below) per well. Wells were washed 3 times with PBS/0.05% Tween 20 (repeated between each step detailed below). Blocking was performed with 2% dried milk powder in carbonate/bicarbonate buffer for 2 hours at 37°C, and 50µl of the appropriate dilution of dog serum in PBS/0.05% Tween20/2% dried milk powder was added to each well. All samples were run in duplicate. For detection of total IgG, 50ul of rabbit anti-dog IgG conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich) was used at 1:1000 dilution for 1 hour incubation at 37°C, while for antibody subtyping, goat anti-IgG1-HRP conjugate at 1:500 dilution, or sheep anti-IgG2-HRP conjugate at 1:10,000 dilution (Bethyl Laboratories) were added. Conjugate and antigen concentrations stated above were selected based on the results of preliminary experiments using checkerboard titrations. 100µl substrate solution (Tetramethylbenzidine (TMB); Sigma-Aldrich) was then added, the reaction was stopped after 20 minutes incubation at room temperature in darkness using 50µl 0.5 M H$_2$SO$_4$, and the optical density of reaction product was read using an automated ELISA plate reader (Multiskan EX, Thermo Fisher) set at 450nm. Positive and negative controls were included on each plate. The sample-to-positive ratio (s/p) (Tijssen, 1985) for each sample was calculated as the mean raw absorbance at 450nm of duplicate test samples relative to a strongly seropositive reference positive control (from a polysymptomatic Brazilian dog with parasitologically confirmed *Leishmania* infection) which was included on every ELISA plate. The method for determination of the cut-off s/p value to define test samples as positive or negative is described in detail in individual chapters. In assays requiring dichotomous output (positive/negative), serum samples were tested at 1:100 dilution, in duplicate. Alternatively, in experiments which required quantification of antigen-specific antibody titre (arbitrary units), titration curves were plotted from doubling dilutions of each serum sample, in duplicate. Negative controls were obtained from sera of 30 UK resident dogs with no

history of foreign travel, presented at veterinary clinics in the UK for unrelated reasons.

2.1.4 rK39 serological dipstick tests

rK39 immunochromatographic dipstick tests (Kalazar Detect Canine Rapid Test: Lot No. HA1047 and HD1037) were obtained from Inbios International Inc. WA, USA, and used to test 20 µl serum samples according to the manufacturer's instructions. The test involves detection of specific antibodies in serum which migrate by capillary action from the pad on the end of the strip, carried by the supplied test buffer. Antibodies bind to immobilized rK39 recombinant *Leishmania* antigen on the stick, where a visible colour change (colourless to red) indicates positive binding. A positive test result was recorded after 10 minutes when a red line appeared in the test area (however faint), in addition to a red line in the migration control area to indicate correct running of the test buffer.

2.1.5 Leishmania immunofluorescent antibody test (IFAT)

Sera were tested by the indirect immunofluorescence test with promastigotes of *L. infantum* zymodeme MON-1, using anti-dog IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich), using standard techniques described elsewhere (Gradoni and Gramiccia, 2004). A series of two-fold serum dilutions were performed, with a conservative cut-off point of 1/40. Positive samples were titrated to the end point, with the IFAT titre stated as the last dilution which gave positive fluorescence. Positive and negative controls were included in each batch of 10 samples, and the negative cut-off titre was validated from the results of IFAT tests carried out on 30 UK non-endemic dogs, all of which gave negative results at 1/40.

2.1.6 Clinical biochemistry and haematology

Blood was collected from vaccinated dogs by jugular or cephalic venepuncture in 2 ml EDTA anti-coagulated and plain serum gel tubes. Samples were sent by same day courier at +4°C to a commercial laboratory (Microanalysi, Athens), and processed for routine biochemical tests (urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK) and total bilirubin) using a standard Aeroset dry chemistry analyzer (Abbott-Toshiba, USA) and red/white blood cell counts using a PCE-210 automatic blood cell counter (Erma Inc., Japan).

2.1.7 DNA extraction

In the present study, DNA was extracted from canine skin biopsies using a commercial kit (DNeasy, Qiagen) according to the manufacturer's recommendations. DNA extraction was also performed on 100µl aliquots of bone marrow, which were digested in 100µl PK buffer (50mM KCl, 10mM Tris pH8.0, 0.05% Tween-20, 100µg/ml Proteinase K). After overnight incubation at 56°C, a phenol-chloroform protocol was used to extract DNA as follows: in a phase lock gel tube (VWR international, UK), 100µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed, followed by centrifugation at 13,000rpm for 5 minutes to separate the aqueous phase. In a new phase lock tube, 100µl of chloroform: isoamyl alcohol (24:1) was added and mixed, and the sample was centrifuged as before. 500µl of 30% polyethylene glycol (PEG 6000)/1.6M NaCl solution was then added to the aqueous phase, before standing for 1 hour at room temperature. Samples were centrifuged as before, and the DNA pellet was washed with 70% ethanol and air dried before elution in 100µl molecular biology grade H_2O. Total DNA quantity was measured by optical absorbance at 260nm using a Nanodrop 1000 spectrophotometer (Thermo Scientific, UK) and purity was estimated from the ratio of optical absorbance at 260:280 nm ($Abs_{260/280}$). Extracted DNA was stored at -20°C until required.

2.1.8 Conventional polymerase chain reaction (PCR) procedures

2.1.8.1 Nested ITS-1 PCR

DNA extracts were subjected to nested PCR targeting the internal transcribed spacer-1 region (ITS-1) of the ribosomal RNA (rRNA) gene (Parvizi *et al.*, 2005). Using a conventional thermal cycler (iCycler, BioRad), first round reactions were carried out to amplify the complete ITS1 region, with primers IR1 and IR2 (Cupolillo *et al.*, 1995) (see Appendix I for primer sequences). 2µl DNA extract was added in a 20µl reaction mix, with primers at 1µM, 2.5mM $MgCl_2$, 0.2mM dNTPs, and 1.25U Truestart Taq (Fermentas). Second round PCRs were performed in separate tubes, using 1µl of a 1:10 dilution of first round PCR product in molecular biology grade H_2O. In the second round, primers ITS1F and ITS2R4 were used to amplify ITS1 (together with the 5.8S rRNA gene) (Parvizi *et al.*, 2005; Parvizi and Ready, 2008). Second round primers were used at 1µM, with 1.5mM $MgCl_2$, and all other reaction conditions as described above. Cycling conditions for both amplification rounds were: 1 cycle of 95°C for 1

min, followed by 37 cycles of 95°C for 30 sec; 58°C for 30 sec; 72°C for 90 sec, followed by 1 cycle of 72°C for 10 min. PCR products were electrophoresed on 1.5% agarose gel as described below to look for a second round reaction product of 480bp. The positive control was DNA taken from a highly symptomatic Brazil dog with confirmed *L. infantum* infection by *in vitro/in vivo* parasitological culture, as determined from the results of a previous study (Quinnell *et al.*, 1997). Negative DNA controls from non-endemic (UK) dogs were included in each run, in addition to contamination controls for PCR solutions and sham extractions.

2.1.8.2 Nested kDNA PCR

A nested *Leishmania* genus-specific PCR (Noyes *et al.*, 1998) was used to amplify a variable region of kinetoplast minicircle DNA (kDNA). First round reactions were carried out with external primers CSB2XF and CSB1XR. 2µl DNA extract was added to make a total 20µl reaction mix, with primers at 1µM, 2.0mM $MgCl_2$, 0.2mM dNTPs, and 1U Truestart Taq (Fermentas). Second round PCR was performed in a separate tube using 1µl of a 1:10 dilution of first round PCR product in molecular biology grade H_2O with primers 13Z and LiR and all reaction conditions as described above. Cycling conditions for both rounds were: 1 cycle of 95°C for 1 min, followed by 40 cycles of 95°C for 30 sec; 55°C for 30 sec; 72°C for 90 sec, followed by 1 cycle of 72°C for 10 min. Electrophoresis was carried out as described below to enable visualization of PCR products. The described kDNA nested PCR is not species-specific, however species can be identified from reaction product sizes of approx. 750bp (*L. tropica*), 680bp (*L. infantum*), or 560bp (*L. major*). Positive and negative controls were as described above.

2.1.8.3 *Leishmania* OligoC-TesT kit

OligoC-TesT kits were provided by the manufacturer (Coris Bioconcept, Gembloux, Belgium) and were used according to product recommendations, for genus-specific detection of *Leishmania* DNA (Deborggraeve *et al.*, 2008). The test targets the *Leishmania* 18S rRNA gene to carry out PCR amplification of a 115-bp product, alongside which an internal PCR control template is co-amplified with the same primers, allowing detection of PCR inhibition. 5µl DNA extract was amplified in a total 50µl reaction volume with 1U Taq polymerase (Truestart Taq, Fermentas, UK) using a conventional thermal cycler (iCycler, BioRad, UK). Thermal cycling

conditions were: 1 cycle of 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 sec; 60°C for 60 sec; 72°C for 60 sec, followed by 1 cycle of 72°C for 5 min; 94°C for 30sec.

Detection of specific PCR product was carried out within 30 minutes of completion of the PCR, using the supplied double-sided dipsticks which replace detection by gel electrophoresis. Briefly, 40µl PCR product was mixed with 40µl of the supplied migration buffer in individual assay tubes. *Leishmania* and internal control PCR amplicons hybridize with gold-labelled detection probes and specific biotinylated capture probes in the lower part of the strip, and migrate upwards to capture areas, where they accumulate on Neutralite avidin lines and become visible to the naked eye as a red line. Following the manufacturer's recommendations, a positive result was recorded after 10 min incubation at 55°C when both the *Leishmania* test line and the internal control line were visible, together with migration control lines which indicate correct running of the test strip buffer. A positive result was also recorded when only the *Leishmania* test line and migration control lines were visible. Negative results were recorded when only the internal control line and migration control lines were visible. PCR inhibition was recorded when migration control lines were visible but neither *Leishmania* test nor internal control lines were visible. Positive and negative controls, and sham DNA extractions, were also tested. Positive results were obtained with control DNA extracted from cultured parasites of *L. infantum* MHOM/FR/78/LEM75, *L. tropica* MHOM/SU/74/K27, *L. major* MHOM/SU/73/5-ASKH, and *L. donovani* MHOM/IN/80/DD8. Negative control DNA from UK dogs and sham extractions were OligoC-TesT negative. The lower limit of detection claimed for the OligoC-TesT kit is 10fg *Leishmania* DNA (Deborggraeve *et al.*, 2008).

2.1.9 Agarose gel electrophoresis of PCR products

DNA amplified in conventional PCR was visualized through the use of 1.5% agarose gels (Fermentas) made with 1× TAE, to which ethidium bromide was added during the casting process to a final concentration of 0.025µg/ml. 10µl samples of PCR product with 2µl 6× loading buffer (Fermentas) were pipetted into each gel lane. Gels were run submerged in 1×TAE for 1 hour at 120V. A 100bp molecular size marker ladder (Generuler 100bp Plus; Fermentas) was run alongside samples to allow determination

of PCR product sizes. DNA was visualized, and bands were photographed, by fluorescence on a UV transilluminator.

2.1.10 Real-time quantitative PCR (qPCR)

Primers and a fluorophore-labelled probe were used in a previously validated protocol specific for a conserved region of *L. infantum* kDNA, which produces a 120-bp amplicon (Francino *et al.*, 2006). Primers LEISH-1 and LEISH-2 and Leishprobe (a FAM-labelled TaqMan MGB probe: Applied Biosystems, UK) were added at 900nM and 250nM respectively, in a 25µl total reaction volume with 2× TaqMan Universal PCR Master Mix containing UNG Amperase (Applied Biosystems, UK) to avoid carry-over contamination, and 5µl sample DNA at 1:10 dilution (bone marrow samples) or 1:100 dilution (skin biopsies). Optimal dilution rates for sample DNA were determined by preliminary optimization experiments. All DNA samples were amplified in duplicate. The thermal cycling profile was 50°C for 2 min, 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 60s, carried out on an Applied Biosystems 7500 Fast Real-Time PCR system. Quantification of *Leishmania* DNA was performed using an absolute method, by comparison of Ct values with those from a standard curve constructed from 10-fold dilutions of *L. infantum* DNA extracted from cultured parasites, from 1×10^{6} to 0.1 parasite equivalents/ml (strain MHOM/MA/67/ITMAP-263), which were run in triplicate on every plate. Pre-developed TaqMan Assay reagents (Applied Biosystems, UK) were used to amplify the 18S rRNA gene as an internal reference of canine genomic DNA, to normalize *Leishmania* parasite load to canine gene copy number using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and to detect PCR inhibition. The positive control was DNA from a bone marrow sample taken from a polysymptomatic Brazil dog with parasitologically confirmed ZVL. Negative controls were DNA extracted from blood samples of 10 UK dogs with no history of foreign travel.

2.1.11 Entomological monitoring

Entomological monitoring to detect the presence of sandflies in the area surrounding kennels used for vaccine trials in Crete was carried out using standard techniques (Alexander, 2000). Light trapping was done on 2 consecutive nights at monthly intervals throughout the trial, using a battery operated CDC mini-light trap hung adjacent to kennels at a height of 0.5m above ground level. Sticky paper trapping

using 5 sheets of castor oil coated A4 paper attached to the front of the kennels and 5 sheets hung on a line from nearby vegetation was carried out continuously, with inspection of sheets daily, and changing of sheets weekly. Insects caught by means of light or sticky trapping were identified using standard entomological dichotomous keys.

2.1.12 Statistical analysis

Comparison of mean cytokine levels in quantitative ELISAs, and antibody titres, were performed using non-parametric Wilcoxon rank sum tests. Differences between vaccine group biochemical and haematological parameters were tested for using one-way ANOVA, with Scheffe multiple comparison tests where appropriate. Qualitative agreement between paired (dichotomous) test results in individual dogs was assessed using Cohen's kappa (k) coefficient (Cohen, 1968) where agreement has been characterized as excellent (k=1.00–0.81), good (k=0.80–0.61), moderate (k=0.60–0.41), weak (k=0.40-0.21), or negligible (k=0.20–0.00) following others (Landis and Koch, 1977). When comparing the sensitivity of different diagnostic tests to detect ZVL infection, differences in proportions of positive samples were compared pairwise by calculation of McNemar's Chi-square (χ^2). In comparisons of ELISA and PCR tests (with dichotomous results), Pearson's χ^2 and Fisher's Exact tests were used as appropriate to compare the proportions positive between different groups. Receiver operating characteristic (ROC) curve analysis of the sensitivity and specificity of diagnostic tests is reported as the area under-the curve (AUC) statistic ranging from 0 to 1. Using this AUC value, diagnostic test performance was classified as excellent (0.90-1), good (0.80-0.89), fair (0.70-0.79), poor (0.60-0.69), or worthless (0.50-0.59) (Tape, 2004). In longitudinal analyses, binning of longitudinal time points was used where indicated to equalize group sizes. When examining large longitudinal data sets to measure trends and correlations between measured parameters, it is likely that multiple non-independent observations were made in the same dog at different time points. This feature of panel data is known as autocorrelation. Associations were thus confirmed using generalized linear modelling (GLM) and generalized estimating equation regression models (XTGEE) as appropriate, using robust standard errors, accounting for autocorrelation by clustering on dog ID, and controlling for confounding variables such as time from patent infection and clinical score. Statistical

significance was set at $P \leq 0.05$; all analysis was carried out using STATA v9.0 (StataCorp, 2005).

2.1.13 Ethics

Samples from Greek dogs were collected in the field with the written informed consent of dog owners (see Appendix III for example consent forms). Trials were undertaken to confirm safety in the target population of genetically diverse outbred dogs following EMeA scientific guidelines for veterinary medicinal products (EMeA, 1994; 1995), EEC directive 86/609/EEC (EEC, 1986) and with approval from local government and the University of Warwick Biological Ethics Committee. Dogs were cared for by fully trained animal house staff under veterinary supervision. Kennels were approved by Hellenic Government Veterinary Officers and compliance with relevant legal requirements under Greek laws (160/1991) relating to animal welfare certified by the Hellenic Republic Ministry of Rural Development & Food: General Veterinary Authority K.A.F.E. Department 'A' (see Appendix IV). Written informed consent was gained from dog owners prior to commencement of all trials. Animals remained the legal property of owners, and were returned after completion of the study. In the absence of a Cretan ethical committee for animal procedures, protocols conformed to the spirit of UK Home Office requirements for United Kingdom research establishments, and with ethical approval from the University of Warwick Biological Ethics Committee. Institutional approval for the use and modification of kennels for the vaccine trials was granted by the University of Crete Scientific Board (see Appendix IV).

Ethical issues pertaining to all Brazilian dog sampling have been described before (Quinnell *et al.*, 1997; Courtenay *et al.*, 2002b). UK dog sera were collected from two UK veterinary practices, with consent of owners, using surplus from samples taken for veterinary clinical diagnostic purposes.

2.2 General laboratory reagents

Buffer solutions and reagents used in this study include:

- PBS/Tween 20: 138mM sodium chloride, 2.7mM potassium chloride (pH7.4), 0.05% (v/v) Tween 20.
- $1 \times$ TAE buffer: 40 mM Tris acetate, 1 mM EDTA (pH8.3).
- PK buffer: 50mM potassium chloride, 10mM Tris, (pH8.0), 100µg/ml proteinase K, 0.05% (v/v) Tween 20.
- PEG buffer: 30% (w/v) PEG (polyethylene glycol) 6000, 1.6M sodium chloride.
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

Commercially available reagents used in the study are summarized in Table 1. Primers used for polymerase chain reaction (PCR) amplification of DNA are listed in Appendix II.

Table 1: Commercially sourced reagents used in the study

Reagent	Purpose	Manufacturer
RPMI cell culture medium	Cytokine assays	Sigma-Aldrich
L-glutamine-penicillin-streptomycin solution (200 mM L-glutamine, 10,000 u/ml penicillin, and 10 mg/ml streptomycin)	Cytokine assays	Sigma-Aldrich
Concanavalin A	Cytokine assays	Sigma-Aldrich
Chloroform: isoamyl alcohol (24:1)	DNA extraction	Sigma-Aldrich
Phenol:chloroform: isoamyl alcohol (25:24:1), 10mM Tris, pH8.0, 1mM ETDA	DNA extraction	Sigma-Aldrich
DNeasy blood and tissue kit	DNA extraction	Qiagen
Proteinase K	DNA extraction	Sigma-Aldrich
TaqMan Universal PCR Master Mix (with UNG amperase)	Real-time PCR	Applied Biosystems
TaqMan Ribosomal RNA control reagents	Real-time PCR	Applied Biosystems
Truestart Taq polymerase	PCR	Fermentas

Reagent	Purpose	Manufacturer
Loading dye solution (6x)	Gel electrophoresis	Fermentas
Low melting point agarose	Gel electrophoresis	Fermentas
Generuler 100bp Plus DNA ladder	Gel electrophoresis	Fermentas
Goat anti-dog IgG1 antibody HRP conjugate	ELISA	Bethyl Laboratories
Sheep anti-dog IgG2 antibody HRP conjugate	ELISA	Bethyl Laboratories
Rabbit anti-dog IgG antibody HRP conjugate	ELISA	Sigma-Aldrich
Rabbit anti-dog IgG antibody FITC conjugate	IFAT	Sigma-Aldrich
Tetramethylbenzidine (TMB) substrate	ELISA	Sigma-Aldrich
Carbonate/bicarbonate coating buffer	ELISA	Sigma-Aldrich
Canine IL-10 MAb (Clone 138111)	Cytokine ELISA	R&D Systems
Canine IL-10 Biotinylated Affinity Purified Polyclonal Ab	Cytokine ELISA	R&D Systems
Canine TNF-α DuoSet	Cytokine ELISA	R&D Systems
Canine IFN-γ ELISA Duoset	Cytokine ELISA	R&D Systems
Streptavidin-HRP substrate	Cytokine ELISA	R&D Systems
Leishmania OligoC-TesT kit	PCR	Coris Bioconcept (provided FOC)
Kalazar detect dipsticks	Serology	Inbios (provided FOC)

2.3 General equipment

Agarose gels were prepared in a 15 x 15 cm gel cast, and subsequent electrophoresis was performed in an MS Choice 15 horizontal gel tank (Geneflow, UK) with electric current supplied by a Consort EV215 power pack (Jencons, UK). DNA bands stained with ethidium bromide were visualized on an UV transilluminator and photographed on a Geneflash system (Syngene Bio Imaging).

Centrifugation was performed with a Minispin Plus microfuge (Eppendorf) for 0.5-1.5ml microfuge tubes, and in a 5810R bench-top centrifuge (Eppendorf) with a swing-out rotor for centrifugation of 96-well PCR plates.

Optical absorbance in ELISA assays was measured using a Thermo Fisher Multiskan EX linked to a desktop PC running Ascent plate reading software.

DNA quantity and purity was measured in 2µl sample aliquots by optical absorbance at 260/280nm using a Nanodrop 1000 spectrophotometer (Thermo Scientific, UK).

Conventional endpoint PCR was carried out in a heated-lid thermal cycler (iCycler, BioRad, UK). Real time PCR was performed in an Applied Biosystems 7500 Fast PCR system, and output plots were analyzed using Applied Biosystems sequence detection software version 1.4.

2.4 Materials & samples sourced from study collaborators

2.4.1 Source of Leishmania antigens, DNA and MVA vaccines

Recombinant proteins used for *in vitro* immunology assays, and to test intradermal reactivity *in vivo*, were prepared by Novexin Ltd (Babraham, UK) under conditions conforming to Good Laboratory Practice (GLP) using constructs originally prepared by the Cambridge Institute for Medical Research (Stober *et al.*, 2005) by cloning TRYP or LACK into the expression vector pET-15b (Novagen) and transformation into *Escherichia coli* BL21 (DE3) host cells. Recombinant protein was purified by affinity column chromatography using 1ml HisTrap FF columns (GE Healthcare). Immobilised target proteins were washed with buffer containing NV polymer to dissociate and remove endotoxin contamination before being eluted with 10 mM Tris-HCl (pH 8.5), 0.5 M NaCl and 250 mM imidazole, and desalted into low-LPS PBS using PD10 desalting columns (GE Healthcare). Proteins were diluted in pyrogen-free PBS for intradermal inoculation into dogs. Crude freeze-thawed *Leishmania infantum* antigen (CLA) was prepared from stationary phase promastigotes of *L. donovani* LV9

as described previously (Stober *et al.*, 2005), by re-suspension in 10 mM Tris-HCl (pH 8.5), 0.5 M NaCl, 1 mM PMSF, and 50 µg/ml leupeptin, and freeze-thawing three times over liquid nitrogen. Recombinant rK39 antigen, prepared as previously described (Burns Jr *et al.*, 1993) from *L. chagasi* (MHOM/BR/82/BA-2,C1), was provided by Prof. K.P. Chang (Rosalind Franklin University of Medicine and Science, Chicago).

DNA and recombinant MVA TRYP and LACK vaccines were manufactured by our collaborators as described below, and in previous research (Stober *et al.*, 2005).

To produce DNA vaccines, LACK (amino acids 143–312) and TRYP (full-length, amino acids 1–199,) were amplified from cDNA clones lmk5 (accession number W88311) and lmf30 (accession number T67356), respectively, obtained from a *L. major* substrain LV39 (MRHO/SU/59/P) cDNA library. Both genes were inserted downstream of the CMV promoter into the expression vector pcDNA3 (Invitrogen Life Technologies). Empty pcDNA3 was used as vector control. Plasmid DNA for use in vaccines was purified using EndoFree Plasmid Maxi kits (Qiagen) with pyrogen-free material, and the final pellet was resuspended for injection in pyrogen-free PBS. This work was carried out by the Cambridge Institute for Medical Research, and DNA vaccines were supplied as frozen aliquots for use in the present study.

To manufacture recombinant MVA vaccines, the viral expression vector pMJ601 was modified by replacing the early 7.5-kDa promoter and lacZ with the early/late 7.5-kDa promoter and the selectable marker gene vaccinia virus K1L, respectively, to generate pMJ601K1L. LACK and TRYP were cloned into the thymidine kinase locus of pMJ601 vector downstream of the synthetic strong promoter. rMVA was obtained by infecting permissive hamster Syrian kidney cells (BHK-21) with wild-type MVA and simultaneously transfecting BHK-21 cells with LACK, TRYP, or vector plasmids using the FuGENE 6 transfection reagent (Roche Diagnostics). After 48h, infected cells were harvested, and rMVA was selected by plaque assay in nonpermissive rabbit kidney epithelial cells (RK13). Coinsertion of the vaccinia virus K1L gene with the *Leishmania* antigen confers rMVA the ability to grow in RK13 cells. This selection procedure was repeated three times, and the insertion of LACK and TRYP into the viral genome was confirmed by PCR. For vaccinations, semipurified stocks of rMVA grown in RK13 cells were prepared by ultracentrifugation through a sucrose cushion, resuspended in 10 mM Tris-HCl (pH 9.0). Expression of protein from MVA-infected culture lysate was demonstrated by Western blotting using sera from DNA-vaccinated

mice. The expected protein bands at 18kDa for LACK and at 22 kDa for TRYP were observed. This work was carried out by the Centro de Biología Molecular Severo Ochoa, and recombinant MVA vaccines were supplied for the present study as frozen aliquots.

2.4.2 Source of positive control Leishmania DNA for PCR procedures

Positive control DNA from cultured *Leishmania* promastigotes, for use in conventional PCR procedures, was provided by Dr. I. Mauricio at the London School of Hygiene and Tropical Medicine. Species and strains obtained were: *L. infantum* MHOM/TN/82/IPT1(LEM235) & MHOM/FR/78/LEM75, *L. tropica* MHOM/SU/74/K27, *L. major* MHOM/SU/73/5-ASKH, and *L. donovani* MHOM/IN/80/DD8. To quantify *L. infantum* parasite burdens using real-time qPCR, a standard curve was constructed using serial dilutions of DNA extracted from known numbers of cultured promastigotes, which were enumerated using a haemocytometer. DNA extracted from *L. infantum* strain MHOM/MA/67/ITMAP-263 was obtained from Dr. A. Tomas (Instituto de Biologia Molecular e Celular, Portugal) for this purpose.

2.4.3 Clinical samples from naturally ZVL infected Brazilian dogs and foxes

We used samples taken from a previously described large-scale longitudinal field study of dogs in the municipality of Salvaterra, Marajó Island, Pará state, Brazil. In that study, blood, bone marrow, and ear biopsy samples were obtained from naturally exposed outbred dogs at approximately 2 month intervals for up to 24 months post initial natural exposure to infection in the field. These dogs were previously tested by serology, bone marrow PCR and parasitological culture (*in vitro* and *in vivo* in hamsters), and clinically examined, as described below and elsewhere (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001a; Courtenay *et al.*, 2002b). A sympatric population of free-living crab-eating foxes (*Cerdocyon thous*) were also trapped for testing as above (Courtenay *et al.*, 2002a).

In the previous research, bone marrow samples were aspirated from the iliac crest of anaesthetized dogs or foxes whilst full-thickness skin biopsies were taken from the pinna of each animal's left ear using a 5mm biopsy punch, as described (Quinnell *et al.*, 2001a). Both sample types were collected into 200µl buffer (15 mM Tris, pH 8.3, 1 mM EDTA, 150 mM NaCl) containing 1% SDS, incubated at 65 °C for 2 h and

stored at -20°C. 20ml venous blood was collected from each animal, and serum samples were similarly stored at -20°C. These samples were kindly made available for the current study from the archived material, by Dr. R. Quinnell at the Institute of Integrative and Comparative Biology, University of Leeds.

2.4.4 Previous serology and parasitology on Brazilian dog and fox samples

In the previous Brazil study, DNA was extracted from bone marrow samples using a phenol-chloroform extraction protocol as described (Quinnell *et al.*, 2001a). PCR was carried out using primers DBY and AJS31 (specific for *L. donovani* group parasites), followed by gel electrophoresis and hybridization with the digoxygenin-labelled oligonucleotide probe B4Rsa to amplify and detect an 805-bp fragment of minicircle kDNA (Smyth *et al.*, 1992; Scrimgeour *et al.*, 1998), and with primers R221 and R332 to amplify a 604-bp fragment of the small ribosomal subunit RNA gene of all *Leishmania* species, for detection using probe R331 (Van Eys *et al.*, 1992). Positives were defined as samples that were positive by both primer sets. Also in the previously performed study, bone marrow samples were inoculated onto sterile Difco blood agar slopes or into golden hamsters for *in vitro / in vivo* parasite culture and isolation using standard techniques, as described (Quinnell *et al.*, 1997).

Sera were previously tested for anti-*Leishmania* IgG in enzyme linked immunosorbent assays (ELISAs), using crude parasite antigen (CLA), and defined as positive or negative using a cut-off point calculated as previously described (Quinnell *et al.*, 1997). IgG antibody titre was obtained from doubling dilutions of test samples titrated from 1/50 to 1/800 in duplicate where possible. Titres were expressed with reference to a highly positive control serum which was titrated 2-fold from 1:100 to 1:3,276,800 on every plate, and assigned a number of arbitrary units equal to the reciprocal of the highest dilution at which optical density (O.D.) of the reaction product exceeded the mean +3S.D. of the background (no sample) wells.

2.4.5 Previous clinical examinations of Brazilian dogs and foxes

Dogs and foxes in the previously described study in were clinically examined and scored using a semi-quantitative method by scoring on a scale 0 (absent) to 3 (intense) six typical clinical signs of leishmaniasis (alopecia, dermatitis, chancres, conjunctivitis, onychogryphosis, and lymphadenopathy), as described (Quinnell *et al.*, 2001a). A total clinical score of 0-2 was classed as asymptomatic, 3-6 as

oligosymptomatic, and ≥7 as polysymptomatic, based on inspection of background clinical presentations.

2.4.6 Previous xenodiagnosis carried out on Brazilian dogs and foxes

Xenodiagnosis experiments on dogs and mark-recaptured foxes in Brazil were carried out previously, as described (Courtenay *et al.*, 2002a; Courtenay *et al.*, 2002b). Briefly, dogs or anaesthetized foxes were placed into individual cages sheathed in sandfly-proof netting. Unfed female *Lutzomyia longipalpis* laboratory-reared sandflies (2–3 days after emergence) were introduced into the cage and were allowed to feed overnight (dogs) or for 1-2 hours in darkness (foxes) under black hessian with access to the whole body. Four to five days after the blood meal, fully-fed flies were dissected and examined by microscopy for visible promastigote life stages in the thoracic midgut, indicative of parasite development to the infectious stage. The number of engorged infected/uninfected flies per cage was recorded, to calculate the proportion of flies infected by each animal at each repeat time point.

Chapter 3: Safety and immunogenicity of prime/boost DNA/ modified vaccinia virus Ankara *Leishmania* vaccines in dogs.

3.1 Introduction

Currently the sole commercially available *Leishmania* vaccine (Leishmune[®]), based on a purified parasite preparation with saponin adjuvants, is licensed for use in dogs only in Brazil (Dantas-Torres, 2006). Although trials showed high vaccine efficacy (Borja-Cabrera *et al.*, 2002), transient adjuvant-related side effects (Parra *et al.*, 2007) may reduce uptake and compliance among vets and dog owners. Development of additional novel vaccine candidates is advisable, since the next generation vaccines/vaccine antigens should be available to combat the evolution of parasite strains which are capable of infecting vaccinated individuals. We should also continue to improve on methods of delivery that will elicit lasting immunological memory in the absence of side effects. Experimental DNA vaccines are the subject of increasing numbers of human and veterinary clinical trials, since they elicit the T-cell memory required for long term protection (Mendez *et al.*, 2001), are extremely safe, easy to standardize, and are highly stable for storage and distribution purposes in tropical environments where cold chain may be unavailable (Dumonteil, 2007). As a development of DNA vaccination, DNA/rVV prime boost protocols are a promising avenue for development of a vaccine against canine ZVL, as discussed more fully in Chapter 1.

Analysis of expressed sequence tags from cDNA libraries of *Leishmania major* (Levick *et al.*, 1996) led to the discovery and functional characterisation (Levick *et al.*, 1998) of tryparedoxin peroxidase (TRYP, also known as thiol specific antioxidant or TSA (Webb *et al.*, 1998)). DNA alone or DNA/MVA prime/boost vaccine delivery highlighted TRYP as a highly effective inducer of protective immunity against virulent challenge with *Leishmania major* in susceptible BALB/c mice (Stober *et al.*, 2005). These findings are consistent with studies using TRYP protein/adjuvant combinations in mice and non-human primates (Campos-Neto *et al.*, 2001). Importantly, TRYP was shown to be far superior as a protective vaccine to the previously described *Leishmania* homolog of the receptor for activated C kinase (LACK) (Mougneau *et al.*, 1995), the functional correlate for this being higher IL-10 from regulatory T cells elicited by LACK and a higher IFN-γ:IL-10 ratio associated

with TRYP (indicative of a type-1 pro-inflammatory response driven by IFN-γ secreting Th1-type CD4+ cells) compared to LACK vaccination (Stober *et al.*, 2005). To date, no research has been published describing the immunological responses of dogs to DNA/MVA TRYP as a potential vaccine against ZVL.

Following the previous successful safety, immunogenicity and efficacy studies of the prime/boost DNA/MVA TRYP vaccine against *L. major* in mice (Stober *et al.*, 2005; Stober *et al.*, 2007), this study aimed to demonstrate safety and immunogenicity of DNA/MVA TRYP and LACK in a cohort of 22 uninfected, unexposed outbred dogs followed-up for 4 months. The need for development of new vaccines in genetically diverse outbred individuals is highlighted by research suggesting an important genotypic component in immune responses (Altet *et al.*, 2002; Quinnell *et al.*, 2003b), which may influence vaccine efficacy.

3.2 Methods

3.2.1 Study population and experimental set-up

A cohort of 22 young (median age 18 months, range 4-24 months) uninfected outbred dogs from a ZVL endemic area (Crete, Greece) were enrolled for vaccination with DNA/MVA TRYP, LACK or control, and followed-up for 4 months post prime/boost vaccination between June and November 2007. Dogs were recruited with informed consent from owners (see information sheet and owners declaration forms; Appendix III) in villages of the Heraklion prefecture within 15km radius of the city of Heraklion, on the criteria of being negative to all diagnostic tests: (1) Indirect immunofluorescent antibody test (IFAT) (Gradoni and Gramiccia, 2004), (2) Crude *Leishmania* parasite antigen (CLA) ELISA (Quinnell *et al.*, 1997), and (3) ITS-1 PCR of buffy coat DNA (Parvizi *et al.*, 2005). The sample comprised 59% mixed breeds, the remainder including local breeds (Cretan / Hellenic hounds) (n=4), Belgian Shepherd (n=2) and pit bull terrier (n=1), at a male:female ratio of 1.2:1.

Dogs were housed in pairs, or individually (adjacent and within sight of each other), in kennels located at the University Hospital of Crete, Heraklion, which were modified for the purpose to conform with EC regulatory standards and UK Home Office Code of Practice for housing of laboratory dogs (UK Home Office, 1989). Prior to commencement of trials, all dogs received routine vaccination for distemper, canine

parvovirus, canine adenovirus and leptospirosis (Hexadog, Merial), in addition to oral antihelminthic treatment with praziquantel/ fenbendazole (Caniquantel Plus, New Vet AE). To rule out exposure to *Leishmania* wild type during the transmission season (May – October), dogs were fitted with deltamethrin-impregnated collars (Scalibor, Intervet) and checked daily for collar loss, or treated instead with fortnightly doses of topical 10% imidacloprid / 50% permethrin solution (Advantix, Bayer AG). Kennels were monitored continuously for sandfly activity by routine light trapping and sticky traps (Alexander, 2000), as described in Chapter 2. No sandflies were detected at the kennels during the trial. After completion of trials, all dogs were returned to their owners.

3.2.2 Vaccine administration

Dogs were randomized to receive intramuscular injections, from blinded operators, in the craniolateral aspect of the right quadriceps femoris, with DNA TRYP or LACK (100µg; n=4, or 1000µg; n=5), or control plasmid DNA (1000µg; n=4) on day 0, followed 28 days later by 10^8 pfu MVA TRYP or LACK vaccine (or empty MVA vehicle as control). This prime/boost regime is similar to that employed in previous canine studies (Ramiro *et al.*, 2003; Ramos *et al.*, 2008), in which administration of plasmid DNA (100µg) and recombinant vaccinia virus ($10^7 – 10^8$ pfu) were carried out 14 days apart. Safety and immunogenicity were measured as described below.

3.2.3 Safety

Dogs were kept under veterinary surveillance post "prime" and "boost" to detect the occurrence of potential adverse reactions. Safety was assessed by daily clinical examinations for 4 days post-vaccination (as detailed in European Medicines Agency (EMeA) requirements (1994)), with defined clinical end-points (local pain on palpation; inflammation; ulceration; alopecia; apathy; fever; diarrhoea; anorexia). Body weight and condition score were recorded weekly. Pre- and post-vaccine haematological and biochemical parameters were measured by collection of blood samples at 2 days before and 2 days after each vaccination. Blood was collected by jugular or cephalic venepuncture in 2ml EDTA anticoagulated and plain serum gel tubes. Samples were sent by same day courier at +4°C to a commercial laboratory (Microanalysi, Athens), and processed for routine biochemical tests (urea, creatinine,

aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK) and total bilirubin) as described in Chapter 2.

3.2.4 Immunogenicity

3.2.4.1 Cytokine assays

Immunogenicity was assessed by measurement of cytokine levels (IFN-γ, TNF-α, and IL-10) expressed by antigen stimulated lymphocytes in whole blood assays (WBA) (Shifrine *et al.*, 1978), measured pre-vaccination (day 0) and on days 26, 42, 70, 98 and 126 following first vaccination, as described in Chapter 2. Background levels in unstimulated control wells were deducted from antigen-stimulated values to quantify antigen-specific cytokine production (with negative values recorded as zero). The mean values for background levels of IFN-γ, TNF-α and IL-10 were 65pg/ml (range 0-313), 47pg/ml (range 0-527), and 162pg/ml (range 0-982). TRYP and LACK antigens were not available to measure pre-vaccination (day 0) cytokine levels, therefore cytokine measurements for these antigens commenced from day 26 onwards.

3.2.4.2 ELISA

Serological responses to vaccination (total specific IgG, IgG1 and IgG2 subtypes) were measured by anti-TRYP and anti-LACK ELISA in all dogs at all 6 follow-up time points (day 0-126), as described in Chapter 2. Positive and negative controls were included on each plate. The sample-to-positive ratio (s/p) (Tijssen, 1985) for each sample was calculated as the mean raw absorbance at 450nm of duplicate test samples relative to a highly positive reference positive sample (from a parasitologically confirmed polysymptomatic Brazilian dog (Quinnell *et al.*, 1997)) which was included on every ELISA plate. For subtyping experiments, to measure antigen-specific antibody titre in arbitrary units, titration curves were plotted for each serum sample using doubling dilutions from 1:100 to 1:3200 (IgG1) or alternate doubling dilutions from 1:200 to 1:204,800 (IgG2). The cut-off point was calculated as the mean s/p ratio of all dogs at time 0 (pre-vaccination). Using a maximum likelihood method (Microsoft Excel software), a straight line was fitted to the linear portion of the s/p ratio titration curve, and the reciprocal of the dilution rate at the point of intersection with the cut-off value was calculated as an estimate of antibody titre.

3.2.4.3 Intradermal tests

Cellular immune responses *in vivo* were measured at day 156 by intradermal skin testing (Pinelli *et al.*, 1994) using 0.1µg TRYP and LACK recombinant antigen (prepared as described in Chapter 2) in 0.1ml sterile pyrogen-free PBS (or 0.1ml PBS alone, as a control) injected intradermally at the right inner thigh, as described in Chapter 2.

Summary of vaccine administration regime and sampling protocol
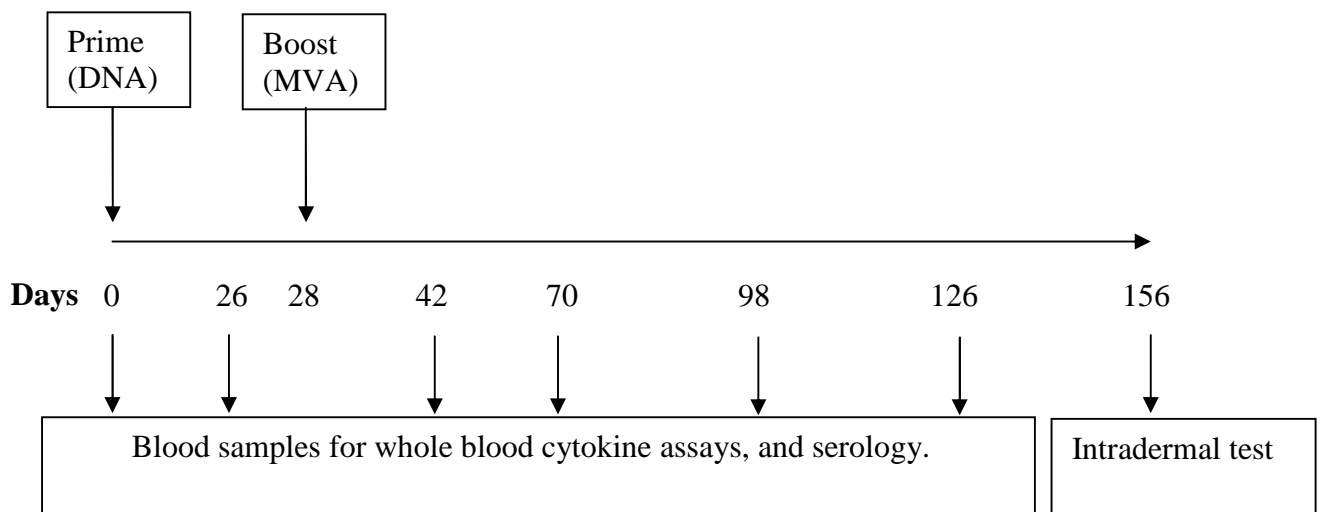
3.3 Results

3.3.1 Safety

3.3.1.1 Clinical examination

Examination post-vaccination detected no adverse clinical side effects except transient pain on palpation of the injection site in one low dose LACK dog on the morning following second vaccination. No swelling, alopecia or systemic signs were recorded in any animal. Mean body weights of all vaccine groups increased slowly throughout the trial (Figure 1), partly due to growth of young dogs in each group. One female animal in the TRYP low dose group was vaccinated in the early stages of gestation, before the pregnancy was apparent on clinical examination. Subsequently to discovery of the pregnancy, this bitch was monitored closely throughout an uneventful gestation, and delivered normal puppies. Data from this animal were excluded from all subsequent analyses.
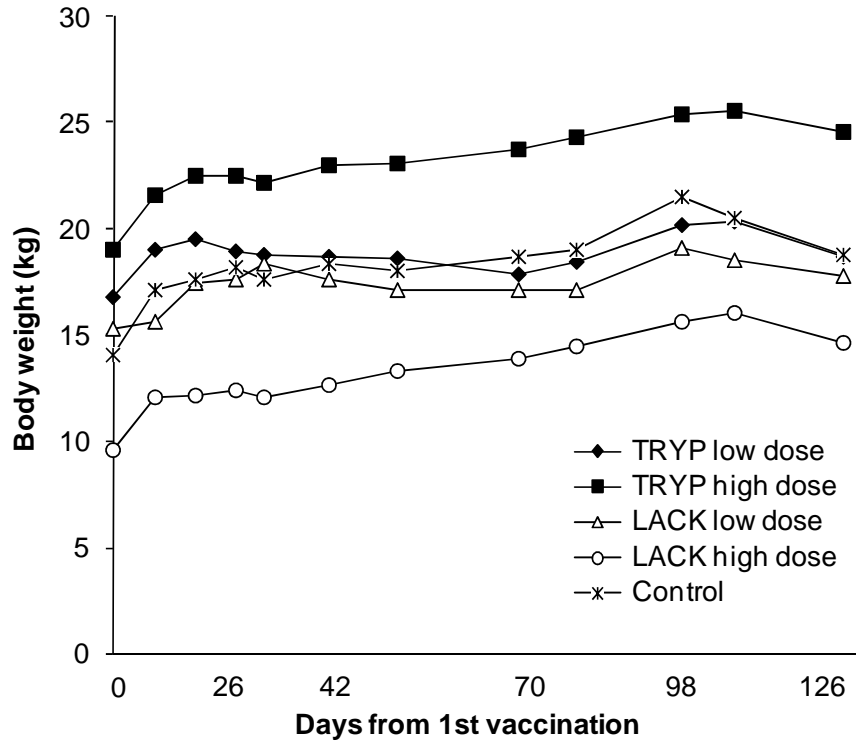
Figure 1. Mean body weight (kg) of vaccine and control dogs.

Mean body weight (kg) at the indicated time points after 1st vaccination on Day 0 with TRYP or LACK (low or high dose) DNA vaccine, or control placebo DNA. 2nd vaccination with MVA TRYP, MVA LACK or placebo (as appropriate) was carried out on Day 28.

3.3.1.2 Clinical biochemistry and haematology

Between group comparison of blood biochemical (AST, ALT, creatinine, urea, total bilirubin and CPK) and haematological parameters (total red blood cell count) pre- and post-prime and boost vaccinations showed no statistically significant differences between TRYP, LACK and control groups (ANOVA; $P \geq 0.11$). Comparison between group mean white blood cell counts at time 0 (before 1st vaccine) approached significant difference (ANOVA; $P=0.053$), however no statistically significant differences between individual vaccine groups were identified using the Scheffe multiple comparison test, and no subsequent post-vaccine between-group differences were found ($P \geq 0.20$) (Table 2).

3.3.2 Immunogenicity

3.3.2.1 IFN-γ cytokine response

Mean IFN-γ levels in response to WBA stimulation with TRYP antigen in TRYP high dose vaccinated dogs (1000µg DNA) were significantly higher than controls at all time points from day 42 onwards. In the TRYP low dose (100µg DNA) group, after removal of an outlier IFN-γ value of 3576 pg/ml at day 126, vaccinated dogs showed higher mean IFN-γ levels than controls at day 42 only (Figure 2). LACK-specific IFN-γ responses in both high and low dose LACK vaccine groups were not significantly different from controls at any time point. As expected, TRYP vaccinated dogs showed no IFN-γ response in LACK stimulated assays, and LACK vaccinated dogs were similarly unresponsive in TRYP stimulated assays. We did not detect any significant difference in TRYP-specific IFN-γ levels between high and low dose TRYP groups at any time point ($P \geq 0.27$; Wilcoxon rank sum test). Similarly, no significant difference was detected between high and low dose LACK dogs' mean IFN-γ levels, in LACK-stimulated WBA ($P \geq 0.45$; Wilcoxon rank sum test). The combined results of high and low dose groups (Figure 3) showed that, overall, mean IFN-γ levels in response to TRYP WBA were significantly higher in TRYP vaccinated dogs than in controls at 3/4 time points post vaccination ($P \leq 0.05$: Wilcoxon rank sum test), whereas no significant difference was seen between LACK vaccinated dogs and controls in LACK WBA.

Mean IFN-γ responses to CLA antigen in all vaccine groups were consistently low (≤ 120pg/ml) or below background (data not shown), showing no significant association with vaccine group.

(A)

Vaccine group	N	Erythrocytes		Total white cells		Lymphocytes	
		Pre	Post	Pre	Post	Pre	Post
TRYP low dose	4	6.72 (5.84-7.60)	6.58 (5.92-7.23)	13.90 (7.88-19.92)	13.05 (9.42-16.67)	4.43 (2.63-6.23)	4.50 (3.25-5.75)
TRYP high dose	5	6.03 (5.27-6.80)	5.93 (4.93-6.93)	12.00 (9.54-14.46)	10.74 (8.27-13.20)	3.84 (2.96-4.72)	3.72 (2.87-4.57)
LACK low dose	4	6.24 (5.38-7.10)	6.41 (5.99-6.82)	10.90 (7.53-14.27)	12.08 (5.57-18.58)	3.53 (2.79-4.26)	3.60 (0.77-6.43)
LACK high dose	5	6.56 (4.94-8.17)	6.35 (5.26-7.44)	12.98 (10.79-15.16)	11.10 (8.62-13.58)	3.98 (1.99-5.96)	3.74 (2.82-4.66)
Control	4	6.25 (3.84-8.65)	6.10 (5.03-7.16)	9.10 (5.60-12.60)	9.68 (6.26-13.10)	3.07 (0.08-6.05)	2.83 (1.49-4.16)

(B)

Vaccine group	N	Erythrocytes		Total white cells		Lymphocytes	
		Pre	Post	Pre	Post	Pre	Post
TRYP low dose	4	6.60 (5.57-7.63)	6.59 (5.76-7.42)	16.03 (10.92-21.13)	14.60 (11.92-17.27)	4.63 (4.10-5.15)	4.10 (2.89-5.31)
TRYP high dose	5	6.69 (5.78-7.60)	6.67 (5.74-7.60)	13.88 (11.00-16.76)	14.40 (11.20-17.60)	4.44 (2.91-5.97)	4.20 (3.10-5.29)
LACK low dose	4	6.45 (6.16-6.73)	6.45 (6.01-6.89)	11.48 (3.88-19.07)	13.92 (8.47-19.38)	3.40 (0.60-6.20)	4.15 (3.13-5.16)
LACK high dose	5	6.56 (5.63-7.49)	6.50 (5.72-7.26)	12.50 (9.35-15.65)	14.20 (12.48-15.91)	4.18 (3.29-5.07)	4.78 (2.88-6.68)
Control	4	6.98 (5.86-8.09)	6.89 (5.63-8.14)	11.38 (8.34-14.41)	11.99 (8.94-15.00)	3.43 (2.49-4.36)	3.70 (2.61-4.78)

(B) **Table 2.** Pre- and post-vaccination clinical haematological parameters. Cell counts ($\times 10^6$/ ml blood; (95% C.I. in italics)) of erythrocytes, total white blood cells and lymphocytes in venous blood samples taken from the cephalic or jugular vein of vaccine trial dogs at 2 days pre- and 2 days post-vaccination. (A) DNA prime, (B) MVA boost.
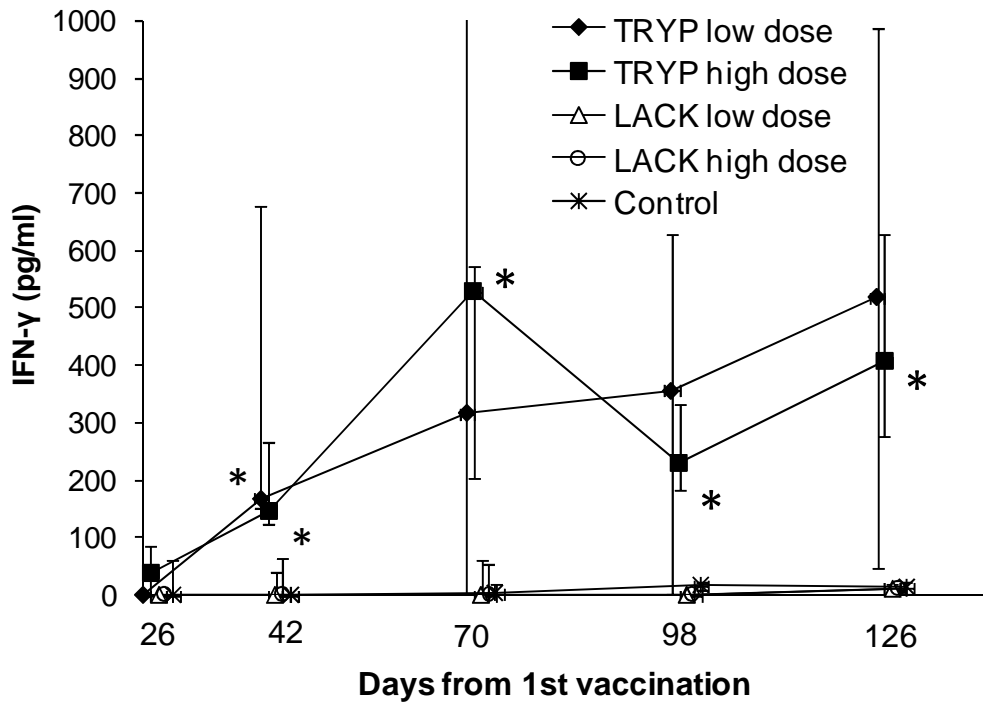
Figure 2. Median IFN-γ in whole blood assays stimulated with TRYP. IFN-γ levels were measured in whole blood cytokine stimulation assays using TRYP antigen, at the indicated time points after 1st vaccination on Day 0 with TRYP or LACK (low or high dose) DNA vaccine, or control placebo DNA. 2nd vaccination with MVA TRYP, MVA LACK or placebo (as appropriate) was carried out on Day 28. Error bars indicate interquartile range. For each time point, the x-axis has been stretched to allow clear visualization of error bars. *Denotes a significant difference between vaccine group and control (Wilcoxon rank sum test; $P \leq 0.05$). One outlier point in TRYP low dose vaccine group at day 126 removed (IFN-γ = 3576 pg/ml); upper limit of interquartile ranges are truncated at 1000 pg/ml on the vertical scale, for clarity.
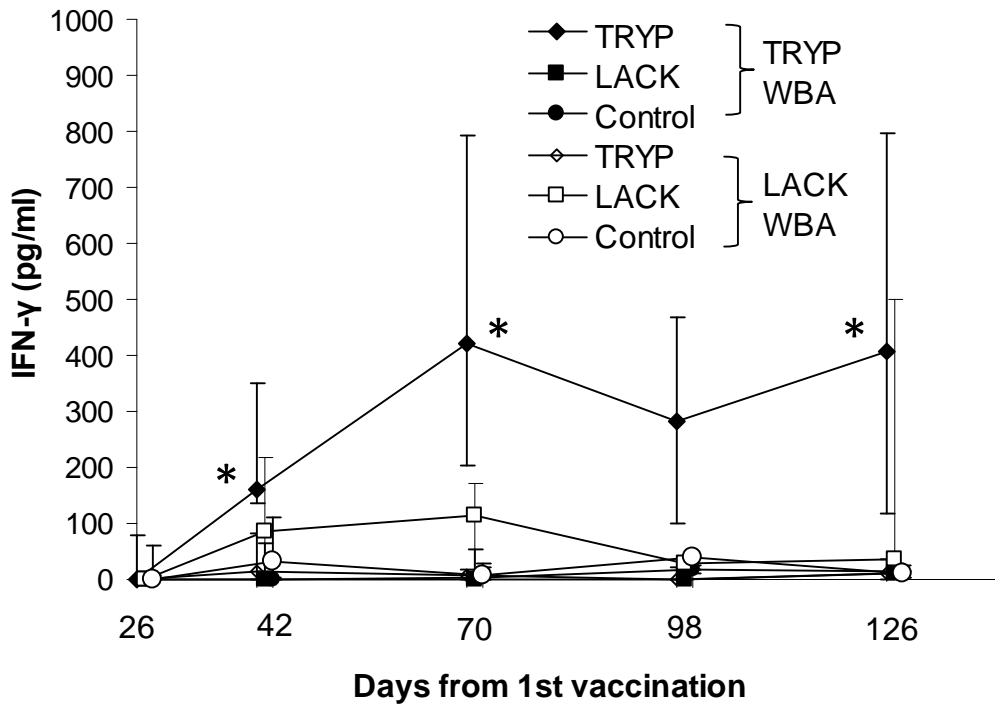
Figure 3. Median IFN-γ in whole blood assays stimulated with TRYP and LACK. IFN-γ levels were measured in whole blood cytokine stimulation assays (WBA) by ELISA using TRYP and LACK antigen, at the indicated time points after 1st vaccination. Error bars indicate interquartile range. Results from TRYP and LACK low and high dose vaccine groups were not significantly different, and are amalgamated here for clarity. Filled points on the graph represent IFN-γ response to TRYP antigen stimulation in whole blood assays, open points with show IFN-γ response to LACK stimulation. *Denotes a significant difference between vaccine group and control (Wilcoxon rank sum test; $P \leq 0.05$).

3.3.2.2 IL-10 cytokine response

No significant differences were observed between high and low dose TRYP ($P{\geq}0.10$) or LACK ($P{\geq}0.09$) vaccine group IL-10 responses, therefore results from the two dose rates were combined for further analysis. Mean IL-10 levels in vaccinated dogs were not significantly different from controls, showing no obvious trend over time apart from a transient increase in mean IL-10 levels at Day 70 in both TRYP and LACK vaccinated dogs (not significantly different from controls: $P{\geq}0.12$; Wilcoxon rank sum test), in response to both TRYP (Figure 4) and LACK antigens (similar results, data not shown). Mean IL-10 responses to CLA antigen were consistently low ($\leq$62pg/ml) or below background, showing no significant association with vaccine group.
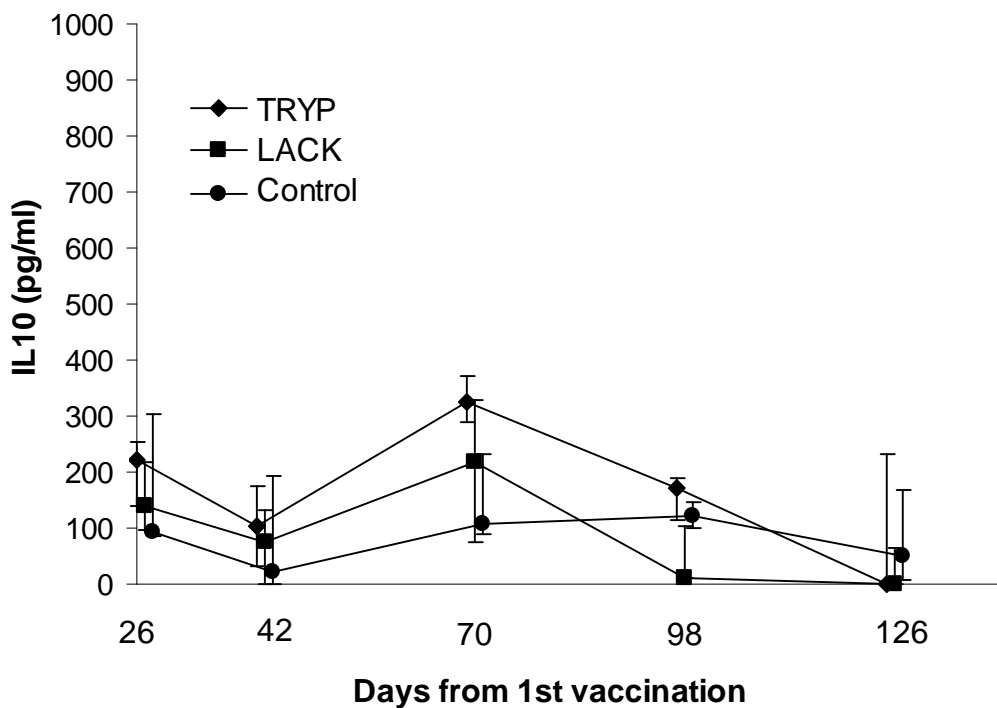
Figure 4. Median IL-10 in whole blood assays stimulated with TRYP.
IL-10 levels were measured in whole blood cytokine stimulation assays with TRYP antigen, at the indicated time points after 1st vaccination. Error bars indicate interquartile range. Results from TRYP and LACK low and high dose vaccine groups are amalgamated for clarity. IL-10 responses to LACK were similar (data not shown).

3.3.2.3 TNF-α cytokine response

None of the vaccinated groups showed significant differences in mean TNF-α level compared with controls at any time point, though a significant upward trend in TNF-α response to TRYP antigen over time was observed in dogs from all groups, including controls, until the end of the study (linear regression: r^2=0.32; P<0.001; n=21 dogs) (Figure 5). In response to LACK antigen, a similar pattern and rising mean TNF-α trend over time was observed (linear regression: r^2=0.36; P<0.001; n=21 dogs; data not shown graphically), moreover TRYP and LACK TNF-α responses within individual samples correlated strongly (r^2≥0.89; P<0.001), irrespective of vaccine group. The mean levels of TNF-α in ConA stimulated wells showed a less pronounced but significant upward trend over time (linear regression: r^2=0.40; P<0.001; n=21 dogs). Mean TNF-α responses to CLA antigen in all vaccine groups at all time points were consistently low (≤100pg/ml) or below background, showing no significant association with vaccine group (Figure 6).
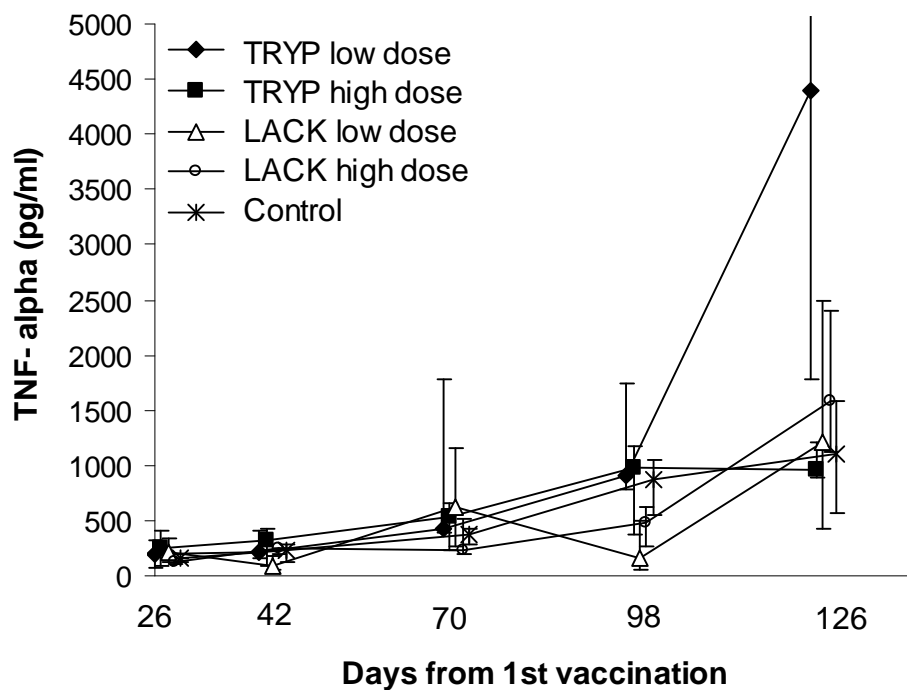
Figure 5. Median TNF-α levels in whole blood assays stimulated with TRYP. TNF- α levels were measured at the indicated time points after 1st vaccination. The between-group differences in TNF-α levels were not significant. The trend of TNF-α responses to LACK antigen were similar to those depicted (data not shown). Error bars indicate interquartile range. The y-axis has been truncated at 5000 pg/ml for clarity.
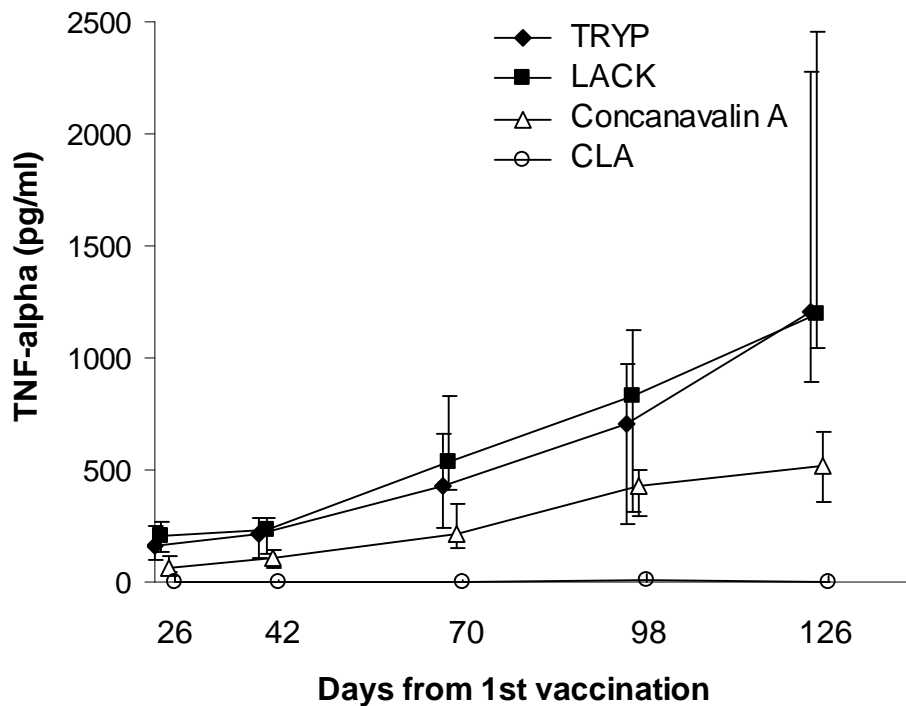
Figure 6. Median TNF-α levels in response to stimulation with all antigens. TNF- α levels were measured in whole blood cytokine stimulation assays with TRYP, LACK, CLA and antigens, and the non-specific mitogen Concanavalin A, at the indicated time points after 1st vaccination. Error bars indicate interquartile range. Results from all dogs (n=21) are amalgamated.

3.3.2.4 Intradermal tests

A positive skin test response to TRYP antigen (>5mm) was observed in 4/5 TRYP high dose dogs and 2/3 TRYP low dose dogs at day 156. The TRYP low dose dog with a negative skin test result corresponded to an animal which had consistently low IFN-γ cytokine assay responses to TRYP, whereas the skin test negative animal in the high dose TRYP group paradoxically showed high IFN-γ responses to TRYP throughout the trial. In LACK vaccinated dogs, there was a positive skin test response to LACK antigen in 2/5 high dose dogs and 0/4 LACK low dose dogs.

3.3.2.5 ELISA IgG1/ IgG2 subtyping

High TRYP-specific total IgG sample-to-positive (s/p) ratios (as defined in section 3.2.4.2) were seen in dogs post TRYP vaccination, however LACK-specific total IgG in LACK vaccinated dogs remained at baseline levels. Measurement of TRYP-specific IgG1 and IgG2 subtypes demonstrated significantly higher levels of IgG2 in both high

and low dose TRYP dogs compared to controls at all time points post vaccination (P ≤0.05; Wilcoxon rank sum test). No difference in IgG2 levels was detected between high and low dose TRYP dogs, therefore data were combined (Figure 7). Data from LACK vaccinated dogs are not shown due to absence of specific antibody response in these dogs. IgG1 levels in TRYP vaccinated dogs were uniformly low, and not significantly different from controls at any time point (Figure 7).
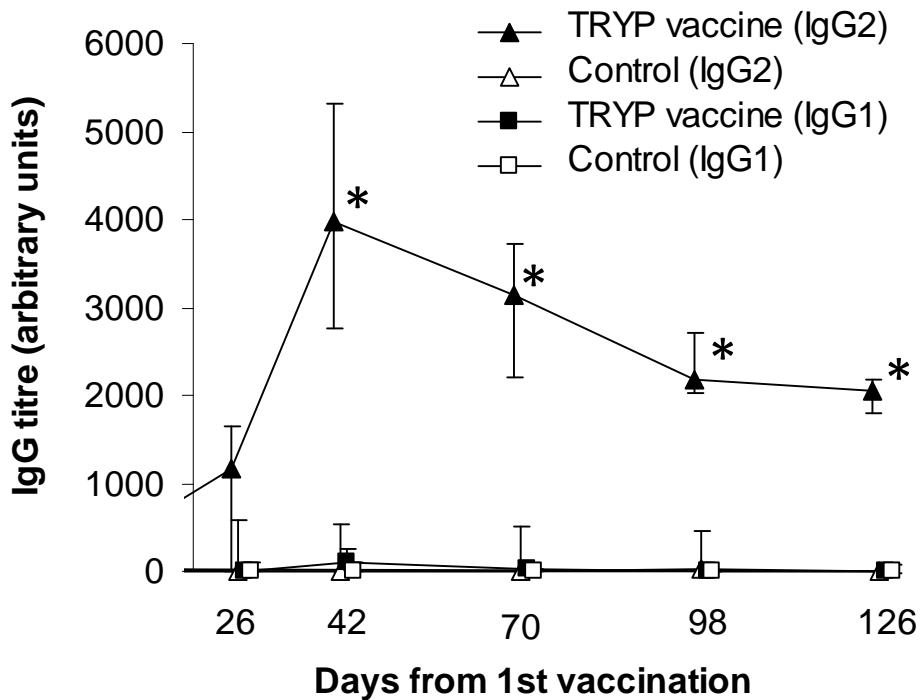
Figure 7. Median TRYP-specific IgG1 and IgG2 antibody class titres by vaccine group.
TRYP-specific IgG responses were measured at the indicated time points by ELISA using HRP conjugated polyclonal antibodies to detect IgG1 and IgG2 subtypes. Dogs were vaccinated with DNA TRYP or control placebo at Day 0. MVA TRYP or placebo was administered at Day 28. N.B. Error bars indicate interquartile range.
* denotes a significant difference between mean IgG levels in vaccinated and control dogs (Wilcoxon rank sum test: P≤0.05).

3.4 Discussion

The data presented in this chapter shows that uninfected, unexposed outbred endemic dogs vaccinated with DNA/MVA TRYP prime/boost vaccine produced higher antigen-specific levels of the signature type-1 cytokine IFN-γ in whole blood cytokine stimulation assays than placebo vaccinated dogs. LACK vaccinated dogs showed a similar trend that was not statistically significant. A majority of TRYP and a minority of LACK vaccinated dogs exhibited *in vivo* delayed-type hypersensitivity responses to intradermal inoculation with the appropriate recombinant vaccine antigen at day 156, indicative of antigen-specific cellular memory recall responses. The elevated antigen-specific IFN-γ level in TRYP vaccinated dogs compares with the reported high levels of IFN-γ associated with protection in murine models against *L. major* (Stober *et al.*, 2005; Stober *et al.*, 2007) and *L. donovani* infection (Kaye *et al.*, 1991; Wilson *et al.*, 2005; Ferreira *et al.*, 2008), and in dogs against *L. infantum* infection and disease (Pinelli *et al.*, 1995; Lemesre *et al.*, 2005; Rafati *et al.*, 2005), and is thus indicative of vaccine-induced protective type-1 immunity and memory phase response.

TRYP vaccinated dogs were also characterized by an IgG2 class dominated response, whereas IgG1 class levels remained low and were not significantly different to control dogs at any time point. Taking IgG2/IgG1 ratio as a proxy measure of Th1/Th2 polarization of the immune response following previous research (Fujiwara *et al.*, 2005; Lemesre *et al.*, 2005; Rafati *et al.*, 2005; Lemesre *et al.*, 2007), these results are further evidence of a type-1 dominated response in the TRYP vaccine group, despite some controversy over the association between canine IgG class ratio and protective cellular immune response (Day, 2007), different to the clear patterns observed in mice (Stober *et al.*, 2005; Stober *et al.*, 2007). In our hands, despite previous evidence of seroconversion to LACK antigen after DNA/MVA LACK prime/boost vaccination in murine trials (Stober *et al.*, 2005), LACK-specific IgG1 and IgG2 did not increase measurably from baseline levels. Strong responses to positive control sera excluded the possibility of LACK ELISA failure. Undetectable specific IgG2 in LACK vaccinated dogs contrasts with high levels in TRYP vaccinated animals, and may be further evidence of superior vaccine immunogenicity of the latter. The absence of significant IgG1 (Th2) responses in vaccinated dogs described here was as expected due to the absence of challenge infection or restimulation with *Leishmania* antigens, in contrast with previous canine trials of DNA/rVV prime/boost vaccines in which

humoral responses were measured post-experimental challenge (Ramiro *et al.*, 2003; Ramos *et al.*, 2008). For the same reason, we detected no antigen-specific increases in IL-10 levels in either vaccine or control groups, making analysis of IFN-γ:IL-10 ratios uninformative until natural challenge experiments are conducted. In murine models vaccinated with the same TRYP vaccine, a high ratio of pre-challenge IFN-γ:IL-10 in draining lymph node cells after *in vivo* crude parasite antigen restimulation was a clear indicator of vaccine success, whereas a low ratio (due to elevated IL-10 levels) predicted failure (Stober *et al.*, 2005). In dogs, the existence of the Th1/Th2/T$_{reg}$ paradigm in relation to *L. infantum* infection as observed for *L. major* in mice (Reiner and Locksley, 1992; Reed and Scott, 1993; Mosmann and Sad, 1996) is not completely resolved (as reviewed (Barbieri, 2006)), and there are conflicting results regarding the role of IL-10, with some studies demonstrating IL-10 elevation in symptomatic naturally or experimentally infected dogs, whereas other work failed to show any association between IL-10 and clinical disease, as discussed in Chapter 1.

In dogs, additional type-1 cytokines associated with protective cellular mediated responses include TNF-α, IL-2, IL-12, and IL-18 (Pinelli *et al.*, 1994; Santos-Gomes *et al.*, 2002; Chamizo *et al.*, 2005), with TNF-α the most frequently measured of these in canine ZVL studies. Our results show that TNF-α produced in WBA in response to both TRYP and LACK increased over time in all vaccine groups and controls (Figures 5&6), and the lack of antigen specificity of this response was evident from the approximately 1:1 relationship between TRYP- and LACK-stimulated TNF-α within individual samples. Macrophages present in WBA may raise innate TNF-α responses to contaminants such as bacterial lipopolysaccharide (LPS) (Kornbluth and Edgington, 1986), however this would not explain the upward trend in mean WBA TNF-α production in relation to the time from vaccination, nor the differing TNF-α levels between dogs, nor the similar upward trend in response to ConA stimulation. LPS contamination is also unlikely due to stringent manufacturing protocols under which TRYP and LACK antigens were produced. An alternative explanation is that non-specific priming of cellular immune responses occurred as a result of exposure to DNA plasmids or MVA vaccine, regardless of the encoded antigen, with a similar effect seen even in placebo dogs exposed to empty plasmids and MVA vehicle. This may result from MVA-induced expansion of polyfunctional, TNF-α producing CD8+

T-cells as previously seen in humans, though in that study, TNF-α production upon re-exposure to antigen was virus specific (Precopio *et al.*, 2007).

We did not detect specific cytokine responses to CLA in the present study, however this does not preclude vaccine efficacy, as indicated elsewhere, for example in mice, where protection afforded by sterol 24-c-methyltransferase vaccine against *L. infantum* correlated with high levels of antigen-specific IFN-γ, but by comparison only very low levels IFN-γ were induced by CLA (Goto *et al.*, 2007). Moreover, a canine trial of HASPB1/H1 vaccine, in which lymphoproliferative responses to CLA were absent post-vaccination, subsequently demonstrated partial protection against high dose experimental challenge with *L. infantum* (Moreno *et al.*, 2007).

In conclusion we have shown that vaccination of the important reservoir host of ZVL, the domestic dog, with prime/boost DNA/MVA TRYP vaccine is free from adverse side effects and shows appropriate immunogenicity consistent with protective efficacy. The combination of *in vitro* and *in vivo* test results clearly demonstrates that DNA/ MVA TRYP vaccine induces a type-1 dominated pro-inflammatory cellular immune response which is necessary for protection against *Leishmania* challenge, and that immune memory persists for at least four months post-vaccination in the absence of restimulation or infection. Further research is required to replicate the trials reported here in larger numbers of dogs, to identify and characterize cell surface markers of cell populations mediating IFN-γ and TNF-α release, and to validate vaccine formulations such as freeze-dried MVA which would reduce reliance on cold chain. In accordance with the 3R's principles of animal research (Russell and Burch, 1959), this pilot study enables sample size calculations to be carried out, based on detection of minimum expected differences in IFN-γ levels between vaccinated and control dogs of 446pg/ml (standardized difference = 1.39) (Whitley and Ball, 2002), from the experimental data reported here. To achieve 90% power at the 0.05 significance level to detect this difference will require approx. 40 animals (assuming unequal group sizes, ratio of vaccinated to control dogs = 5:1) (Whitley and Ball, 2002). Accounting for a pessimistic estimate of 15% loss to follow-up, it would be necessary to enrol 48 dogs (or 2 cohorts of 24), i.e. 40 vaccinated dogs and 8 controls. Subsequent to adequately replicated safety and immunogenicity trials in unexposed dogs, community based field trials will next be required to test DNA/MVA TRYP vaccine efficacy for prevention of ZVL infection and disease in naturally exposed dogs in *Leishmania* endemic areas.

Chapter 4: Selection of appropriate serological tests to measure the incidence of natural *Leishmania infantum* infection during DNA/MVA prime/boost canine vaccine trials.

4.1 Introduction

A growing number of canine experimental vaccines against *L. infantum* are under development and being taken forward into field efficacy trials. To capture the dynamics of vaccine efficacy over time, measurement of infection incidence (or force of infection) by repeated longitudinal testing is required (Quinnell *et al.*, 1997; Courtenay *et al.*, 2002b; Gradoni *et al.*, 2005). Serological tests are technically straightforward and may be more appropriate for large scale surveys than parasitological methods which rely on time consuming, and often invasive sampling methods, and are generally insensitive relative to serology. However, false positive serological test results due to the presence of vaccine-induced antibodies would invalidate incidence measures, depending on the magnitude and time course of the vaccine-induced immunoglobulin response. This is a particular issue with vaccines which incorporate crude or purified parasite derivatives, such as autoclaved *L. major* (Lasri *et al.*, 1999) or excreted/secreted *L. infantum* fractions (Lemesre *et al.*, 2005). Some investigators have used end-point seroconversion (e.g. at 16 months post vaccination) as an indicator of natural infection for calculation of vaccine efficacy (Mohebali *et al.*, 2004a), on the unconfirmed assumption that vaccine-induced antibody levels would decay rapidly. Dogs vaccinated with the fucose mannose ligand (FML) vaccine (Leishmune®, Fort Dodge, USA), currently licensed for use only in Brazil, may also test positive to serological tests for *L. infantum* (Borja-Cabrera *et al.*, 2002), rendering these tests ineffective for surveillance and intervention, though flow cytometric procedures to distinguish antibody responses to Leishmune® from natural infection have been described (Andrade *et al.*, 2009).

Second generation vaccines which employ prime/boost DNA/rVV protocols expressing *Leishmania* antigens have been successful in eliciting cellular immune responses in murine and canine infection models, with concurrent high titres of vaccine antigen-specific IgG (Ramiro *et al.*, 2003; Stober *et al.*, 2006; Stober *et al.*, 2007; Ramos *et al.*, 2008; Carson *et al.*, 2009). To date, no studies have characterized differences in standard serological test results between DNA/rVV vaccinated

unexposed dogs and naturally infected dogs. Therefore, the first aim of the present study was to test two widely used ELISAs, one based on crude *Leishmania* antigen (CLA), the other based on the recombinant antigen rK39, to confirm that antigen specific canine IgG responses to natural *L. infantum* infection differ from those mounted after DNA/rVV vaccination. rK39 is a 39 amino acid repetitive immunodominant B-cell epitope in a kinesin-related protein, conserved between *L. infantum* and the *L. donovani* complex (Burns Jr *et al.*, 1993). Recombinant rK39 ELISA has been used successfully as a diagnostic antigen for serodiagnosis in human visceral leishmaniasis (VL) patients (Qu *et al.*, 1994; Singh *et al.*, 1995; Badaro *et al.*, 1996; Houghton, 1998; Zijlstra *et al.*, 1998; Pedras *et al.*, 2008) and in canine cases of ZVL (Ozensoy *et al.*, 1998; Rhalem *et al.*, 1999; Zerpa *et al.*, 2000; Scalone *et al.*, 2002; do Rosario *et al.*, 2005; Mettler *et al.*, 2005; Porrozzi *et al.*, 2007; Taran *et al.*, 2007).

Longitudinal studies to measure vaccine efficacy require a diagnostic test with high sensitivity to detect early stage infection, particularly since the period from infection to clinical disease may vary from months (Quinnell *et al.*, 1997) to more than a year (Rioux *et al.*, 1979), and asymptomatic dogs may comprise up to 50% of infected animals in cross-sectional surveys (Lanotte *et al.*, 1978; Fisa *et al.*, 1999; Sideris *et al.*, 1999). However, longitudinal studies using serological tests to detect asymptomatic recently infected dogs reveal that their comparative sensitivity/specificity varies during the course of natural canine *L. infantum* infection (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001a; Solano-Gallego *et al.*, 2001; Scalone *et al.*, 2002; Oliva *et al.*, 2006; Otranto *et al.*, 2009b). Furthermore, longitudinal follow-up of experimentally challenged dogs (Rosypal *et al.*, 2005; Talmi-Frank *et al.*, 2006) is unlikely to reflect immunological events in natural infection caused by the bite of infected sandflies (Rogers *et al.*, 2004; Poot *et al.*, 2005; Peters *et al.*, 2009). Therefore the second aim of the present study was to quantify the sensitivities of CLA and rK39 ELISAs as examples of two routine tests to detect early stage infection in longitudinal analysis of a cohort of naturally infected dogs, for which the time course of infection was parasitologically well characterized.

4.2 Methods

4.2.1 Study animals and samples

Blood samples were obtained from three groups of dogs: (i) an unexposed group in the UK, (ii) a DNA/rVV vaccinated, unexposed group in Crete, Greece, and (iii) a naturally exposed group in Brazil. These groups are described in more detail below.

(i) 30 unexposed outbred UK dogs with no history of foreign travel that had attended two UK veterinary clinics during June–December 2007 for unrelated clinical reasons.

(ii) 22 unexposed outbred Cretian dogs <24 months old (median 18 months, range 4-24 months; 59% mixed breeds; male:female ratio=1.2:1) enrolled in trials of experimental DNA/rVV *Leishmania* vaccines previously validated in mice (Stober *et al.*, 2006; Stober *et al.*, 2007), reported in full in Chapter 3. Briefly, vaccination was carried out on day 0 with plasmids encoding *Leishmania* antigens (DNA "prime") and on day 28 with $1x10^8$ pfu modified vaccinia virus Ankara (MVA) (rVV "boost"). Dogs were DNA/MVA vaccinated with either tryparedoxin peroxidase (TRYP; n=9), *Leishmania* homolog of the receptor for activated C kinase (LACK; n=9), or control placebo (n=4). Two different DNA dose rates were tested: low dose 100μg DNA (n=4) and high dose 1000μg DNA (n=5). Dogs were blood sampled pre-vaccination (time 0) and at 26, 42, 70, 98 and 126 days after first vaccination.

(iii) 81 outbred dogs from a previously described large-scale longitudinal field study in Brazil (Quinnell *et al.*, 1997), from which blood and bone marrow samples were obtained at approximately 2 month intervals for up to 24 months post initial natural exposure. These dogs were previously tested by CLA ELISA (*L. infantum*), bone marrow PCR and parasitology (*in vitro* and *in vivo* bone marrow culture in hamsters), and clinically examined, as described in Chapter 2 and elsewhere (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001a; Courtenay *et al.*, 2002b), with subsequent storage of serum at -20°C.

4.2.2 ELISA

In the present study, serum samples were tested using an ELISA based on the recombinant antigen rK39, For comparison we also tested a CLA ELISA, which is reported to be more sensitive (Mettler *et al.*, 2005) and is widely used for serological diagnosis. ELISA methods were as described in Chapter 2. ELISA cut-off points were established by testing 30 unexposed non-endemic UK dogs (described in section

4.2.1). Conservative cut-off values were calculated from the UK dogs' mean $\log_{10}$ s/p ratio +3 S.D. and then back-transformed to obtain the raw s/p ratio cut-off value to classify all dogs.

Serum samples from vaccinated dogs were additionally tested by the indirect immunofluorescence test (IFAT) as described in Chapter 2. The negative cut-off titre was validated from the results of IFAT tests carried out on 30 UK non-endemic dogs, all of which gave negative results at 1/40.

For the Brazilian dogs, results were available from previous diagnostic tests as listed in 4.2.1 (iii), above (Quinnell *et al.*, 1997).

4.2.3 Serological responses in exposed, and vaccinated unexposed dogs

To compare CLA and rK39 ELISA sensitivity required populations of dogs that were positive and negative for *Leishmania* infection. Using sera from Brazilian dogs naturally exposed to *Leishmania* in the field, samples were defined as 'true' negatives at all longitudinal sampling time points before first detection of infection (t0) by bone marrow PCR, and/or *in vitro/ in vivo* culture, based on previous tests (Quinnell *et al.*, 1997). Samples were designated as 'true' positives at all sampling time points after first detection of infection. For calculations of sensitivity, a single post-infection sample was selected at random from each Brazilian dog, giving 63 'true' positive samples. Similarly, we selected a single pre-infection sample at random from each dog to obtain 62 'true' negatives. This method allowed estimation of CLA and rK39 ELISA sensitivity and specificity, removing potential statistical bias due to autocorrelation (i.e. non-independent repeat samples from the same dog).

4.2.4 Serological test performance in the early stages of infection

Sensitivities of rK39 and CLA ELISAs in early stage infection were measured using additional longitudinal sample series from dogs selected from the larger Brazilian cohort study. A total of 128 samples were aligned relative to the longitudinally assigned time of first detection of infection t0, as described above, to represent up to 4 consecutive time points: -2, t0, +2 and +4 months from t0.

4.3 Results

4.3.1 Comparison of serological responses between vaccinated and infected dogs

Cut-off values for the rK39 and CLA ELISAs were first calculated from the backtransformed mean $\log_{10}$ s/p ratio +3 S.D. of 30 UK unexposed dogs giving s/p values of 0.259 and 0.422 respectively. Similarly, IFAT cut-off was established at the first serial dilution (1/40), as none of the 30 UK dogs sampled showed positive results at or above this dilution. All samples at all time points from TRYP and LACK vaccinated unexposed dogs fell below the designated rK39 and CLA ELISA cut-off values, and were therefore classed as negative, with no misclassification errors. There were no significant differences between mean s/p ratios of low and high dose TRYP or LACK vaccine groups at any time point (Wilcoxon rank sum test: $P \geq 0.14$), thus aggregated results are shown (Figure 8). None of the vaccinated dogs had an increased rK39-specific IgG titre post-vaccination, and responses were all lower than the maximum response in UK negative controls. In contrast, two TRYP-vaccinated dogs showed a transitory modest rise in anti-CLA response at 42 and 70 days post-vaccination, but these responses had declined by day 98, and did not exceed the cut-off at any follow-up time point. Weak positive IFAT titres of 1/40 – 1/80 were detected in samples from 4 vaccinated dogs at day 28 and, 1 dog at days 42 and 70. Measurement of ELISA sensitivity and specificity, using the same cut-off points to classify the 62 'true' negative and 63 'true' positive dogs in Brazil (Figure 8), showed significantly higher sensitivity of CLA (81%; 51/63) over rK39 ELISA (61.9%; 39/63: $\chi^2=5.60$; $P=0.018$). Specificity was marginally lower for CLA than rK39 ELISA to correctly identify 'true' negatives (69.4% (43/62) vs 82.3% (51/62), respectively; $\chi^2=2.81$; $P=0.093$). The coefficient of agreement between ELISAs indicated moderate agreement (kappa=0.53; S.E.=0.09; $P<0.001$).

4.3.2 ELISA sensitivity in longitudinal samples from naturally infected dogs

To test the relative sensitivities of the rK39 and CLA ELISAs with time from first parasitological detection of infection t0, Brazil cohort dog samples (n=128) were examined between t-2 to t+4 months (Table 3). This analysis showed that the sensitivity of both ELISAs increased with time from t-2 months, and the cumulative sensitivity of the rK39 ELISA by t+4 was not significantly different to the CLA ELISA (Fisher's Exact test; $P=0.13$). Analysis of the results for individual follow-up

time points showed that the sensitivity of rK39 ELISA was significantly lower relative to CLA ELISA at t-2, t0 and t+2 months ($\chi^2 \geq 3.79$; $P \leq 0.05$), whilst at t+4 months the rK39 ELISA sensitivity was not significantly different from that of the CLA ELISA (Fisher's Exact test; $P=0.42$). Agreement between rK39 and CLA ELISAs was moderate (kappa=0.47; S.E.=0.08; $P<0.001$). Clinical scores in the longitudinally sampled dogs increased over time, with the proportion of symptomatic dogs (total clinical score >2) rising from 0.03 at t-2 to 0.38 at t+4 months. The possibility that missing data introduced bias in our results was tested for and excluded using logistic regression in a generalized linear model (GLM), which showed no significant association between number of different parasitological tests (bone marrow PCR, and/or *in vitro/ in vivo* culture) performed on a sample, and infection status (z=1.45; $P=0.15$).
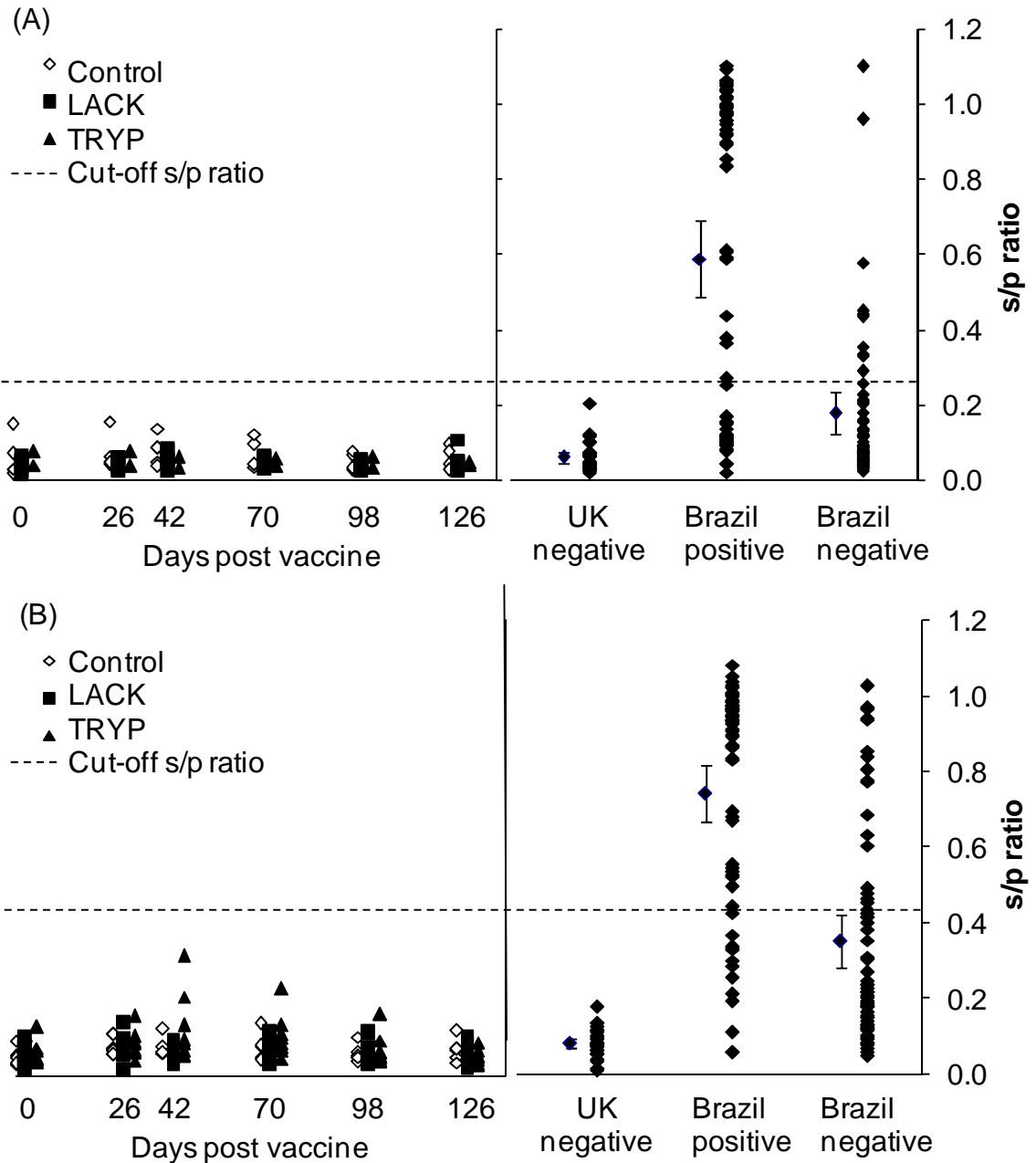
Figure 8 . Comparison of IgG responses to rK39 (A) and crude *Leishmania* antigen (CLA) (B) in vaccinated and naturally infected dogs.

Total IgG response was measured by (A) rK39 ELISA and (B) CLA ELISA. Sample-to-positive (s/p) ratios of individual vaccine control and TRYP/LACK vaccinated dogs (left), with s/p ratios of UK negative, and Brazil positive and negative dogs (filled diamonds, right) as defined in text (error bars in the right hand panel show 95% C.I. around mean s/p ratios of UK and Brazilian dogs). Brazil positive dogs were defined as those with at least one previous positive sample by bone marrow PCR and /or culture. Brazil negative dogs were defined as all those sampled before the time of infection. Cut-off s/p values are rK39 = 0.259 and CLA = 0.422, representing the back-transformed mean +3S.D. of the $\log_{10}$ s/p ratios of 30 UK negative dogs. Vaccine trial dogs were vaccinated at day 0 (DNA TRYP/LACK) and day 28 (MVA TRYP/LACK).

Table 3. Proportion of Brazilian dogs positive in CLA and rK39 ELISAs, relative to time of first detection of infection (t0).
Infection was defined by positivity to bone marrow PCR, and/or *in vitro/in vivo* parasitological culture. Binomial 95% C.I. are shown in parentheses.

Test	t-2 months	t0	t+2 months	t+4 months	Cumulative
CLA	0.34	0.76	0.90	0.92	0.95
	(0.19-0.52)	(0.59-0.88)	(0.74-0.98)	(0.75-0.99)	(0.82-0.99)
rK39	0.09	0.54	0.63	0.81	0.84
	(0.02-0.23)	(0.37-0.71)	(0.44-0.80)	(0.61-0.93)	(0.68-0.94)
N	35	37	30	26	37
***P*-value**	0.009**	0.05**	0.015**	0.42	0.13

N: Number of samples tested
** denotes a statistically significant difference between CLA ELISA and rK39 test sensitivity at the indicated time point, at the stated level of probability (*P*-value), using χ^2 or Fisher Exact tests as appropriate.

4.4 Discussion

Characterization of vaccine-induced antibody responses in the target species is an essential component of vaccine development. Here, we compared IgG responses to CLA and rK39 antigens in *L. infantum* naturally infected dogs, with those of DNA/MVA TRYP and LACK vaccinated dogs. CLA is likely to contain significant quantities of both TRYP and LACK (Mougneau *et al.*, 1995; Levick *et al.*, 1998), while rK39 is derived from an unrelated gene in the parasite genome with no evidence for cross-reactive epitopes at the level of amino acid sequence identity (Omnigene BLAST analysis at www.geneDB.org). We therefore expected to observe CLA ELISA seroconversion in vaccinated dogs and in naturally infected animals, and rK39 seroconversion only in the latter. Surprisingly, we found that both CLA and rK39 ELISA s/p ratios in vaccinated dogs were statistically similar to unexposed UK dogs, and below the calculated cut-off values for seroconversion. A similar lack of specific IgG for CLA, in response to vaccination with recombinant *Leishmania* proteins HASPB1, Histone 1 and polyprotein MML has been reported previously (Moreno *et al.*, 2007). As described in Chapter 3, strong antigen-specific IgG responses to TRYP (but not LACK) after vaccination of dogs in the present study demonstrated that weak

or absent IgG responses were not due to vaccine failure, and whilst LACK-vaccinated dogs did not mount significant IgG responses to LACK antigen, vaccine immunogenicity was indicated by measurable cytokine responses to LACK.

Although all CLA ELISA s/p ratios in vaccinated dogs were below the cut-off value, 2 dogs showed a transitory CLA IgG response to vaccination. In contrast, seroconversion above the cut-off value is consistently seen in natural infections, as reported here and elsewhere (Quinnell *et al.*, 1997; Solano-Gallego *et al.*, 2005). This important distinction would be apparent from the results of repeated testing of dogs in longitudinal vaccine efficacy field trials. Low IFAT titres above the cut-off point were observed in 6 post-TRYP-vaccination samples (n=4 dogs) suggesting that IFAT would not be a suitable serological test to measure natural infection incidence in these vaccinated dogs. The cut-off value of 1/40 used in the present study may be conservative, as laboratories elsewhere specify cut-off values ranging from 1/20 to 1/200 (Abranches *et al.*, 1983; Sideris *et al.*, 1996; Acedo-Sanchez *et al.*, 1998; Mettler *et al.*, 2005; da Silva *et al.*, 2006; Oliva *et al.*, 2006). The second aim of the current study was to identify which serological test would give the most sensitive measure of infection incidence. Whilst there is little doubt that sensitivity increases with time from infection (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001a; Courtenay *et al.*, 2002a; Courtenay *et al.*, 2002b), precise incidence estimates will depend on the test's sensitivity to diagnose early stage infection. Our longitudinal comparisons of relative test sensitivities show that the post-infection increase in rK39 ELISA sensitivity lags behind that of CLA by up to 6 months. In contrast, a previous study of 6 experimentally infected beagles found that rK39 ELISA seroconversion preceded CLA seroconversion (Talmi-Frank *et al.*, 2006). This apparent difference could be due to the use of *i/v* high dose challenge in the latter study, *c.f.* natural intradermal inoculation by sand fly bite here. In cross-sectional analysis, CLA ELISA was the more sensitive test (81% vs 61.9%), and rK39 ELISA was marginally more specific (82.3% vs 69.4%). Absolute values of sensitivity and specificity for both ELISAs are lower than published estimates (Reithinger *et al.*, 2002; Scalone *et al.*, 2002; Mettler *et al.*, 2005), though in longitudinal testing the sensitivities of both CLA and rK39 ELISAs after t+4 were relatively high (92% and 81%, respectively). ELISA specificities calculated here will likely be underestimates since our 'gold' standard for leishmaniasis diagnosis (bone marrow PCR and/or *in vitro/in vivo* culture) was itself imperfect. The sensitivity of parasitological culture is low, whilst that of PCR is

known to decline after the earliest stages of infection (Quinnell *et al.*, 2001a). Hence, samples that were 'gold' standard negative but tested rK39 or CLA ELISA seropositive do not necessarily represent false positives. Further evidence of discordance between PCR/culture and serological test results exists in other research (Reithinger *et al.*, 2002; Otranto *et al.*, 2009b). On the other hand, it is always necessary to rule out false positives due to serological cross-reactivity with antibodies to other endemic canine parasitic infections. A CLA ELISA false positive rate of up to 85% has been observed in studies testing serum from small numbers of dogs infected with *Trypanosoma cruzi* (do Rosario *et al.*, 2005; Ferreira *et al.*, 2007), consistent with data from human infections (Reed *et al.*, 1987). Limited data exist showing CLA ELISA false positives in dogs infected with *Leishmania braziliensis* (5/9), *Babesia canis* (1/24), *Ehrlichia canis* (1/3), and *Dirofilaria immitis* (4/10) (Mancianti *et al.*, 1996; do Rosario *et al.*, 2005; Ferreira *et al.*, 2007). Since the geographical distributions of many of these canine parasites overlap with that of ZVL (Trotz-Williams and Trees, 2003; Solano-Gallego *et al.*, 2006), significant cross-reactivity may preclude the use of CLA ELISA to gain accurate estimates of infection incidence in some areas. However, signal strength is likely to be lower with less related pathogens, and many studies support high diagnostic value CLA ELISA in diverse settings. It is recommended therefore that pre-intervention survey sampling in vaccine trial field sites should be done to characterize the prevalence of potentially cross-reacting canine pathogens in CLA ELISA. Conversely, rK39 ELISA appears to show high specificity (Scalone *et al.*, 2002; do Rosario *et al.*, 2005) though cross-reactions have been documented with *L. braziliensis* and *Leptospira interrogans* (Porrozzi *et al.*, 2007); due to the small numbers of animals tested in the latter study, further large scale studies are required to investigate the specificity of rK39 ELISA.

In conclusion, our results confirm the important requirement for efficacy field trials: seroconversion to either CLA or rK39 ELISA can be used to measure the incidence of natural infection in DNA/MVA TRYP or LACK vaccinated dogs. CLA ELISA provides maximum serological sensitivity in early stage infection, whereas rK39 as a defined recombinant antigen with fewer potentially cross-reactive epitopes is likely to reduce false positives. The imperfect agreement between CLA and rK39 ELISAs suggests that when monitoring infection incidence, a combination of both rK39 and CLA ELISAs should ideally be employed.

Chapter 5: Comparison of monoclonal and polyclonal antibodies for detection of canine IgG1 and IgG2, and associations with infection outcome in *Leishmania infantum* naturally infected dogs.

5.1 Introduction

In experimental murine models of leishmaniasis, the profile of IgG class titres against crude *Leishmania* antigen (CLA) has been well validated as a proxy measure of T-cell activation. In particular, a high ratio of specific IgG2a:IgG1 has been associated with a Th1-type pro-inflammatory cytokine response (production of IFN-γ by CD4+ helper T lymphocytes), which in turn promotes resistance to disease caused by *Leishmania major* infection. Conversely, a comparatively low IgG2a:IgG1 ratio results from preferential activation of a non-protective Th2 response involving production of IL-4 and progression to clinical disease (Snapper and Paul, 1987; Heinzel *et al.*, 1989; Coffman *et al.*, 1993; Germann *et al.*, 1995). Measurement of IgG class expression as a proxy for Th1 response is highly attractive as an additional measure of vaccine immunogenicity in dogs, the reservoir host of zoonotic visceral leishmaniasis (ZVL). However, the interpretation of IgG class profiles in relation to clinical outcomes in dogs is currently unclear: some studies show greater elevations in IgG2 than IgG1 level in dogs with asymptomatic compared to symptomatic infection (Deplazes *et al.*, 1995; Nieto *et al.*, 1999; Iniesta *et al.*, 2005; Carrillo *et al.*, 2007; Ramos *et al.*, 2008), whilst others show the opposite association (Cordeiro-da-Silva *et al.*, 2003; Reis *et al.*, 2006b; Cardoso *et al.*, 2007; Iniesta *et al.*, 2007). One reason for these inconsistent results may be a lack of specificity of the commercially available polyclonal conjugate antibodies used in those studies (Day, 2007). For example, four subfractions of IgG from normal canine serum were found to cross-react with both IgG1 and IgG2 polyclonal antibodies (Mazza *et al.*, 1993). It was suggested that higher specificity for canine IgG classes might be achieved using a previously described panel of monoclonal antibodies (Mazza *et al.*, 1994a; Mazza *et al.*, 1994b; Day, 2007), hereafter referred to as 'monoclonal' antibodies. Only two studies to date have employed these monoclonal antibodies to measure IgG subtypes in dogs with ZVL and both have involved cohorts of naturally exposed outbred dogs in Brazil. In the first of these investigations, the IgG2:IgG1 ratio was lower in clinically ill and

PCR positive dogs, but signs of disease were associated with generalized elevation in all four IgG classes, and great variation between individual dogs meant that relative IgG class levels were not a useful marker of resistance or susceptibility (Quinnell *et al.*, 2003a). In the second investigation a similar broad elevation in all four classes was found in seropositive, parasite positive and symptomatic dogs, but seropositive and asymptomatic dogs had a serological response dominated by IgG1 to the exclusion of IgG2. Moreover, this second study included a group of naïve dogs vaccinated with the commercially available Leishmune® vaccine and these animals had a striking serological response dominated by IgG2 (Oliveira *et al.*, 2009).

The current study tests the association between IgG1 and IgG2 classes and clinical/parasitological outcomes using commercially available polyclonal antibodies (generated in goats and sheep, respectively) on archived samples from the study described above (Quinnell *et al.*, 2003a) which were previously tested using monoclonal antibodies. This study is important to establish (1) whether IgG1 and IgG2 classes measured by polyclonal antibodies are related to clinical/parasitological outcomes in this population, and if so, (2) whether choice of antibodies will bias associations with these outcomes. Analysis was performed to detect associations between absolute levels and ratios of IgG1 and IgG2 classes, and a range of infection outcome measures (clinical signs of leishmaniasis, bone marrow PCR, and parasitological culture). This study represents the first direct comparison between evaluation of canine IgG classes with the polyclonal and monoclonal antibodies.

5.2 Methods

5.2.1 Sample selection

Samples were generated during a longitudinal study comprising a cohort of naturally exposed Brazilian dogs, as described in Chapter 2 and previously (Quinnell *et al.*, 1997). In that study, *Leishmania*-specific total IgG titres and IgG class levels were quantified by ELISA, using the monoclonal antibody mAb B6 for IgG1 measurement, and mAb E5 for IgG2, as described (Quinnell *et al.*, 2003a). Clinical scores were calculated as described in Chapter 2: dogs with total scores 0-2 were considered asymptomatic, while total scores>2 were classed as symptomatic. Dogs were categorized as infected at all time points after first detection of infection by *in vitro*/*in*

vivo culture, and/or crude parasite antigen total IgG ELISA, and/or bone marrow PCR, as described in Chapter 2 and previously (Quinnell *et al.*, 1997).

In the current study, previously tested stored sera from infected dogs were selected from this population for re-testing using commercially available polyclonal antibodies (anti-IgG1 and anti-IgG2 HRP conjugates: Lot No. A40-120P-13 & A40-121P-14, respectively; Bethyl Laboratories, Montgomery, Tx. USA). To avoid possible bias due to autocorrelation (i.e. non independent repeat samples from the same dog) one serum sample was selected at random from the larger longitudinal sample series from each of 60 infected dogs, yielding samples at varying stages of infection with a median 70.5 days (range: 0-535 days) from first detection of infection. The previously determined serological, parasitological and clinical status of the dogs at the time of collecting these cross-sectional samples for the current study are shown in Table 4.

Table 4. Serological, parasitological and clinical status of 60 longitudinally confirmed infected dogs at the time of cross-sectional sampling.

	PCR	CLA ELISA	Culture	Clinical signs
Positive	20	55	12	22
Negative	15	5	30	37
Not tested	25	0	18	1

5.2.2 IgG Class ELISAs

ELISA to measure CLA specific IgG1 and IgG2 titres with polyclonal anti-dog IgG1 and IgG2 (Bethyl Laboratories) was carried out as described in Chapter 2. Samples were run in duplicate, at doubling dilutions as described below.

To calculate IgG class levels from assays with polyclonal antibodies, we used the methodology previously employed on the same samples tested by monoclonal antibodies (Quinnell *et al.*, 2003a). A highly positive control serum was titrated 2-fold from 1:100 to 1:3,276,800 on every plate, and assigned a number of arbitrary units equal to the reciprocal of the highest dilution at which OD exceeded the mean +3S.D. of the background (no sample) wells. A standard line was fitted using a log-logit

transformation (Peterman and Butler, 1989) to plot dilutions of the standard from 1:200 to 1:3,276,800. Test sample IgG1 and IgG2 levels were quantified from titration curves plotted over doubling dilutions from 1:100 to 1:6400. The geometric mean number of antibody units was estimated from the range of dilutions at which the test sample titration curve was parallel to the standard. All subsequent analysis was carried out on log-transformed data expressed in arbitrary units.

5.3 Results

The values of Pearson's correlation coefficient, calculated for pairwise comparisons of IgG class level measurements, are shown in Table 5. Anti-CLA IgG1 units measured by polyclonal and monoclonal antibodies were positively correlated, though the r^2 value was not particularly high (Pearson's correlation coefficient=0.75; r^2 =0.56, P<0.001; (Figure 9A). IgG2 units measured by the two antibodies were also significantly correlated, but with a lower r^2 value (Pearson's correlation coefficient=0.54; r^2 =0.29, P<0.001) (Figure 9B). In the study using monoclonal antibodies, samples with titres above or below the linear range of the standard curve were truncated and assigned the maximum or minimum values of the linear range - this is seen clearly in Figure 9B, where the maximum upper value for monoclonal antibodies IgG2 $\log_{10}$ titre is 3.20. To assess the extent to which this biased correlations, analysis was repeated after removing samples truncated at the upper maximum and lower minimum values for IgG1 and IgG2 titre. This procedure did not substantially alter the results of analyses (IgG1: r^2=0.52, Pearson's correlation coefficient=0.72; IgG2: r^2=0.26, Pearson's correlation coefficient=0.51; P<0.001 in both analyses).

There was a generally high positive correlation between IgG1 and IgG2 units (regardless of antibody type), and between each class and total IgG (Table 5), reflecting a general elevation in anti-CLA IgG. The lowest correlation was seen between IgG2 measured by monoclonal antibodies, and all other IgG levels (Pearson's correlation coefficient ≤0.54). As expected, the absolute values for class levels obtained using monoclonal and polyclonal antibodies differed, since values were expressed in arbitrary units relative to positive controls. There was no significant correlation between IgG2:IgG1 ratios using monoclonal and polyclonal antibodies to measure levels in each individual sample (r^2<0.01, P=0.52; Pearson's correlation coefficient=-0.08).

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Table 5. Correlation between total CLA-specific IgG, and IgG class $\log_{10}$ units measured using monoclonal and polyclonal antibodies.

	Total IgG	Poly IgG1	Poly IgG2	Mono IgG1	Mono IgG2
Total IgG	1.00				
Poly IgG1	0.79	1.00			
Poly IgG2	0.84	0.87	1.00		
Mono IgG1	0.90	0.75	0.85	1.00	
Mono IgG2	0.50	0.47	0.54	0.51	1.00

Numerical values indicate Pearson's correlation coefficient calculated from all possible pairwise comparisons of monoclonal and polyclonal class log-transformed units, and total IgG ($\log_{10}$ units) ($P \leq 0.01$ in all cases).
Poly: polyclonal antibodies; Mono: monoclonal antibodies.
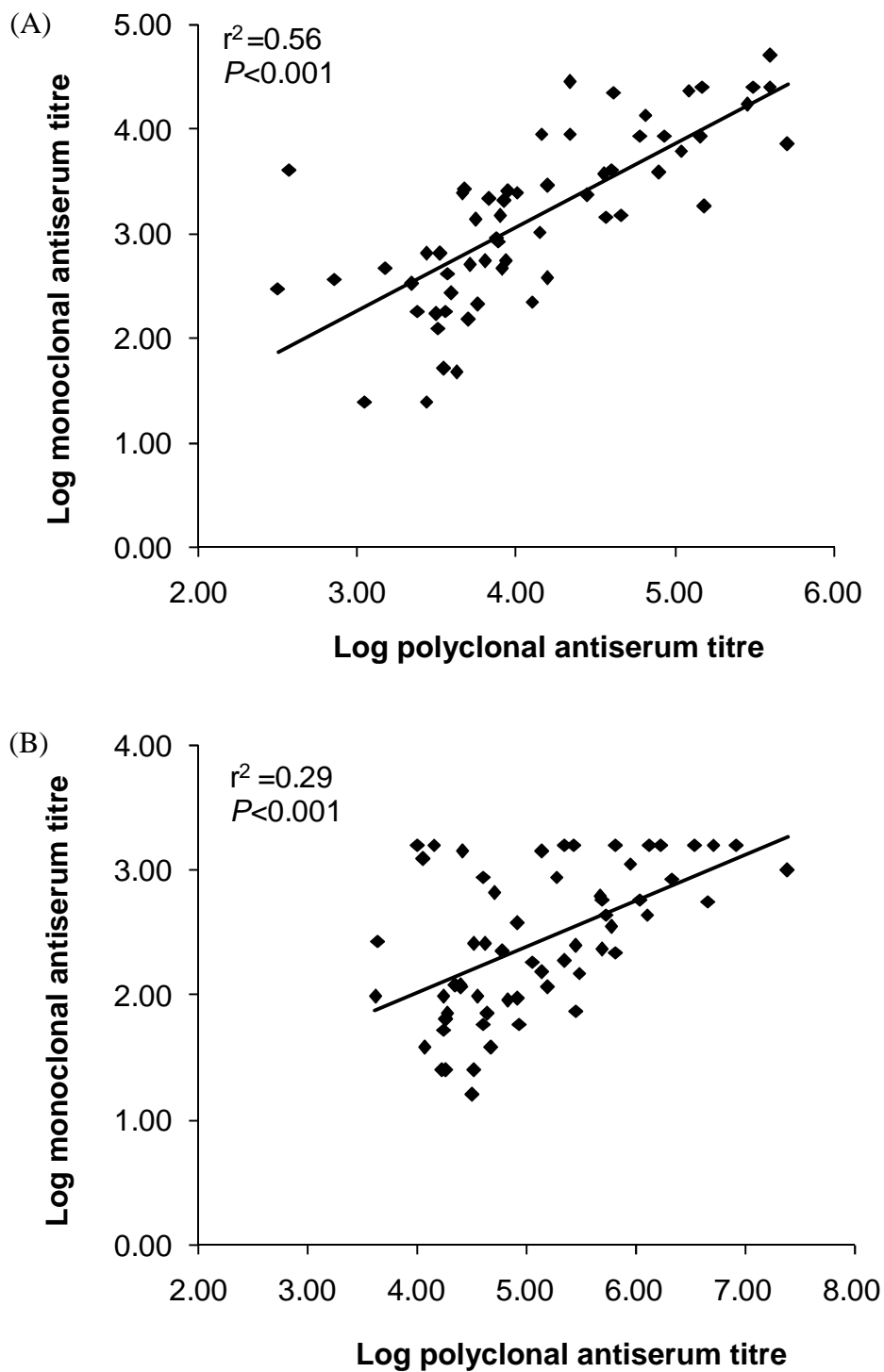
Figure 9. Relationship between polyclonal and monoclonal IgG1 (A) and IgG2 (B) class titres in naturally infected dogs.
IgG1 and IgG2 were measured using both monoclonal and commercial polyclonal HRP conjugate antibodies. Both scales represent $\log_{10}$ arbitrary units as described in the text.

When dogs were stratified by clinical signs (i.e. asymptomatic or symptomatic), mean IgG2:IgG1 ratios measured by either monoclonal or polyclonal antibodies did not differ significantly between clinical groups (z≤1.11, P≥0.27; Table 6). This result indicates an absence of IgG class polarization associated with the dichotomised clinical outcome. However, mean $\log_{10}$ units of both IgG1 and IgG2, measured by both antibodies, were 1.11-1.18 times higher in clinically symptomatic than in asymptomatic dogs (Wilcoxon rank sum test: polyclonal antibodies IgG1, z=2.32, P=0.02; IgG2, z=2.16, P=0.03: monoclonal antibodies: IgG1, z=2.27, P=0.02; IgG2, z=3.00, P<0.01; Table 6). When dogs were stratified based on bone marrow PCR positivity, mean $\log_{10}$ IgG1 and IgG2 levels measured by polyclonal antibodies were each significantly higher (by a factor of 1.16-1.17) in PCR positive animals than in PCR negative animals (z=2.50, P=0.01; z=2.27, P=0.02, respectively). Conversely, using monoclonal antibodies, mean $\log_{10}$ IgG1, but not IgG2, was 1.28 times higher in PCR positive dogs (z=2.52, P=0.01; z=0.25, P=0.80, respectively). Analysis for associations between bone marrow culture positivity and $\log_{10}$ class level found that only IgG1 measured by monoclonal antibodies was significantly elevated (by a factor of 1.19) in culture-positive dogs compared with culture-negatives (z=2.00, P=0.05).

Table 6. Associations between infection status and IgG1/IgG2 $\log_{10}$ units by polyclonal and monoclonal antibodies.

Values represent *n*-fold difference in the mean $\log_{10}$ units of dogs that were positive for clinical signs of leishmaniasis, or test positive by PCR or culture, relative to negatives.

	IgG1		IgG2		IgG2:IgG1 ratio		
	Poly	**Mono**	**Poly**	**Mono**	**Poly**	**Mono**	**N**
Clinical signs	1.11*	1.16*	1.11*	1.18**	0.99	1.04	59
PCR	1.16**	1.28**	1.17*	1.03	1.00	0.75	35
Culture	1.08	1.19*	1.10	1.00	1.00	0.77	42

Poly: polyclonal antibodies; Mono: monoclonal antibodies.

N: no. of dogs tested.

Clinical signs, PCR, Culture: Dogs showing clinical signs of leishmaniasis (total score>2), or that were test-positive by PCR or *in vitro/in vivo* parasitological culture, respectively.

* $P \leq 0.05$, **$P \leq 0.01$: Statistical significance of the association between IgG units/ratio and clinical status/diagnostic test result by Wilcoxon Rank Sum test.

5.4 Discussion

The results of this study suggest that reported differences in the immunological specificity of monoclonal and polyclonal antibodies are sufficient to cause inconsistent associations between IgG class levels and parasitological outcomes (Table 6). Similarly, the lack of a correlation between IgG2:IgG1 ratios measured in each sample using the different antibodies, suggests that results of studies using these different reagents will not be directly comparable. This confirms the concerns expressed by others (Day, 2007). The latter study also cautioned that it is unsafe to assume cross-species functional similarity between human, dog and mouse IgG subclasses, when criteria for numbering the canine IgG classes 1-4 were based solely on similar relative serum concentrations and electrophoretic mobility to human IgG 1-4 fractions. Whilst levels of anti-CLA IgG1 and IgG2 measured using each antiserum were positively correlated, there were also strong positive correlations between IgG1, IgG2 and total IgG levels. Thus correlation between monoclonal and polyclonal reagents for the same class may not reflect similar specificity; rather, it may reflect a general upregulation of antibody responses of all classes. Moreover, both antibodies detected significantly higher IgG1 and IgG2 levels in clinically symptomatic dogs compared with asymptomatic animals. This was consistent with results of the original larger longitudinal study, in which elevation of all four IgG classes was detected by monoclonal antibodies in dogs with clinical disease (Quinnell *et al.*, 2003a) and consistent with the more recent study of symptomatic dogs (Oliveira *et al.*, 2009). However the findings of the present study with respect to asymptomatic dogs conflict with those of Oliveira *et al.* (2009). Asymptomatic animals in the present cohort had a similar ratio of IgG1:IgG2 compared with symptomatic dogs, but in the population examined by Oliveira et al. (2009) the serological response of asymptomatic dogs was almost exclusively attributed to IgG1. Therefore, current findings using polyclonal antibodies did not confirm polarization of IgG class expression according to clinical status and therefore do not support the hypothesis that differential IgG1/IgG2 class expression is a useful proxy measure of resistance or susceptibility in endemic populations of dogs naturally infected with *L. infantum*. There were clearly differences in the level of characterization of the asymptomatic populations examined in these two studies. Those in the present investigation were very well investigated using a range of diagnostic procedures, whilst the asymptomatic population in the study of Oliveira et al. (2009) was a randomly sourced serosurvey group. Moreover,

although both populations were from endemic areas in Brazil, these were not geographically identical. In the present study, IgG class ratios were also not useful as predictors of parasitological infection status or PCR positivity. The collective results reported here suggest that class responses to leishmaniasis in dogs may be more similar to those in humans than in mice: in human patients with clinically and parasitologically confirmed visceral leishmaniasis, a general upregulation in all four IgG class titres has been reported with no particular class polarization (Elassad *et al.*, 1994).

Due to the complexity of cytokine interactions during the course of natural infection, a simplified canine model to investigate correlations between class expression and cytokine responses is desirable. Such models already exist in the form of immunogenicity trials of canine ZVL vaccines in unexposed dogs, where vaccine effectiveness relies on preferentially stimulating a Th1 cellular immune response. In the DNA/MVA prime/boost vaccination of naïve dogs carried out as described in Chapter 3, peak production of the Th1-type cytokine IFN-γ in response to vaccination occurred concurrently with significant elevation of IgG2 (measured by commercially available polyclonal antiserum), and in the absence of detectable IgG1 or the Th2/Treg type cytokine IL-10. This observation is of note, as in the study of Oliveira et al. (2009) vaccination of naïve dogs with the commercially produced Leishmune[®] vaccine also led to a markedly skewed serological response dominated by IgG2, in that case detected by monoclonal antibody. Given the relatively poor correlation between IgG2 polyclonal and monoclonal antibodies observed in the present study, this finding is of interest. Thus, in addition to more extensive comparison of monoclonal and polyclonal IgG class specificity in larger groups of naturally exposed dogs, it is recommended that canine trials of vaccines that elicit Th1 responses in the absence of infection should be used as a test bed to examine the extent of IgG class bias, in parallel with cytokine measurement and the use of both polyclonal and monoclonal antibodies.

Chapter 6: Evaluation of an immunochromatographic rK39 dipstick test for detection of infection in longitudinal follow-up of dogs with naturally acquired ZVL

6.1 Introduction

Diagnosis of ZVL infection in the reservoir host, the domestic dog, is usually carried out using serological tests based on crude or recombinant parasite antigens. One of the most widely tested recombinant antigens for serological detection of *Leishmania*-specific IgG is rK39, as described more fully and used in ELISA format in Chapter 4. The rK39 antigen has been incorporated into commercially available rapid diagnostic tests (RDT's), in the form of immunochromatographic dipsticks. In recent years, increasing numbers of RDT's have become available for field diagnosis of human and animal diseases, such as malaria, Chagas disease and babesiosis. Published work describes a number of currently commercially available rK39 dipsticks for use in humans, and 3 dipstick tests specifically for veterinary use (DiaMed-Vet-IT Leish (Diamed AG, Switzerland), Kalazar Detect Canine Rapid Test (Inbios, USA) and visceral *Leishmania* dipstrip (Cypress Diagnostic Company, Belgium)). These tests display a coloured band to indicate a positive result within minutes of addition of serum, are ideal for resource-poor field environments, and do not require a high level of operator technical skill making them potentially very useful for large-scale field surveys or vaccine trials against canine leishmaniasis. The use of rK39 dipstick tests for diagnosis of VL/ZVL has been described by previous research in both humans (Sundar *et al.*, 1998; Zijlstra *et al.*, 2001; Schallig *et al.*, 2002; Chappuis *et al.*, 2003; Veeken *et al.*, 2003; Chappuis *et al.*, 2005; Diro *et al.*, 2007; Sundar *et al.*, 2007; Boelaert *et al.*, 2008) and in dogs (Mancianti *et al.*, 2002; Reithinger *et al.*, 2002; da Costa *et al.*, 2003; Mohebali *et al.*, 2004b; Otranto *et al.*, 2004; Toz *et al.*, 2004; Mettler *et al.*, 2005; Otranto *et al.*, 2005; Lemos *et al.*, 2008). Although these studies have aimed to quantify rK39 dipstick sensitivity in naturally infected cases, tests have been performed in cross-sectional samples, generally from groups of clinically symptomatic or confirmed parasite-positive humans or dogs. Little information is available to characterize test performance in longitudinal samples from infected dogs, particularly to examine test sensitivity in the early stages of natural infection. Experimental longitudinal work is limited to one study which describes rK39 ELISA results during follow-up of just 4 artificially challenged dogs (Scalone *et al.*, 2002).

The association between clinical disease and rK39 dipstick test positivity, which has been suggested by results from a number of canine studies which stratified dogs by clinical status (Mettler *et al.*, 2005; Lemos *et al.*, 2008), also requires further investigation using longitudinal data to control for duration of infection.

To address these research needs, in the present study we describe the results of longitudinal follow-up of uninfected dogs exposed to natural infection in an endemic area (Brazil), tested by rK39 dipstick, crude *Leishmania* parasite antigen (CLA) ELISA, PCR, microscopic examination and *in vitro / in vivo* culture of bone marrow, and clinically examined, at bi-monthly intervals. Data were analysed to compare the sensitivity of rK39 dipsticks against CLA ELISA, and to look for associations between rK39 dipstick test positivity, clinical status, and PCR positivity.

6.2 Methods

6.2.1 Selection of animals

Clinical samples of a cohort of 54 confirmed positive dogs were selected from archived material from a larger Brazilian longitudinal population study described in Chapter 2 and previously (Quinnell *et al.*, 1997; Quinnell *et al.*, 2001a; Quinnell *et al.*, 2001b; Courtenay *et al.*, 2002b; Quinnell *et al.*, 2003a), and aligned relative to the longitudinally assigned time of patent infection t0 (defined as the first time point of detection of *Leishmania* infection by bone marrow PCR and/or *in vitro/in vivo* culture, as previously defined in Chapter 4), as indicated by the results of the original cohort study. Dogs in that study were also clinically examined and scored as described in Chapter 2: a total clinical score of 0-2 was classed as asymptomatic, 3-6 as oligosymptomatic, and ≥ 7 as polysymptomatic.We purposively selected the sample series which had the longest periods of follow up, thereby obtaining samples from 39 dogs which had clinical symptoms of ZVL at ≥ 1 time point during the course of follow-up, and from 15 dogs that remained asymptomatic at all time points, despite parasitological evidence of infection.

A total of 323 samples were thus tested. PCR results were not available for all time points. 53/54 dogs had PCR results on at least one follow-up time point (mean 3.7; range 1-10). In total, PCR results were available from 196/323 samples.

rK39 dipsticks were also tested in 30 unexposed, non-endemic UK dogs with no history of foreign travel that had attended two UK veterinary clinics during June – December 2007 for unrelated clinical reasons.

6.2.2 Serology

CLA ELISA was carried out as described in the previous study, to obtain total anti-CLA IgG titres as described in Chapter 2. (Quinnell *et al.*, 1997).

In the current study, rK39 dipstick tests (Kalazar Detect Canine Rapid Test: Lot No. HA1047 and HD1037) were obtained from Inbios International Inc. WA, USA, and used according to the manufacturer's instructions as described in Chapter 2. rK39 ELISA results were also available for a proportion of samples (181/323) from the work described in Chapter 4, above.

6.3 Results

6.3.1 Cross-sectional analysis

A total of 133/323 serum samples from 38/54 Brazilian dogs tested rK39 dipstick positive, while 16/54 dogs remained rK39 negative throughout the course of sampling. CLA ELISA gave positive results in 246/323 samples from 54/54 dogs. In samples with confirmed infections by parasitological tests (i.e. samples taken on or after t0), the cross-sectional sensitivity for detection of infection by CLA ELISA was 88% (185/210) whereas rK39 dipsticks were significantly less sensitive (56%; 117/210), as shown in Table 7 (χ^2=54.5; $P \leq 0.001$). The proportion of samples positive by rK39 dipsticks was highly correlated with $\log_{10}$ anti-CLA IgG titre as shown in Figure 10 (Logistic regression; χ^2=252; p<0.001). Results were available from rK39 ELISA (see Chapter 4) for 181/323 of the samples which were tested with rK39 dipsticks. As shown in Figure 11, rK39 ELISA s/p ratios were high (≥ 0.770), in all samples which tested positive by rK39 dipsticks (60/181), well above the cut-off point calculated from negative UK control dogs (cut-off=0.259; see Chapter 4). Excluding pre-t0 samples from the subset of samples tested by rK39 dipsticks and rK39 ELISA, the sensitivity of rK39 dipsticks was 46.2% (55/119), lower than the sensitivity of rK39 ELISA (66.3%: 79/119; χ^2=51.6; p$\leq$0.001).

As shown in Table 7, analysis of crude cross-sectional data from 210 post-infection serum samples showed a strong association between rK39 dipstick positivity and

clinical disease: only 37% (44/120) of serum samples from asymptomatic dogs (clinical score<2) were rK39 dipstick positive, compared with 79% (38/48) samples from oligosymptomatic animals (score 3-6) and 94% (34/36) samples from polysymptomatic (score >6) animals (χ^2=50.4; $P\leq0.001$: χ^2 for trend). Clinical examination results from 6 sample time points were unavailable. The sensitivity of CLA ELISA was significantly superior to rK39 dipsticks in asymptomatic and oligosymptomatic dogs (though the difference was less striking in the latter), whereas in polysymptomatic dogs the sensitivity of the two tests was similar. Both CLA ELISA and rK39 dipsticks were more sensitive in PCR positive dogs (89% and 61%, respectively) compared with PCR negative animals (79% and 32%, respectively), though CLA ELISA was the more sensitive test in both PCR groups.

All 30 UK non-endemic dogs were negative by rK39 dipsticks (specificity =100%), though specificity was not tested here in dogs with non-*Leishmania* parasitic infections.

6.3.2 Longitudinal analysis

In longitudinal follow-up, rK39 dipstick and CLA ELISA sensitivity increased with time from patent infection as shown in Figure 12. Sensitivities of both CLA ELISA and rK39 dipstick were low at 4-6 months before the time of parasitological detection of infection t0, though CLA ELISA (42%) was superior to rK39 dipstick (4%). CLA ELISA was significantly more sensitive than the rK39 dipstick test at all subsequent time points tested up to the end of the study (>16 months after t0), although larger differences in sensitivity between the two tests were seen in the early stages of infection from t-6 to t+4 months ($\chi^2\geq9.0$; $p\leq0.003$) than were seen after 6 months from t0 ($\chi^2\geq4.6$; $p\leq0.03$). Maximum rK39 sensitivity was 76.3% (at t+6-8 months) in comparison with maximum CLA sensitivity of 100% (>16 months post-t0).

In longitudinal follow up, 15 dogs remained asymptomatic throughout the study, whereas 39 developed clinical signs. In clinical cases, the proportion of dogs with signs of ZVL increased with time from t0 and declined somewhat after t+10 months, a trend that was temporally associated with the proportion of these dogs which were positive by rK39 dipsticks (Figure 13). Among animals which remained asymptomatic throughout the period of follow-up, the proportion of dipstick positive dogs at each time point was consistently low, though an isolated peak prevalence of 50% rK39 dipstick positivity was observed at t+10 months (Figure 13). Controlling

for within-dog autocorrelation and duration of exposure to infection in the field, rK39 dipstick positivity was significantly associated with symptomatic infection (O.R.= 5.3; 95% C.I.= 2.17-12.63; z= 3.69; $P \leq 0.001$; n=47 dogs; 167 observations), and with PCR positivity (O.R.= 3.9; 95% C.I.= 1.73-8.84; z= 3.27; $P=0.001$). In dogs which tested positive by rK39 at ≥ 1 time point, 34/38 remained positive at all points after rK39 seroconversion, whereas in 4 dogs, a single rK39 negative time point occurred during the period of post rK39 seroconversion follow-up (mean 353 days; range 137-472 days), showing the low frequency of reversion to rK39 seronegative.

Table 7. Cross-sectional analysis of rK39 dipstick and CLA ELISA test results

P-values shown are the results of χ^2 tests comparing the raw proportions of positive serum samples in each PCR/ clinical examination category by CLA ELISA and rK39 dipstick tests. Except where indicated, pre-t0 samples were excluded from analysis.

	CLA ELISA +	rK39 dipstick +	*P*-value
Confirmed infections (post-t0)	88.1% (185/210)	55.7% (117/210)	≤0.001
Pre-t0 samples	54.0% (61/113)	14.2% (16/113)	≤0.001
PCR +	89.2% (99/111)	61.2% (68/111)	≤0.001
PCR -	79.4% (27/34)	32.4% (11/34)	≤0.001
Polysymptomatic	97.2% (35/36)	94.4% (34/36)	0.56
Oligosymptomatic	95.8% (46/48)	79.2% (38/48)	0.01
Asymptomatic	84.2% (101/120)	36.7% (44/120)	≤0.001
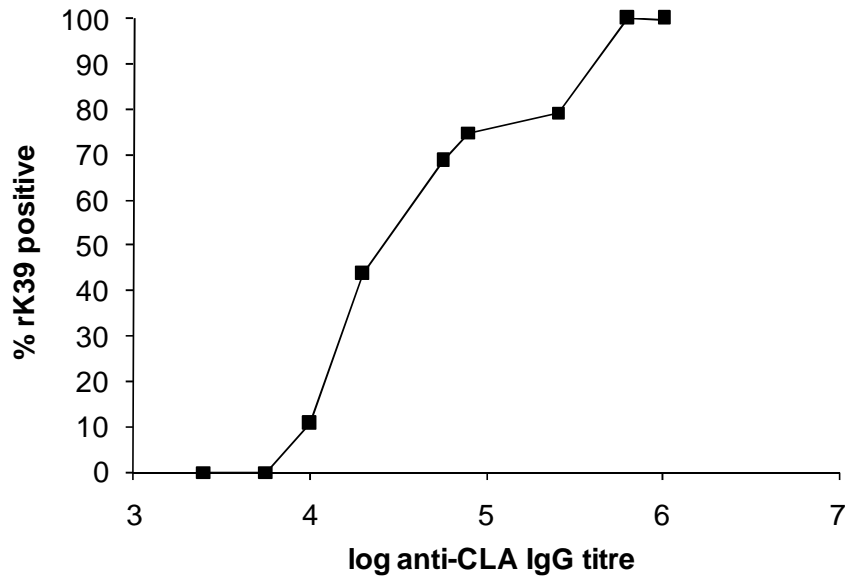
Figure 10. rK39 dipstick positive serum samples (%) by $\log_{10}$ anti-CLA IgG titre. CLA ELISA titre is expressed in $\log_{10}$ arbitrary units (relative to a positive control symptomatic Brazil dog) calculated as described in Chapter 2.
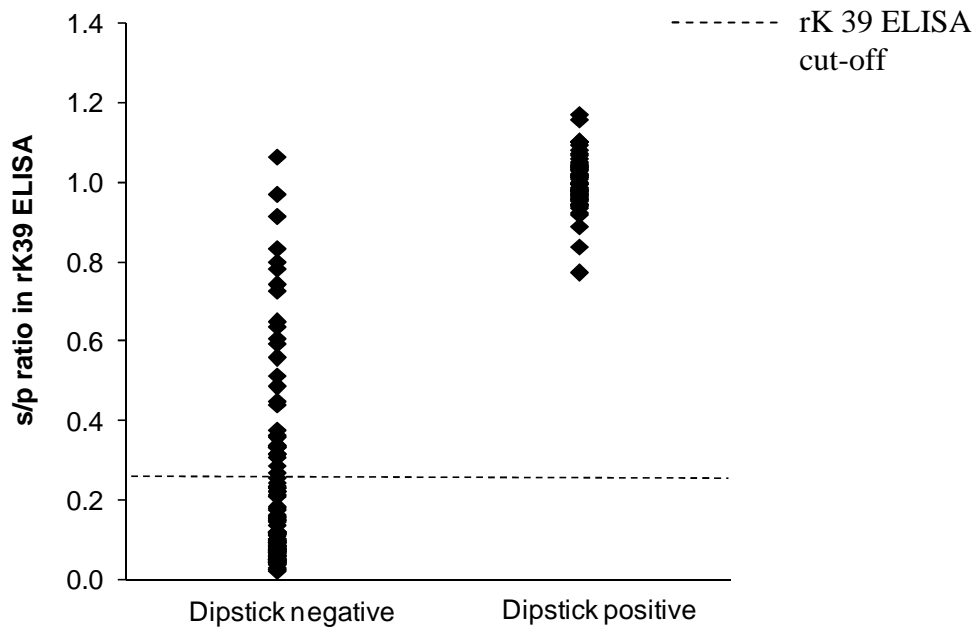
Figure 11. Comparison between rK39 dipstick and rK39 ELISA results. rK39 ELISA s/p ratios (see Chapter 4) were available from 181/323 dogs which were tested with rK39 dipsticks. rK39 ELISA cut-off was calculated from the back-transformed mean +3S.D. of the $\log_{10}$ s/p ratios of 30 UK negative dogs, as described in Chapter 4.
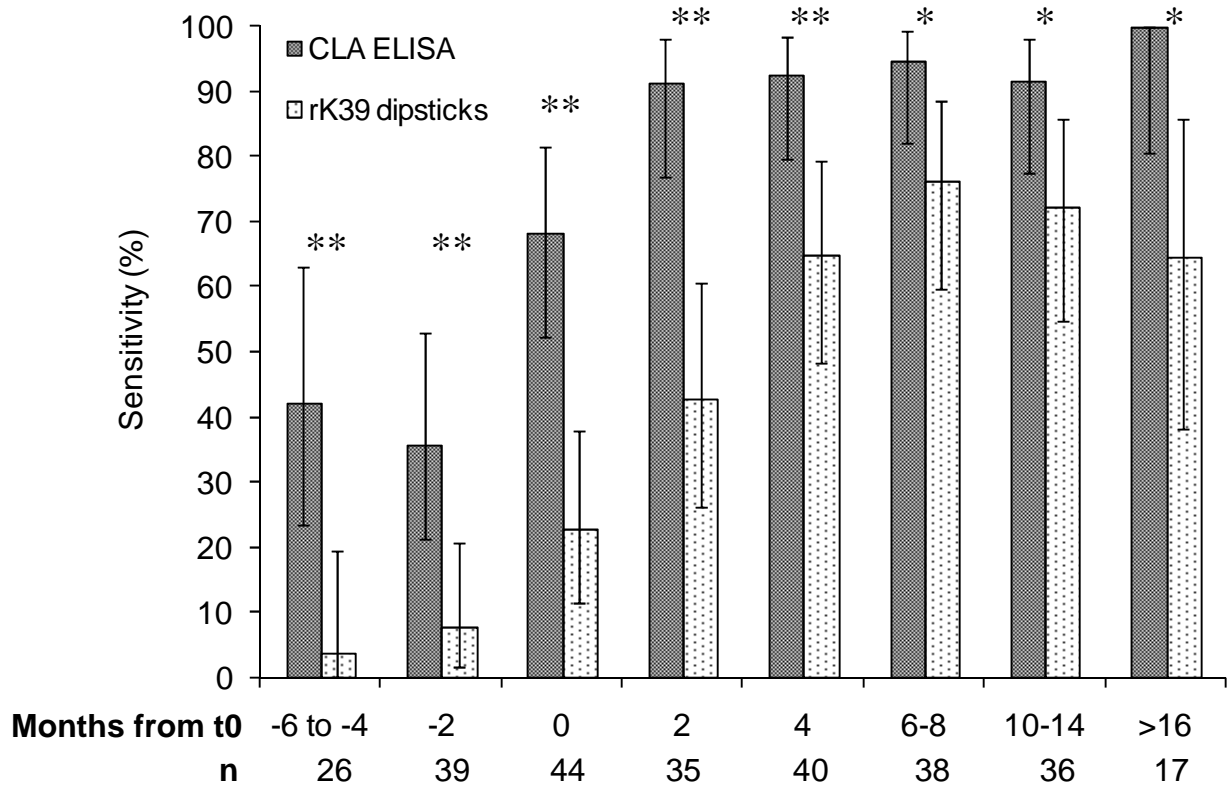
Figure 12. Test sensitivity (binomial 95% C.I.) of CLA ELISA and rK39 dipsticks. Dogs (n=275) were aligned by time from first detection of infection by *in vitro/in vivo* culture and/or PCR (t0). Binning by time points was used as indicated to equalize group sizes. N.B. 40 samples from uninfected animals (i.e. without parasitologically confirmed time of infection t0) were excluded, as were samples taken more than 6 months pre-t0 (n=8). Asterisks indicate significant differences between the sensitivity of CLA ELISA and rK39 dipsticks.

n: no. of dogs tested at each time point.

** $P \leq 0.01$; * $P \leq 0.05$ (χ^2 test).
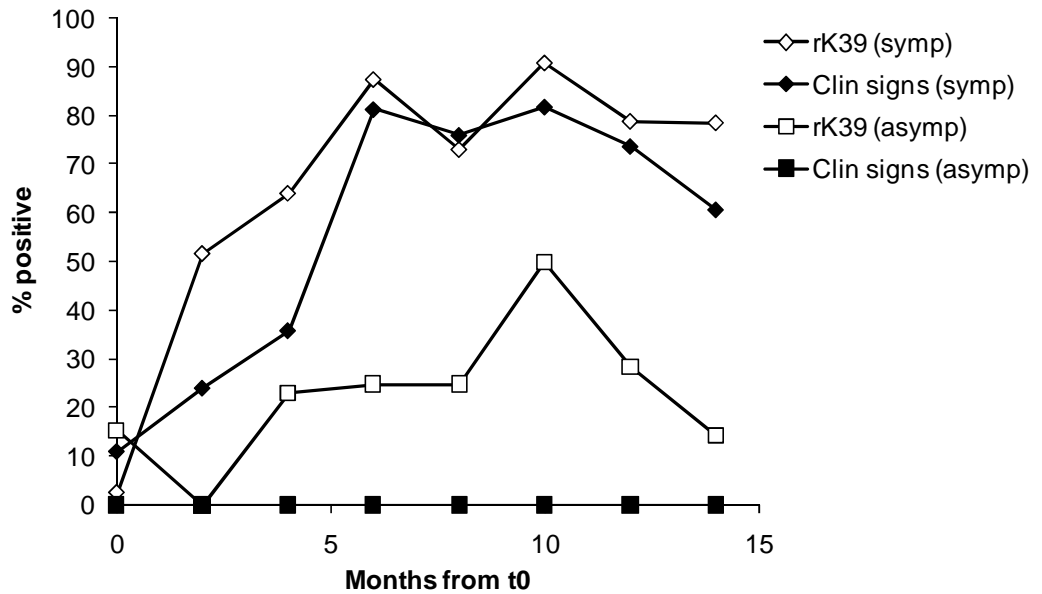
Figure 13. Temporal relationship between rK39 dipstick positivity and clinical disease.
Open shapes show the proportion of samples positive by rK39 dipsticks, filled shapes show the proportion of dogs with clinical signs of leishmaniasis (clinical score >2).

symp: dogs which developed clinical signs of disease (clinical score >2) at any time point (n=39).
asymp: Dogs which never showed clinical signs of ZVL throughout the entire period of follow-up (clinical score ≤2) (n=15).

6.4 Discussion

Published figures for rK39 dipstick sensitivity in dogs vary from 71% to 96%, differences which were partly due to differing selection criteria for 'true' positive dogs for calculation of test sensitivity in these previous studies. For example, Reithinger *et al.* estimated rK39 dipstick sensitivity as 72% using crude parasite ELISA and/or buffy coat PCR to define positives (Reithinger *et al.*, 2002), whereas in dogs with microscopically parasite-positive lymph node smears (a 'gold' standard of comparatively low sensitivity), rK39 dipstick sensitivity was 97% (Otranto *et al.*, 2005). However, in two previous cross-sectional studies which used similar selection criteria for 'true' positive dogs (clinical signs of ZVL and serology) widely differing

estimates of sensitivity were again obtained: rK39 dipsticks were 71% sensitive in a study of dogs in Iran (Mohebali *et al.*, 2004b), whereas in dogs sampled in Brazil, rK39 dipsticks were 94% sensitive (da Costa *et al.*, 2003). It is likely that variations in rK39 dipstick sensitivity also reflect differences in infection duration (which cannot be quantified in cross-sectional studies). In human VL patients, reports of varying rK39 dipstick sensitivity (71-100% (Jelinek *et al.*, 1999; Brandonisio *et al.*, 2002; Guimaraes Carvalho *et al.*, 2003; Sundar *et al.*, 2006) have also resulted from analyses of cross-sectional samples of patients with infections of unknown duration. The present study is the first to report longitudinal analysis of rK39 dipstick results in naturally infected dogs for which the time course of infection was well characterized. As described above, rK39 dipstick sensitivity was low in the early stages of infection (<10% sensitivity in samples taken before t0), and increased with time from t0 to a maximum of 76% 6-8 months post-t0. This provides a partial explanation for the large differences in sensitivity estimates obtained from previous cross-sectional surveys. Another possible cause of inconsistent results in previous studies is variation in rK39 dipstick sensitivity in geographically distinct strains of *Leishmania*: such variations in dipstick sensitivity for diagnosis of human VL have been recorded between East Africa and the Indian subcontinent (Boelaert *et al.*, 2008), and further investigation is needed to determine if similar diagnostically relevant differences exist between Old and New world *L. infantum* strains in dogs. Comparison between studies is further complicated by the use of rK39 dipsticks from several different manufacturers in the previously reported work. Unfortunately in the present study, it was not possible to source rK39 dipsticks from more than one manufacturer to allow direct comparisons of sensitivity to be made.

In the present study, CLA ELISA was significantly more sensitive than the rK39 dipstick test in *Leishmania*-infected dogs at all time points, though differences between the two tests were less marked at ≥ 6 months from t0, due to the relative improvement of rK39 sensitivity over time. Similarly high estimates of crude parasite ELISA sensitivity in previous canine research (do Rosario *et al.*, 2005; Mettler *et al.*, 2005; Ferreira *et al.*, 2007) (range 95%-100%) tend to confirm the results shown here. Sensitivity of the rK39 ELISA described in Chapter 4 was also superior to that of the rK39 dipstick, which contrasts with a previous cross-sectional study which found no significant difference in the sensitivity of rK39 ELISA when compared to Diamed-Vet-IT Leish assay (Mettler *et al.*, 2005), though the number of dogs tested in that

study was comparatively low. As shown in Chapter 4, rK39 ELISA was itself inferior to CLA ELISA. Thus, CLA ELISA is still the most appropriate test for accurate detection of early stage infection and hence calculation of infection incidence in dogs enrolled in vaccine trials, subject to the considerations discussed in Chapter 4.

Previous studies employing the rK39 antigen have suggested an association between rK39 positive test results and presence of active clinical disease in both humans (Badaro *et al.*, 1996) and in dogs (Rhalem *et al.*, 1999; do Rosario *et al.*, 2005; Mettler *et al.*, 2005; Porrozzi *et al.*, 2007; Lemos *et al.*, 2008) and confirmed by our results reported here. In longitudinal analysis, the increase in the proportion of symptomatic dogs after t0 was temporally associated with the proportion positive by rK39 dipsticks, which was followed by a decline in both proportions from t+10 months, due to recovery or death of diseased animals. The low frequency of reversion to rK39 negative in animals with ≥1 positive rK39 dipstick result suggests that the latter explanation is more likely. rK39 dipstick sensitivity increased in association with the severity of clinical signs to 94% sensitivity in polysymptomatic dogs, and dipstick sensitivity was also highly associated with anti-CLA IgG titre, which is an important prognostic indicator of disease severity in dogs (Reis *et al.*, 2006b). rK39 dipstick test positivity was restricted to those samples with high rK39 ELISA s/p ratios, which is of interest in view of evidence from human VL cases that anti-rK39 IgG is a prognostic indicator for relapse after therapy (Kumar *et al.*, 2001). Thus, in a veterinary clinical setting, rK39 dipsticks are likely to be highly sensitive for detection of clinically symptomatic cases of canine leishmaniasis, which present a diagnostic challenge for veterinarians due to the non-specific nature of canine clinical signs of ZVL, and may also be useful for identifying relapses. The dipstick test format allows rapid and straightforward testing of animals in the field, which will be useful for busy veterinarians in practice, and provides an immediate result without the delays associated with laboratory testing of samples. Where dog culling is the primary control measure, delays in sample processing have been identified as one reason for failure of this strategy to reduce disease incidence (Courtenay *et al.*, 2002b), and the use of dipstick tests could help to improve this situation. There may also be significant improvements in owner compliance when presented with an unequivocal, visually interpretable result at the time of testing. The rK39 antigen also has the potential advantage of lower cross-reactivity with other pathogens likely to be co-endemic in areas affected by ZVL. The specificity of the Inbios rK39 dipsticks tested

here was 100% in 30 UK negative dogs. However, false positive rK39 dipstick results have previously been detected in small numbers of dogs with *Ehrlichia canis* or *Trypanasoma cruzi* infection (Lemos *et al.*, 2008), and in negative control dogs from areas that are not endemic for *Leishmania* or either of these diseases (Reithinger *et al.*, 2002). In other work, the specificity of anti-rK39 antibodies for infection with the visceralizing *L. donovani* complex (Burns Jr *et al.*, 1993) has been questioned due to cross-reactions in rK39 ELISA with sera from 3/9 dogs infected with cutaneous leishmaniasis (*L. braziliensis* (Porrozzi *et al.*, 2007)). More extensive characterization of rK39 antigen specificity is therefore required, before recommendations can be made for the use of these tests in areas where co-infection with the above pathogens is likely. In conclusion, we have shown that rK39 dipstick sensitivity was inferior to that of CLA and rK39 ELISA in naturally infected dogs in longitudinal follow-up, and that rK39 dipstick positivity is highly associated with clinical disease, making this test more appropriate for clinical diagnosis than for use in vaccine trials against canine ZVL.

Chapter 7: Comparison of *Leishmania* OligoC-TesT PCR with conventional and real-time PCR for the detection of canine *Leishmania infantum* infection

7.1 Introduction

For accurate measurement of infection incidence in vaccine trials against canine leishmaniasis, the ideal diagnostic test should show high sensitivity in early stage infections, and should also be cost-effective, technically simple and robust. Serological methods are the most technically straightforward of the available tests for diagnosis of canine ZVL infection, but these methods have been reported to lack sensitivity in asymptomatic and early stage infection (Dye *et al.*, 1993; Quinnell *et al.*, 1997; Miró *et al.*, 2008). Detection of *Leishmania* parasites in canine clinical samples has traditionally been performed by means of microscopic examination of stained tissue specimens, or by parasitological culture, which are known to be insensitive. Polymerase chain reaction (PCR) for amplification of defined parasite DNA sequences is highly sensitive in animals with clinical disease, and has higher sensitivity than serology in asymptomatic animals and early-stage infections, as reported in Chapters 4&6, and in previous research (Quinnell *et al.*, 2001a; Solano-Gallego *et al.*, 2001; Iniesta *et al.*, 2002; Lachaud *et al.*, 2002a; Oliva *et al.*, 2006). However, the technical complexity of PCR and the requirement for trained laboratory personnel may reduce its practicality for use in developing countries most affected by ZVL. Furthermore, there is a lack of standardization in the selection of target *Leishmania* DNA sequences and experimental PCR protocols used in laboratories worldwide, which complicates objective comparisons of test sensitivity and specificity. In order to address some of these issues, a commercially available PCR test kit (*Leishmania* OligoC-TesT) has been developed and validated for detection of *Leishmania* parasite DNA in human specimens (Deborggraeve *et al.*, 2008; Espinosa *et al.*, 2009). Sensitivity of the test ranged from 77.8%-100% in clinical samples from patients with visceral leishmaniasis in Sudan and Kenya, with a limit of detection of 1 parasite in 180µl blood (Deborggraeve *et al.*, 2008). The OligoC-TesT has not yet been independently validated for use in dogs. The aims of this study were therefore: (1) to measure the sensitivity of the OligoC-TesT compared with three conventional PCR procedures (nested PCR for amplification of kinetoplast DNA (kDNA), nested PCR of internal transcribed spacer region-1 (ITS-1) of the ribosomal RNA (rRNA)

gene, and kDNA/rRNA PCR followed by hybridization with specific oligonucleotide probes), and compared with real-time quantitative PCR (qPCR), (2) to compare the sensitivity of the OligoC-TesT, and PCR methods listed above, in samples from infected dogs in the presence or absence of clinical signs of leishmaniasis, and (3) to determine the analytical sensitivity of the OligoC-TesT relative to canine bone marrow parasite burdens measured by real-time qPCR. Here we use samples collected in a previous longitudinal study on a cohort of *L. infantum* naturally infected domestic dogs in Brazil.

7.2 Methods

7.2.1 Sampling and selection of dogs

Bone marrow samples from naturally exposed outbred dogs were tested. These samples came from a previous longitudinal field study in Brazil, described in Chapter 2 and previously (Quinnell *et al.*, 1997). Bone marrow samples (for PCR followed by hybridization with specific oligonucleotide probes, and *in vitro/in vivo* parasitological culture, as described in Chapter 2) were obtained at approximately 2 month intervals. Dogs were clinically examined at each time point and assigned a semi-quantitative clinical score: dogs with total scores 0-2 were considered asymptomatic, scores 3-6 were defined as oligosymptomatic, and scores 7-18 were defined as polysymptomatic (Quinnell *et al.*, 2001a). In the current study we used archived bone marrow samples from dogs which showed evidence of infection at any time after initial exposure, where infection was defined as positivity by serology (crude parasite ELISA) and/or *in vitro/in vivo* culture. Samples were aligned by time point of first detection of infection (t0). The criteria for defining t0 in the current chapter (serology and/or parasitological culture) were different from those used in Chapters 4&6 (PCR and/or parasitological culture), which was necessary to ensure independence of the defining criteria for infection from the experimental methods on test. Thus, the 'gold standard' for defining infection was, in each case, an amalgam of all other available independent methods. The number of OligoC-TesT kits available allowed testing of 163 samples (from 67 infected dogs), with a mean 2.4 time points per dog (range 1-5 time points), from 2 months before first detection of infection (t-2) to 12 months post infection (t+12). 147 samples were from dogs with confirmed infection (i.e. samples taken on or after t0) and 15 samples were taken 2 months before confirmed infection,

when dogs are likely to be in the pre-patent period (Quinnell *et al.*, 1997). 1 sample was not included in the subsequent data analysis due to PCR inhibition (see below). Clinical scores were unavailable for 2.5% (4/163) of the samples.

7.2.2 DNA extraction

In the present study, DNA extraction was performed on 100μl aliquots of bone marrow, using a phenol-chloroform protocol as described in Chapter 2. The median $Abs_{260/280}$ of DNA extracts was 1.81 (interquartile range=1.74-1.85), indicating high purity of the extracted DNA.

7.2.3 Nested ITS-1 and kDNA PCR

In the present study, DNA extracts were subjected to nested PCR targeting the ITS-1 region of the rRNA gene of all *Leishmania* species, as described in Chapter 2 (Parvizi *et al.*, 2005; Parvizi *et al.*, 2008). A nested PCR was used to amplify a variable region of *Leishmania* kDNA, as described in Chapter 2. The latter PCR protocol was not species-specific for *L. infantum*, however species can be discriminated based on product size (Noyes *et al.*, 1998). Positive and negative controls were as described previously.

7.2.4 Real-time quantitative PCR

LEISH-1 and LEISH-2 primers and the fluorophore-labelled Leishprobe were used in a previously validated protocol specific for a conserved region of *L. infantum* kDNA, as described in Chapter 2 and previously (Francino *et al.*, 2006). All bone marrow DNA samples were amplified in duplicate at 1:10 dilution in water (appropriate dilution factors were identified from serial 10-fold dilutions in preliminary optimization experiments). Quantification of *Leishmania* DNA was performed using an absolute method, by comparison of Ct values with those from a standard curve constructed from 10-fold dilutions of *L. infantum* DNA extracted from cultured parasites, from 1×10^6 to 0.1 parasite equivalents/ml (strain MHOM/MA/67/ITMAP-263), which were run in triplicate on every plate. Parasite burdens were $\log_{10}$ (n+1) transformed for analysis.

Pre-developed TaqMan Assay reagents (Applied Biosystems, UK) were used to amplify the 18S rRNA gene as an internal reference of canine genomic DNA, to detect PCR inhibition. Negative controls were DNA extracted from blood samples of

10 UK dogs with no history of foreign travel. The inter-assay coefficient of variability (CV) of the *Leishmania* real-time qPCR, estimated by running a reference sample in duplicate on every plate, was 1.90%. The mean intra-assay CV of reference sample duplicates was 0.29% (range 0.12%-0.52%).

7.2.5 OligoC-TesT kit

Kits were provided by the manufacturer (Coris Bioconcept, Gembloux, Belgium) and were used according to product recommendations, for genus-specific detection of *Leishmania* DNA, as described in detail in Chapter 2, and previously (Deborggraeve *et al.*, 2008). Briefly, pre-formulated master mix was used to amplify sample DNA in a single round conventional PCR protocol. This step was followed by detection of specific PCR product using double-sided dipsticks which replace the requirement for gel electrophoresis. An internal positive control sequence co-amplifies with the target to allow detection of PCR inhibition, while migration control lines indicate correct running of the test strip buffer. A single inconclusive OligoC-TesT result (1/163) due to PCR inhibition was excluded from further data analysis.

7.3 Results

7.3.1 Overall test sensitivity in infected dogs

In bone marrow samples taken from dogs on or after the time of first detection of infection by serology and/or *in vitro/in vivo* culture (t0), the overall sensitivity of the OligoC-TesT was 70.3% (102/145) (Table 8). The proportion of positive samples by the OligoC-TesT was significantly greater than the proportion detected by the PCR/hybridization method (60.7% (88/145); McNemar's χ^2=8.17; P=0.007) and by the ITS-1 nested PCR (53.8% (78/145); McNemar's χ^2=22.2; P<0.001), but was not significantly different to the proportion positive by kDNA nested PCR (72.4% (105/145); McNemar's χ^2=0.36, P=0.69). The most sensitive method was real-time qPCR, which detected *L. infantum* DNA in 91.0% (132/145) of the samples tested, significantly more than any of the other procedures (McNemar's $\chi^2 \geq$25.14, P<0.001 in all pairwise comparisons).

7.3.2 Sensitivity in symptomatic and asymptomatic dogs

The proportions of positive test results in bone marrow samples from dogs which were stratified into 3 clinical groups (oligo- poly- and asymptomatic, as defined above) are shown in Table 8. Comparing symptomatic (oligo- and polysymptomatic groups combined) with asymptomatic dogs, test sensitivity was significantly higher in bone marrow samples from the former group, using OligoC-TesT, kDNA PCR, ITS-1 PCR and PCR/hybridization ($\chi^2 \geq 3.91$; $P \leq 0.05$), but was not significantly different between clinical groups for real-time qPCR ($\chi^2 = 2.41$, $P = 0.12$) (Table 8). Controlling for autocorrelation, there was a significant positive association between the severity of clinical signs of leishmaniasis in infected dogs (total clinical score, range 0-18) and the probability of testing positive, using any of the diagnostic PCR methods including real-time qPCR ($z \geq 2.66$, $P \leq 0.008$). Similarly, total clinical score in infected dogs was positively correlated with log-transformed *Leishmania* parasite burdens measured in bone marrow samples by real-time qPCR ($z = 4.33$, $P < 0.001$).

	Test	Polysymp (n=29)	Oligosymp (n=34)	Asymp (n=82)	Overall (n=145)		
% positive samples (95% C.I.)	Real-time qPCR	100 *(88.1-100)*	91.2 *(76.3-98.1)*	87.8 *(78.7-94.0)*	91.0 *(85.2-95.1)*	+	
	kDNA PCR	96.6 *(82.2-99.9)*	76.5 *(58.8-89.3)*	62.2 *(50.1-72.7)*	72.4 *(64.4-79.5)*	=	**
	OligoC-TesT	93.1 *(77.2-99.2)*	73.5 *(55.6-87.1)*	61.0 *(49.6-71.6)*	70.3 *(62.2-77.6)*		**
	PCR/hybridization	82.8 *(64.2-94.2)*	58.8 *(40.7-75.4)*	53.7 *(42.3-64.7)*	60.7 *(52.2-68.7)*	-	*
	ITS-1 PCR	89.7 *(72.6-97.8)*	52.9 *(35.1-70.2)*	41.5 *(30.7-52.9)*	53.8 *(45.3-62.1)*	-	**

Table 8. Percentage of canine bone marrow samples positive by conventional and nested PCRs, OligoC-TesT and real-time qPCR.
95% binomial C.I. are indicated in italics. Samples were taken from dogs with *L. infantum* infection confirmed by ELISA and/or *in vitro/in vivo* culture. The clinical status of dogs was defined as asymptomatic (asymp: total clinical score (ts) ≤2), oligosymptomatic (oligosymp: ts=3-6), or polysymptomatic (polysymp: ts=7-18). Samples taken from dogs with no clinical score data were excluded.
PCR tests which showed higher sensitivity in samples from dogs with clinical signs of ZVL (oligo- and polysymptomatic groups combined) compared with asymptomatic dogs are indicated by asterisks (χ^2 test: *P≤0.05; **P≤0.01).
Results of pairwise comparison of the overall sensitivity of each test with the OligoC-TesT are shown (McNemar's χ^2 test: +, more sensitive than OligoC-TesT; -, less sensitive than OligoC-TesT; =, no significant difference between tests).

7.3.3 Test agreement

Pairwise agreement between different PCR tests in individual samples was only moderate between OligoC-TesT and both ITS-1 (k=0.57, S.E.=0.07, *P*<0.01) and PCR/hybridization (k=0.59, S.E.=0.08, *P*<0.01), reflecting the superior sensitivity of the OligoC-TesT. Agreement was weak between OligoC-TesT and real-time qPCR (k=0.38, S.E.=0.06, *P*<0.01), due to superior sensitivity of real-time qPCR. Despite similar sensitivity of the OligoC-TesT and kDNA PCR, their pairwise agreement was only moderate: 16 samples were kDNA positive/ OligoC-TesT negative whereas for 14 samples the reverse was true (k=0.56, S.E.=0.08, *P*<0.01). In 29/30 of these apparently discordant samples, parasite burden estimated by real-time qPCR was low (<100 parasites/ml; mean=19.5, 95% C.I.=13.5-25.5; one outlier excluded).

A single bone marrow sample that tested negative by real-time qPCR was positive by the OligoC-TesT, PCR/ hybridization and kDNA PCR. Specificity of the procedures on test was not measured in dogs from the endemic area, since all Brazilian dogs in the present study were selected on the criteria of infection at some time point. However, using a small number of non-endemic healthy UK control dogs (n=10) specificity was 100% for all tests. As expected, positive OligoC-TesT results were obtained when testing control DNA from a range of *Leishmania* species: *L. infantum* MHOM/FR/78/LEM75, *L. tropica* MHOM/SU/74/K27, *L. major* MHOM/SU/73/5-ASKH, and *L. donovani* MHOM/IN/80/DD8.

7.3.4 Analytical sensitivity

The detection limit of the OligoC-TesT, as stated by the manufacturer is 0.05 *Leishmania* parasites per PCR (Deborggraeve *et al.*, 2008). The analytical sensitivity of both kDNA nested PCR and ITS-1 PCR, estimated here from 10-fold serial dilutions of DNA from cultured *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263), was 0.2 parasites per PCR, though subjective signal strength (band intensity) in end point PCR was stronger in kDNA PCR. Analytical sensitivity for the previously performed PCR/hybridization reaction was not determined here. In our hands, the detection limit for real-time qPCR was 0.005 parasites per PCR, similar to the previously published estimate of 0.001 parasites per PCR (Francino *et al.*, 2006). When tested in clinical samples (Figure 14), the OligoC-TesT gave positive results in 100% of canine bone marrow DNA samples with estimated *Leishmania* burdens ≥74

parasites/ml (i.e. ≥ 0.4 parasites/PCR); its sensitivity to detect 10-100 parasites/ml (0.05-0.5 parasites/PCR) and <10 parasites/ml (<0.05parasites/PCR) was 50.0% and 21.1% respectively (Figure 15). kDNA PCR showed a slightly lower cut-off of 49 parasites/ml (0.1 parasites/PCR) in bone marrow samples, missing only one positive sample above this threshold (i.e. 98.9% sensitivity), and detecting 57.9% and 21.1% positive samples at 10-100 and <10 parasites/ml, respectively. PCR/hybridization and ITS-1 PCR were less sensitive at low parasite burdens in clinical samples, and did not reach 90% sensitivity until parasite concentration exceeded 1000 per ml sample.

7.3.5 Test sensitivity and time course of infection

Longitudinal results of the three conventional PCR tests, OligoC-TesT, and real-time qPCR on bone marrow samples from the time of detection of infection t0 are shown in Figure 16A. The most striking differences in test sensitivity occurred at t-2 months, when the proportions positive by ITS-1 and PCR/hybridization were both zero, whereas real-time qPCR, OligoC-TesT and kDNA PCR were positive in 73.3%, 53.3% and 46.7%, respectively. The proportion positive was highest in the first 6 months after confirmed infection, and decreased thereafter. Excluding samples at t-2 months, and controlling for clinical score, there was a significant negative correlation between the proportion of positive samples and increasing time from confirmed infection when tested by the OligoC-TesT (z=-2.71, P=0.007), kDNA PCR (z=-2.93, P=0.003), PCR/hybridization (z=-4.05, P<0.001 or real-time qPCR (z=-2.52, P=0.01), a trend which was borderline significant in the case of ITS-1 PCR (z=-1.70, P=0.09). This trend for lower sensitivity with increasing duration of infection, was associated with a similar negative correlation between bone marrow parasite burdens measured by real-time qPCR and increasing duration of infection, also controlling for clinical score (z=-4.48, P<0.001) (Figure 16B).
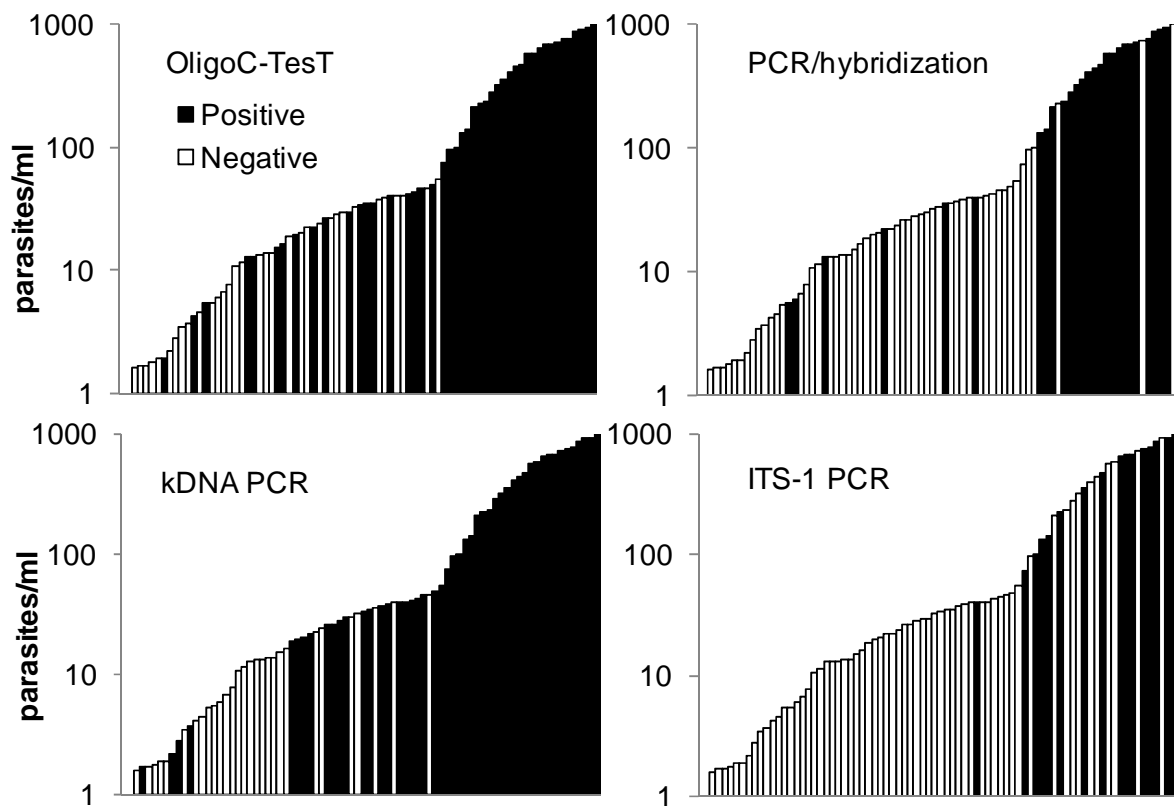
Figure 14. Detection threshold of OligoC-TesT and conventional nested PCRs.
The distribution of positive and negative results in individual bone marrow samples is shown according to parasite burden measured by real-time qPCR.
Samples are sorted in ascending order with respect to parasite burden, truncated at 1000 parasites/ml for clarity.
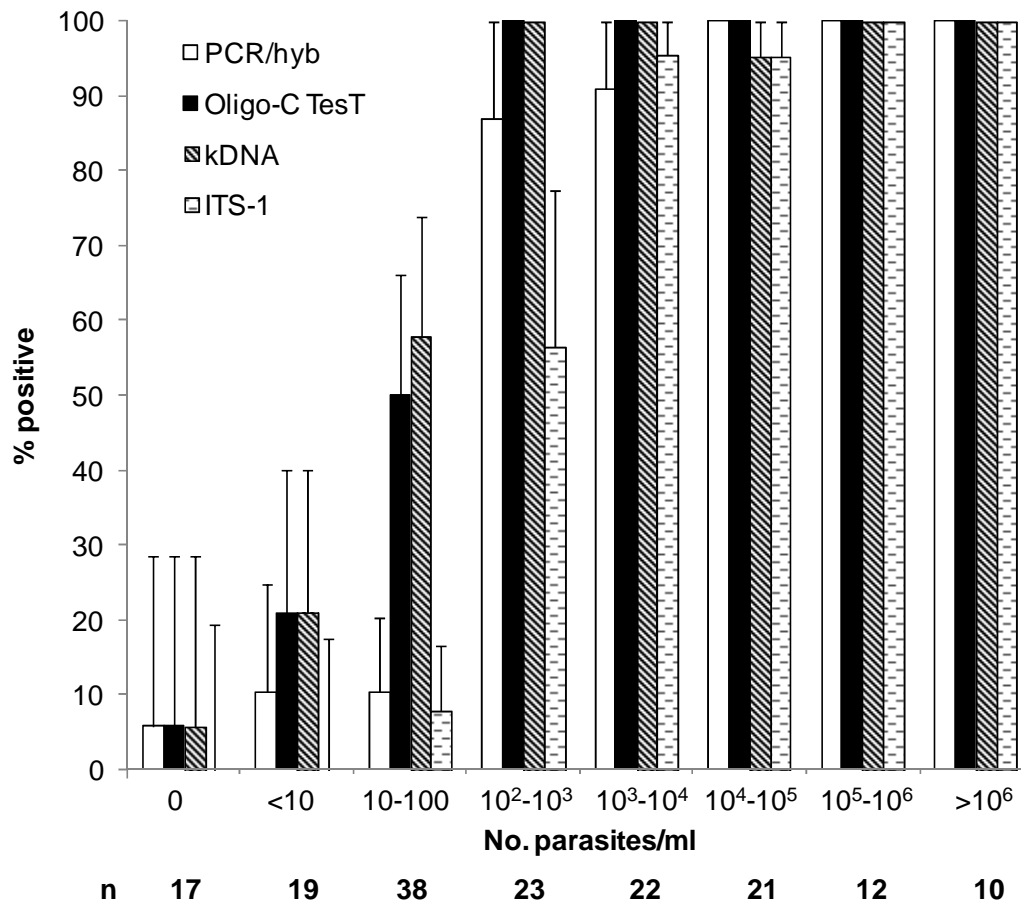
Figure 15. Sensitivity of OligoC-TesT and conventional PCR (95% upper binomial C.I.) in bone marrow samples across a 7- $\log_{10}$ range of parasite burdens. Samples are grouped by parasite burdens measured by real-time qPCR.

n: no. of samples
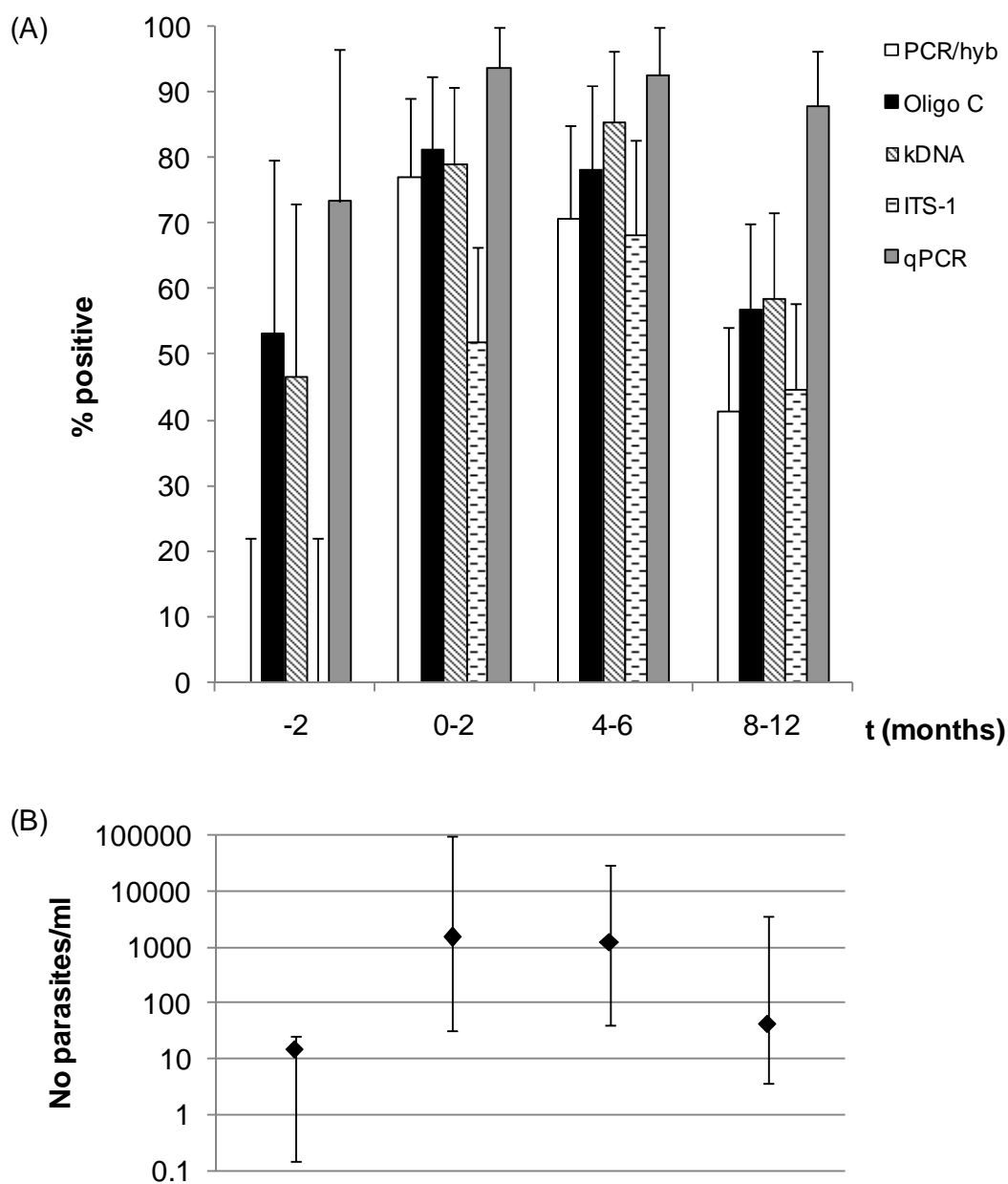
Figure 16. Sensitivity of OligoC-TesT, conventional PCR and real-time qPCR with varying time from infection.
(A) Percentage of positive canine bone marrow samples (95% upper binomial C.I.) by conventional and nested PCRs, OligoC-TesT and real-time qPCR, at time t after first detection of *L. infantum* infection by culture and/or serology (t0). (B) Median parasite burden (parasites/ml sample) measured by real-time qPCR at time t.
Error bars show interquartile range (25%-75%).

7.4 Discussion

This study shows that the overall sensitivity of the OligoC-TesT to detect *Leishmania* DNA in bone marrow aspirates from infected dogs (70%) is comparable to or greater than the sensitivity of the conventional PCR methods tested (range 54-72%). In polysymptomatic clinical cases of canine ZVL, the sensitivity of the OligoC-TesT was 93%, in comparison with 83-97% sensitivity of the conventional PCR methods. This is comparable to previously reported OligoC-TesT sensitivity in humans with symptomatic visceral leishmaniasis (78%-100%) (Deborggraeve *et al.*, 2008). The positive correlation reported here between parasite burdens in canine bone marrow samples and the severity of clinical signs of ZVL was consistent with our finding that the OligoC-TesT was significantly more sensitive in symptomatic compared to asymptomatic dogs. This was similar to the trend shown by the other conventional PCR tests, and corroborates previous work which demonstrated similar relationships (Rodriguez-Cortes *et al.*, 2007; Manna *et al.*, 2009). For the diagnosis of ZVL in suspected clinical cases by veterinarians, therefore, the OligoC-TesT is likely to be highly sensitive. The ease of use of the OligoC-TesT will be an advantage in both veterinary diagnostic and public health contexts. In particular, OligoC-TesT provides high sensitivity without the need for nested PCR protocols, which are prone to contamination problems, or hybridization steps, which increase the processing time and in some cases do not improve sensitivity (Lachaud *et al.*, 2002b).

OligoC-TesT and kDNA PCR, despite similar overall sensitivity, showed only moderate agreement indicating a degree of discordance. Importantly, almost all these instances (29/30) occurred in samples with low parasite burdens (<100 parasites/ml), which is likely to reflect lower analytical sensitivity at the detection limit of both these tests. The apparent lower sensitivity of the PCR/hybridization method could have resulted from a lower DNA extraction efficiency when carried out during the previous study (Quinnell *et al.*, 2001a), whereas all other tests here were performed on repeat DNA extractions from the same archived bone marrow samples. In addition, the PCR/hybridization method relied on agreement between rRNA and kDNA PCR, thus any samples which were positive in one test but not the other would have been classed as negative.

Despite the wide range of PCR protocols that have been used to detect *Leishmania* infection, relatively few studies have directly compared the sensitivity of different

protocols. More sensitive conventional methods than the ones used here have been described (Lachaud *et al.*, 2002a; Lachaud *et al.*, 2002b). Detection limits of 10^{-3} parasites/ml seeded canine blood have been reported using primers targeting kDNA sequences, though as previously discussed, theoretical analytical sensitivity does not always reflect diagnostic performance in clinical samples (Lachaud *et al.*, 2002b). For example, in other work comparing the sensitivity of conventional kDNA PCR with real-time qPCR in canine bone marrow, only samples with parasite loads in excess of 30 parasites/ml were positive by kDNA PCR (Francino *et al.*, 2006). In blood samples from human patients suffering from visceral leishmaniasis, nested PCR was 100% sensitive when parasitaemia exceeded 22 parasites/ml, though parasite burdens as low as 0.12 parasites/ml were detected sporadically (Mary *et al.*, 2004). The nested kDNA PCR tested here showed 99% sensitivity at parasite burdens above 49 parasites/ml (Figure 14), compared with the OligoC-TesT which reliably produced positive results when parasite density exceeded 74 parasites/ml sample. In contrast, sensitivity of nested ITS-1 PCR and PCR/hybridization remained low until bone marrow parasite burdens were 10-100 fold higher. The diagnostic advantage of the OligoC-TesT and kDNA PCR was most apparent in samples with low parasite burden, such as samples from asymptomatic dogs, and dogs in the early stages of infection (2 months before the first detection of infection by serology and/or parasitological culture). The most sensitive test was real-time qPCR which is likely to become the gold standard for parasite detection in population (prevalence) studies, although it is not necessarily the best method for veterinary clinical diagnostic use. As pointed out by others (Lachaud *et al.*, 2002b), the advantages of a highly sensitive test are offset by a reduced positive predictive value for clinically patent disease, which has been correlated with increased transmission of parasites from dogs to sand flies in previous studies (Courtenay *et al.*, 2002b). Conversely, the OligoC-TesT would be appropriate for detection of infection incidence in vaccine trials against ZVL, though the high cost of the test kit (approx. 6-9 GBP per sample (S. Deborggraeve, personal communication)) may make large scale use financially prohibitive until retail costs are reduced.

A negative relationship between the sensitivity of conventional PCR and the duration of infection has been reported in previously published results using the PCR/hybridization test, carried out in larger numbers of samples from these Brazilian dogs. That study showed a decline to c. 50% sensitivity after 300 days post-infection

(Quinnell *et al.*, 2001a). In the present study, we confirmed a similar trend for lower test sensitivity with increasing duration of infection, in three out of the four qualitative PCR methods tested. The fall in sensitivity with increasing time from confirmed infection was associated with lower bone marrow parasite burdens, and a consequent decrease in the number of samples with parasite levels above the detection limit for each test. Lower parasite burdens are likely to reflect both parasite clearance from more resistant animals and loss of highly parasite-positive susceptible dogs from the population through increased mortality rate (Quinnell *et al.*, 2001a). This trend shows interesting parallels with the results of rK39 dipstick serological tests reported in Chapter 6, which showed a similar decline in sensitivity in the later stages of infection.

Species of *Leishmania* other than *L. infantum*, such as *L. amazonensis* and *L. braziliensis* have previously been isolated from dogs (Delgado *et al.*, 1993; Llanos-Cuentas *et al.*, 1999; Reithinger and Davies, 2002; Tolezano *et al.*, 2007; Quaresma *et al.*, 2009), although the epidemiological significance of the dog as a reservoir host for these parasites is unclear. Mixed infections with *L. braziliensis* and *L. infantum* have also been documented (Madeira *et al.*, 2006). It is necessary to confirm that the comparative high sensitivity of OligoC-TesT and kDNA did not result from detection of species of *Leishmania* other than *L. infantum*, since both the OligoC-TesT and kDNA PCR show broad specificity for all *Leishmania spp*. However, product sizes in kDNA PCR were in all cases consistent with *L. infantum*, and enabled exclusion of *L. amazonensis* (but not *L. braziliensis*) (Noyes *et al.*, 1998), and previously reported identification of cultured parasites in 34 canine bone marrow samples from the current study population yielded only *L. infantum* (Quinnell *et al.*, 1997). Moreover, examination of health records from several decades of monitoring the human population in the study site does not provide any evidence to support autochthonous transmission of cutaneous leishmaniasis in this study area (Dr. Lourdes Garcez, Instituto Evandro Chagas, Brazil; personal communication).

In conclusion, we have validated the use of the OligoC-TesT for detection of *L. infantum* DNA in the reservoir host of ZVL, and have shown that the test has comparable or superior sensitivity to the conventional PCR methods tested. Due to the speed and ease of operation and interpretation, and standardized methodology, it is highly suitable for use in endemic areas for veterinary diagnostic purposes and for public health authorities. In regions where multiple species of *Leishmania* are

(potentially) circulating, direct sequencing of PCR product may be necessary to allow species identification (Deborggraeve *et al.*, 2008), however prototype *Leishmania* species-specific oligochromatographic kits are also in development.

Chapter 8: Comparison of quantitative and qualitative PCR as proxy measures for the evaluation of infectiousness and incrimination of potential reservoirs of leishmaniasis

8.1 Introduction

From a public health perspective, strategies targeted at blocking the transmission of vector-borne diseases between hosts require information on the infectiousness of the reservoir host. In the case of ZVL, this requires measurement of the infectiousness of domestic dogs to phlebotomine sand flies which transmit the disease. Unfortunately, to estimate infectiousness directly requires time consuming and labour-intensive xenodiagnosis experiments (Molina *et al.*, 1994; Courtenay *et al.*, 2002b) which involve feeding colony-raised sand flies on infected animals, a procedure which is not amenable to scaling up for use in large-scale survey work. No consensus currently exists as to the best choice of canine tissue sample or test procedure to use as a viable proxy measure of infectiousness, though it is sometimes assumed that infectiousness to biting sand flies correlates with parasite load in skin (Silva *et al.*, 2001). Indeed, previous investigations have suggested that PCR-negative canine skin, lymph node and blood samples are evidence of a successful transmission blocking vaccine (Nogueira *et al.*, 2005), however there is limited data to support such a claim; similarly, the use of parasitological methods (e.g. microscopy, immunohistochemistry, or PCR) as a proxy measure of infectiousness to sand flies has yet to be validated. The increasing accessibility of real-time quantitative PCR (qPCR) offers another potential tool to investigate levels of tissue parasitism. Although previous studies show that parasite burdens differ between simultaneously sampled tissues such as skin, bone marrow and peripheral blood (Manna *et al.*, 2006; Quaresma *et al.*, 2009), it is a reasonable hypothesis that parasitaemia or parasite DNA levels in skin correlate with increased likelihood of parasite uptake by biting sand flies. There is no published data testing this hypothesis by using real-time qPCR to correlate tissue parasite load with infectiousness by xenodiagnosis experiments, and comparing the predictive value of real-time versus conventional PCR for identification of infectious animals. A PCR based proxy measure of infectiousness would be highly desirable for use in large-scale intervention trials including those for up-and-coming experimental vaccines, in understanding the potential importance of

wildlife reservoirs in transmission, and in investigating the entomological mechanisms behind success or failure of interventions (Mazloumi Gavgani *et al.*, 2002; Jalouk *et al.*, 2007). Our first aim was to determine the predictive value of the presence and abundance of parasite DNA in canine tissues (detected by conventional nested kinetoplast (kDNA) and internal transcribed spacer-1 (ITS-1) PCR, and real-time qPCR) for infectiousness of dogs to *Lutzomyia longipalpis* sand flies. Canine skin and bone marrow samples were collected in longitudinal follow-up of naturally exposed dogs, stratified by their infectiousness to sand flies from the results of previous longitudinal xenodiagnosis experiments (Courtenay *et al.*, 2002b). Second, we aimed to characterize the relationship between tissue parasite burdens, infectious status and time since infection in naturally exposed dogs. Third, we aimed to compare parasite burdens in dogs with those of a sympatric population of crab-eating foxes (*Cerdocyon thous*: a putative wildlife reservoir), which showed similar contemporary prevalence of ZVL infection but that were negative in xenodiagnosis experiments (Courtenay *et al.*, 2002a). This study compares the potential for real-time and conventional PCR to contribute to measuring the impact of intervention trials against canine *Leishmania* on disease transmission, and to aid studies seeking to quantify the infectious role of suspected *Leishmania* reservoir hosts.

8.2 Methods

8.2.1 Canine samples

Samples were selected from naturally exposed outbred dogs, from a previously described large-scale longitudinal field study in Brazil (Quinnell *et al.*, 1997), in which bone marrows and biopsies of ear skin were sampled repeatedly at approximately 2 month intervals for up to 24 months post initial natural exposure. For the present study, 157 bone marrow samples were available from 41 dogs, while a subset of 31 dogs also had paired ear biopsy samples (n=102 samples). Xenodiagnosis results were available from matched time points, for 81 bone marrow samples and 51 ear biopsies. Dogs were also categorized as "ever-infectious" or "never-infectious" to sandflies, as defined below (Section 8.2.3), based on all xenodiagnosis experiments (1-12 per dog) conducted previously (Courtenay *et al.*, 2002b). Samples were aligned by the time of first detection of infection (t0), defined as positivity by serology (crude parasite ELISA) and/or parasitological culture of bone marrow *in vitro* or *in vivo* in hamsters, as described in Chapter 2 and previously

(Quinnell *et al.*, 1997). Blood samples from 10 UK dogs with no history of foreign travel provided negative DNA controls for PCR tests in the current study.

8.2.2 Fox samples

Samples were selected from a concurrent longitudinal study of free ranging crab-eating foxes (*Cerdocyon thous*), sympatric with the dog population detailed above, as described (Courtenay *et al.*, 2002a). We selected samples from 16 foxes, from which paired bone marrows and ear biopsies were available on at least one occasion (n=26 pairs of samples), on the criterion of seropositivity by crude parasite ELISA at the time of sampling. Anti-*Leishmania* IgG titres in the seropositive foxes selected were lower than in dogs described above (Wilcoxon rank sum test: z=3.11; *P*=0.002). Clinical signs of ZVL were detected in only one of these 16 foxes (sampled on two occasions). *In vivo* xenodiagnosis experiments to measure fox infectiousness to *Lu. longipalpis* sand flies were repeated on 1-3 occasions, in all 16 foxes. Negative results were obtained in all xenodiagnosis experiments (Courtenay *et al.*, 2002a).

8.2.3 Xenodiagnosis

Xenodiagnosis experiments on dogs and mark-recaptured foxes were carried out previously, as described in Chapter 2 and elsewhere (Courtenay *et al.*, 2002a; Courtenay *et al.*, 2002b). The number of positive (infectious) flies was recorded as a proportion of total number of fed flies at the end of the experiment. The total proportion of fed vs unfed flies was also recorded. The proportion of blood-fed flies was similar for dogs (45%) and foxes (37%). Animals were defined as xenodiagnosis positive ($\geq$1 infectious sandfly detected by dissection) or negative (0 infectious flies) at each individual time point, and also as never-infectious or ever-infectious based on the results of repeated xenodiagnosis carried out on the same animal (ever-infectious animals were those which were positive by xenodiagnosis at ≥ 1 time point).

8.2.4 DNA extraction, nested ITS-1 and kDNA PCR

DNA extraction was performed on 100μl aliquots of bone marrow, using a phenol-chloroform protocol as described in Chapter 2. Ear biopsy samples were extracted using a commercial kit (DNeasy: Qiagen, UK) following the manufacturer's recommendations. Mean $Abs_{260/280}$ in dog and fox bone marrow extracts was 1.83 and 1.89 respectively, and in ear biopsies, 1.88 and 1.94, respectively, showing high purity of the extracted DNA.

DNA extracts were subjected to nested PCRs targeting a) the ITS-1 region of the rRNA gene of all *Leishmania* species, and b) a variable region of *Leishmania* kDNA, as described in Chapter 2. Detection PCR products was carried out by gel electrophoresis to confirm second round reaction products of approximately 480bp and 680bp, respectively. Positive and negative controls were as described in Chapter 2.

8.2.5 Real-time qPCR

Quantitative real-time PCR (qPCR) was carried out using LEISH-1 and LEISH-2 primers and FAM-labelled Leishprobe as described in Chapter 2. Sample DNA (5µl) was added at 1:10 dilution (bone marrows) or 1:100 dilution (ear biopsies) in water (appropriate dilution factors were identified from serial 10-fold dilutions in preliminary optimization experiments). All DNA samples were amplified in duplicate. Positive and negative controls were as described in Chapter 2. Quantification of *Leishmania* DNA using real-time qPCR data was performed using three different methods. Absolute quantification (parasites/ml) was carried out by comparison of sample Ct values with those from a standard curve constructed from 10-fold dilutions of *L. infantum* DNA from 1×10^6 to 0.1 parasite equivalents/ml (strain MHOM/MA/67/ITMAP-263), which were run in triplicate on every plate. Parasite burdens calculated as above were also expressed relative to total DNA concentration measured spectrophotometrically (parasites/ml/100ng DNA), to provide a normalized measurement which could be referred to subsequent studies. Thirdly, parasite load was normalized to the eukaryotic 18S rRNA gene as a reference of total canine DNA, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For this purpose, pre-developed 18S rRNA gene TaqMan Assay reagents (Applied Biosystems, UK) were used, following the manufacturer's recommendations. $2^{-\Delta\Delta Ct}$ values were calculated using Ct values from *Leishmania* and rRNA real-time qPCR for each sample, relative to a reference sample with the lowest parasite burden relative to rRNA gene copy number, using the formula:

$$2^{-\Delta\Delta Ct} = 2^{-\left((Ct_{leish} - Ct_{rRNA})_{sample} - (Ct_{leish} - Ct_{rRNA})_{reference} \right)}$$

All data was $\log_{10} (n+1)$ transformed for analysis. The inter-assay coefficient of variability (CV) of the *Leishmania* real-time qPCR, estimated by running a reference

sample in duplicate on every plate, was 2.48%. The mean intra-assay CV of reference sample duplicates was 0.40% (range 0.03%-1.44%).

8.3 Results

8.3.1 Real-time and conventional PCR for detection of infectious dogs

The sensitivity and specificity of bone marrow and ear biopsy real-time qPCR, kDNA and ITS-1 conventional nested PCR for detection of xenodiagnosis-positive dogs at the time of sampling are shown in Table 9. Results of previous serological tests (crude parasite ELISA) are shown for comparison. Real time qPCR of bone marrow and ear biopsies showed similarly high sensitivity to both of the conventional PCR methods tested, and serology. Specificity of real-time qPCR was low, similar to serology, and lower than the specificity of both the conventional nested PCRs in bone marrows (McNemar's $\chi^2 \geq 10.9$; $P \leq 0.001$) and ear biopsies (McNemar's $\chi^2 \geq 18$; $P \leq 0.001$).

Using parasite burdens (expressed as $\log_{10}$ parasites/ml/100ng DNA) measured by real-time qPCR, categorized as low (0-4.9), medium (5-9.9) and high (≥ 10) (Figure 17), the probability of xenodiagnosis-positivity was highest in the latter group when sampling bone marrows ($\chi^2 \geq 9.66$; $P=0.002$) and ear biopsies ($\chi^2 \geq 9.05$; $P=0.003$). Optimum cut-off points of 9 & 15 $\log_{10}$ parasites/ml/100ng DNA (arrowed, Figure 18, A&B) were selected to give the maximum combined value for sensitivity and specificity for identification of xenodiagnosis-positive dogs, using bone marrow and ear biopsy samples, respectively. These cut-offs improved the diagnostic value of bone marrow real-time qPCR by increasing specificity to approximately the same value as ITS-1 PCR (McNemar's $\chi^2=2.7$; $P=0.22$) and superior to kDNA PCR (McNemar's $\chi^2=18.2$; $P<0.001$: Table 9). Specificity of ear biopsy real-time qPCR using the optimum cut-off point was higher than ITS-1 PCR, though not significantly so (McNemar's $\chi^2=4.45$; $P=0.07$), and was superior to kDNA PCR (McNemar's $\chi^2=9.0$; $P=0.004$). However, the use of cut-off parasite burdens in bone marrows and ear biopsies reduced the sensitivity of real-time qPCR to 89% and 88%, respectively, not significantly different to the sensitivities of ITS-1 and kDNA PCR. ROC curves (1-specificity vs sensitivity) plotted from varying parasite burden cut-off values, gave area under the curve (AUC) values of 0.86 (bone marrows) and 0.94 (ear biopsies), indicating good to excellent diagnostic value of real-time qPCR for detecting infectious dogs at the time of sampling.

Parasite burdens in tissue samples were also estimated from Ct values measured in real-time qPCR using two alternative quantification methods, as $\log_{10}$ parasites/ml, and $\log_{10} 2^{-\Delta\Delta Ct}$ values, calculated as described above. Parasite burdens calculated by all three procedures were highly correlated (adjusted r^2=0.92-0.98; Table 10), thus statistical associations were similar when data were analyzed using the alternative quantification methods (Figures 19 & 20). All subsequent analysis has been carried out on parasite burdens normalized to total DNA (parasites/ml/100ng DNA), and for clarity all parasite burdens will be reported in these units.

8.3.2 Predictive value of parasite burden to identify highly infectious dogs.

We next determined whether tissue parasite burdens were associated with the proportion of fed sandflies that became infected in xenodiagnosis experiments performed at the time of sampling (Figure 18, A&B). The purpose of this analysis was to attempt to identify highly infectious dogs responsible for the majority of disease transmission. There was a poor correlation between the proportion of total fed flies that were infected in xenodiagnosis at a single time point, and parasite burdens in simultaneously sampled bone marrows (adjusted r^2=0.24) and ear biopsies (adjusted r^2=0.27). Ranking dogs by their cumulative contribution to transmission (Figure 21, A&B) illustrates that the dogs identified by the described real-time qPCR cut-off points in bone marrow and ear biopsy samples (arrowed, Figure 18, A&B) were together responsible for 99.4% and 97% of transmission events, respectively. Conventional kDNA PCR of bone marrows and ear biopsies identified dogs responsible 99.7% and 100% of transmission events, respectively, while ITS-1 PCR identified 99.7% and 67.6% (one highly infectious dog was ear biopsy ITS-1 PCR negative). Crude parasite ELISA serology carried out in the previous survey was positive in all infectious dogs.

Table 9. Sensitivity and specificity of conventional and real-time qPCR for detection of infectious dogs.

Columns headed ITS-1 and kDNA show sensitivity and specificity of ITS-1 and kDNA conventional nested PCRs respectively, for identification of xenodiagnosis positive dogs at the time of sampling. Figures in parentheses show 95% C.I.

CLA ELISA: sensitivity and specificity of crude parasite ELISA, for detection of infectiousness in dogs at the time of bone marrow or ear biopsy sampling.

Leishmania real-time qPCR results are shown for dichotomous outcome (qPCR +/-), and using the optimum cut-off bone marrow and ear biopsy parasite burdens (qPCR cut-off: 9 & 15 $\log_{10}$ parasites/ml/100ng DNA, respectively) which were selected to produce the highest combined figure for sensitivity and specificity. AUC: Area under ROC curve (1-specificity vs sensitivity) for varying parasite burden cut-off points to classify dogs as infectious based on results of real-time qPCR. An AUC value of 1 indicates a perfect test, 0.5 indicates a test with no diagnostic value.

Dogs	ITS-1	kDNA	CLA ELISA	qPCR +/-	qPCR cut-off	n
Bone marrow						
Sensitivity (%)	94 (73-100)	94 (73-100)	100 (82-100)	94 (73-100)	89 (66-99)	18
Specificity (%)	71 (59-82)	46 (33-59)	22 (13-34)	24 (14-36)	78 (66-87)	63
					AUC=0.86	
Ear biopsy						
Sensitivity (%)	75 (35-97)	100 (63-100)	100 (63-100)	100 (63-100)	88 (47-100)	8
Specificity (%)	74 (59-87)	63 (47-77)	26 (14-41)	21 (10-36)	91 (78-97)	43
					AUC=0.94	
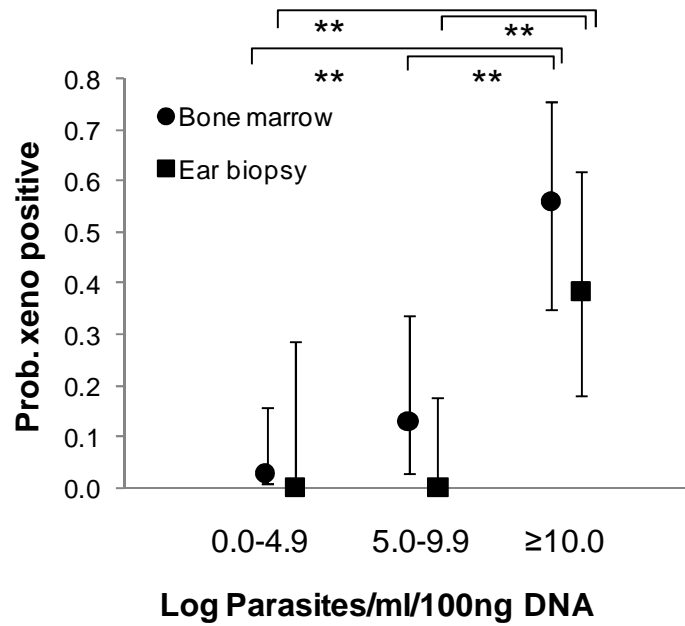
Figure 17. Relationships between bone marrow and ear biopsy parasite burdens and infectiousness to sand flies (1).

Probability of xenodiagnosis-positivity in dogs stratified by parasite burdens in bone marrows and ear biopsies, measured by real-time qPCR in samples taken at the time of xenodiagnosis. Asterisks indicate statistically significant differences between the indicated proportions positive by xenodiagnosis (**: $P \leq 0.01$; *: $P \leq 0.05$; χ^2 test).
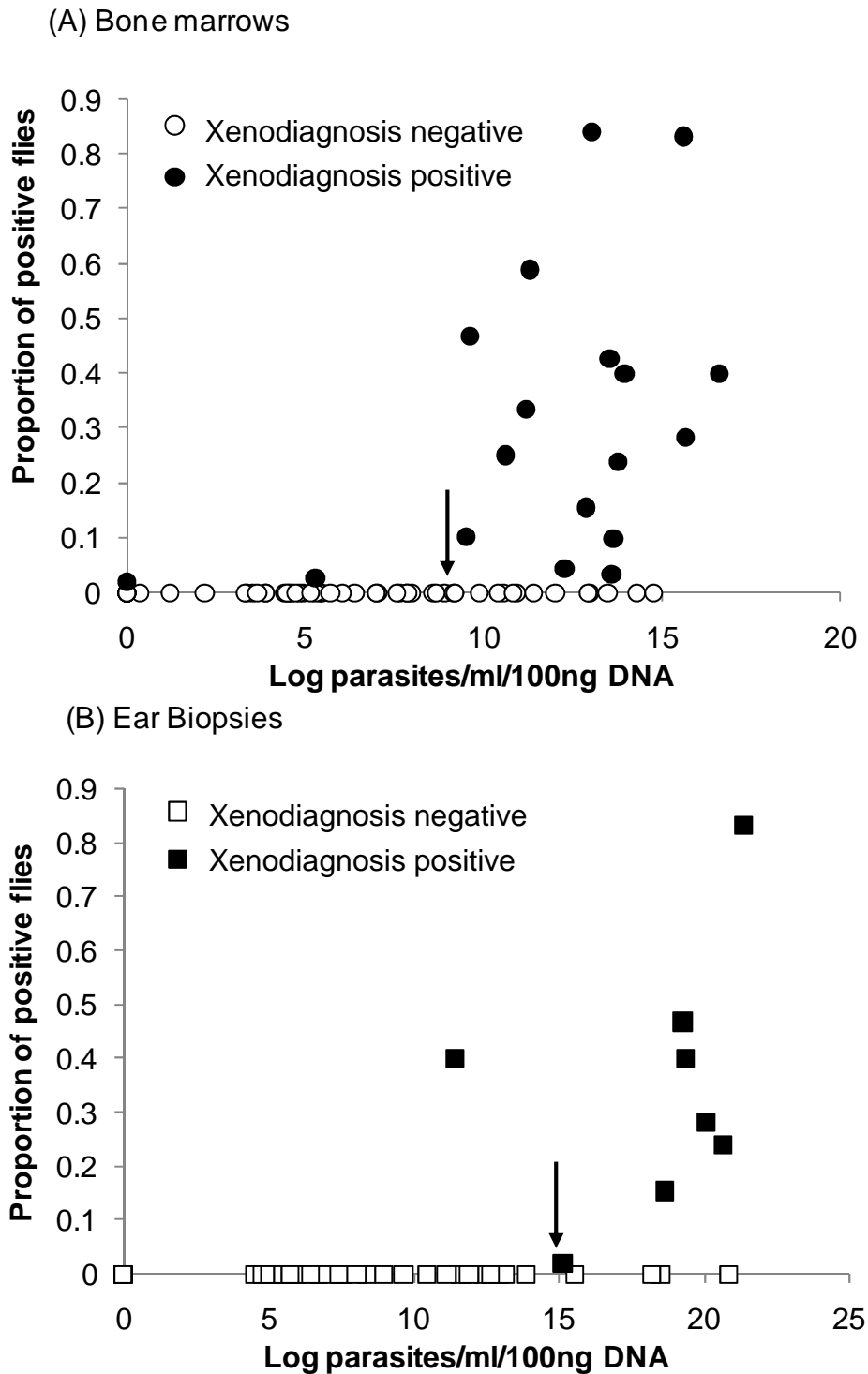
Figure 18. Relationships between bone marrow and ear biopsy parasite burdens and infectiousness to sand flies (2).

Proportion of total fed *Lu. longipalpis* sandflies that were infected in xenodiagnosis experiments, in relation to (A) bone marrow and (B) ear biopsy parasite burdens. Arrows indicate optimum cut-off points selected to maximize real-time qPCR sensitivity and specificity for identification of xenodiagnosis positive dogs based on their bone marrow and ear biopsy parasite burdens (cut-off=9 and 15 $\log_{10}$ parasites/ml/100ng DNA, respectively).

Table 10. Correlation between parasite burdens estimated using absolute and relative quantification methods.

Correlation (adjusted Pearson's r^2 value) between estimates of parasite burden calculated from real-time qPCR data, using absolute parasite burden expressed as $\log_{10}$ parasites/ml, quantification relative to total DNA quantity measured spectrophotometrically ($\log_{10}$ parasites/ml/100ng DNA), and quantification relative to the copy number of the canine rRNA gene ($\log_{10} 2^{-\Delta\Delta Ct}$).

Quantification method	Bone marrow		Ear Biopsy	
$\log_{10}$ parasites/ml				
	0.92		0.95	
$\log_{10}\ 2^{-\Delta\Delta Ct}$		0.93		0.98
	0.97		0.92	
$\log_{10}$ parasites/ml/100ng DNA				
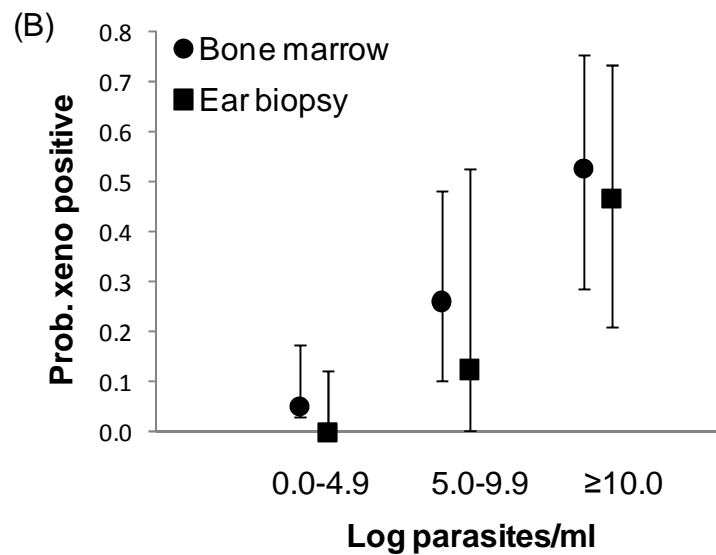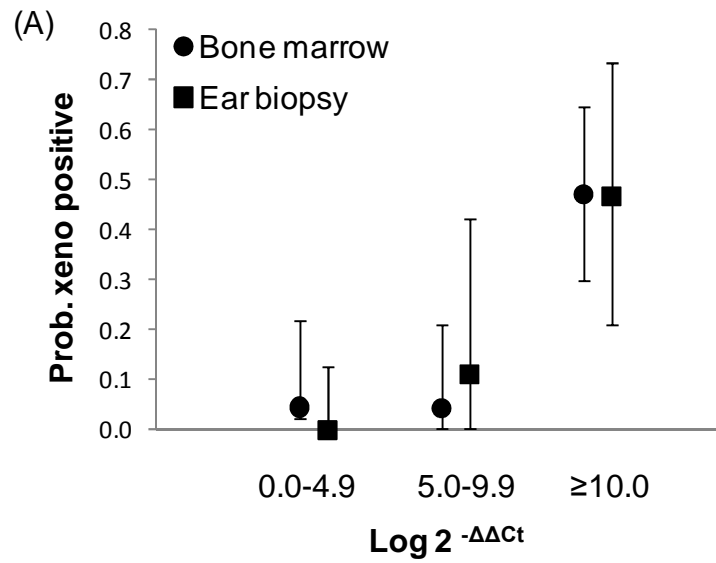
Figure 19. Relationships between tissue parasite burdens measured using alternative quantification methods, and infectiousness to sand flies (1).
(A) & (B) Probability of xenodiagnosis-positivity in dogs stratified by parasite burdens in bone marrows and ear biopsies, measured by real-time qPCR, quantified by the $\log_{10} 2^{-\Delta\Delta Ct}$ method (A) and as $\log_{10}$ parasites/ml (B).
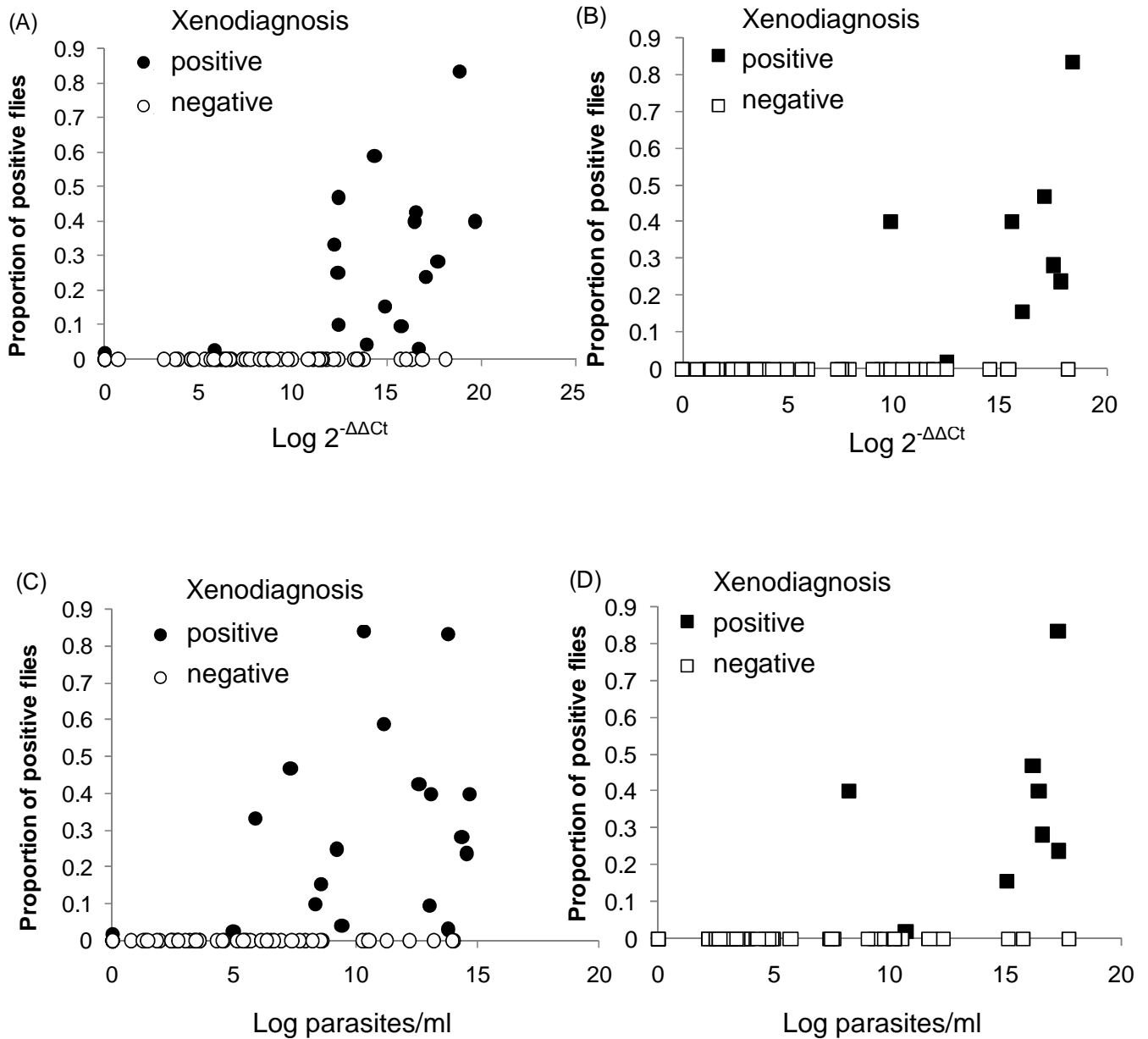
Figure 20. Relationships between tissue parasite burdens measured using alternative quantification methods, and infectiousness to sand flies (2).
Proportion of total fed *Lu. longipalpis* sandflies that were infected in xenodiagnosis experiments, in relation to parasite burdens quantified by real-time qPCR using:
 (A) & (B) $\log_{10} 2^{-\Delta\Delta Ct}$ method, and
 (C) & (D) $\log_{10}$ parasites/ml
 Bone marrow samples are indicated by circles and ear biopsies by squares.

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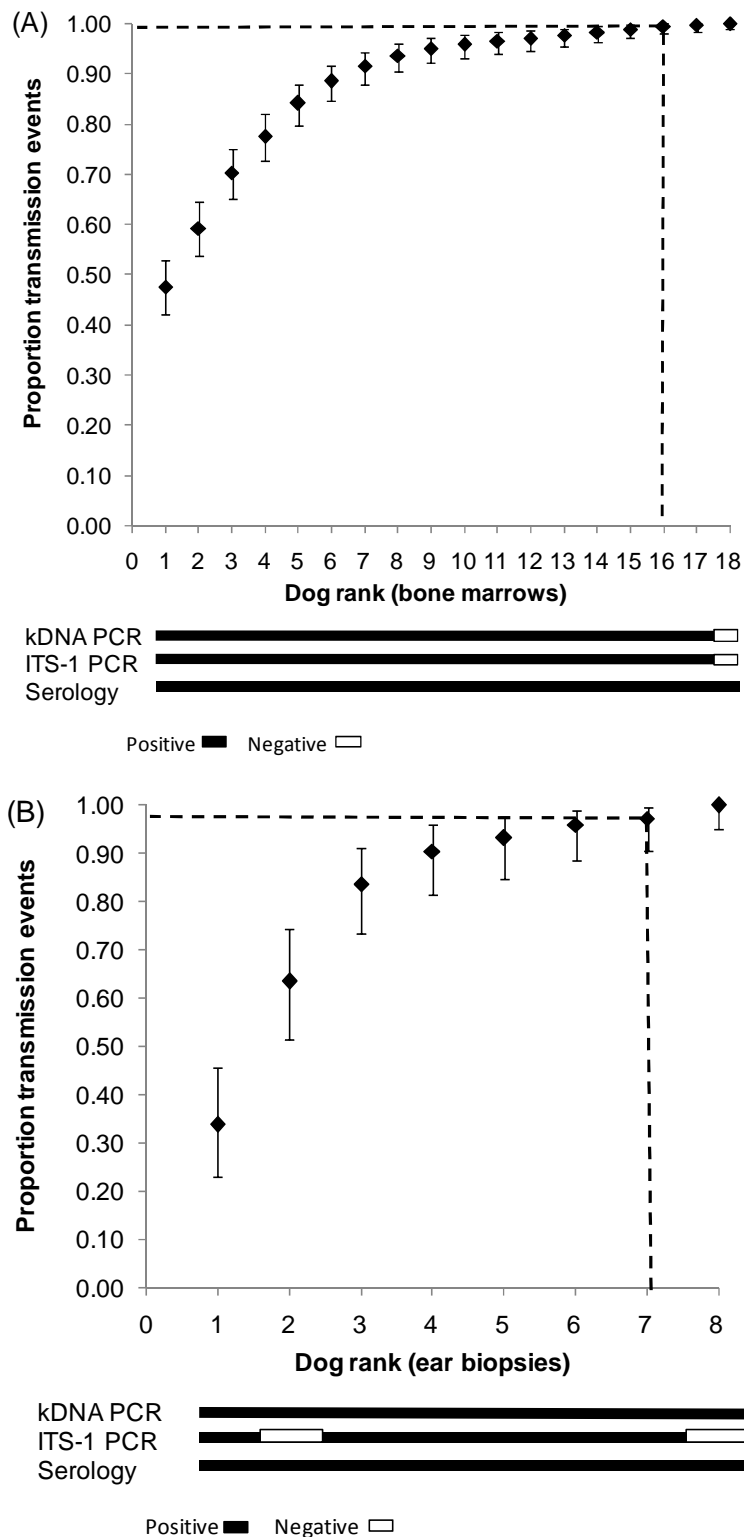
Figure 21. Proportion of transmission events in dogs identified by real-time and conventional PCR positivity

Dogs are ranked from left to right in order of their contribution to total transmission events (no. of sand flies infected overall). Dotted lines indicate the proportion of transmission events attributable to real-time qPCR positive dogs, defined using the cut-off points described above, in bone marrows (A) and ear biopsies (B). Horizontal bars indicate the results of conventional kDNA and ITS-1 PCR, and serology on bone marrows and ear biopsies respectively.

8.3.3 Parasite burden, infectiousness and time since infection.

L. infantum parasite loads in bone marrow and ear biopsy samples estimated by real-time qPCR (median +/- interquartile range), stratified by the infectious status of dogs as ever-infectious or never-infectious, with time from first detection of infection (t0) are shown in Figure 22(A). Parasite levels in samples from ever-infectious animals were higher than never-infectious dogs in bone marrow samples (n=157) at t0 to t+6 mo and t+8 to t+12 mo (Wilcoxon rank sum test: z=-2.95; *P*=0.003; z=-2.99; *P*= 0.003), and in ear biopsies (n=102) at the same time points (Wilcoxon rank sum test: z=-2.28; *P*=0.02; z=-3.88; *P*<0.001, respectively). Controlling for within-dog autocorrelation, there was a significant positive correlation between tissue parasite burden in infected dogs and the probability of being ever-infectious to sand flies, when testing either bone marrows (z=2.11; *P*=0.04) or ear biopsies (z=2.85; *P*=0.004). In fox samples, parasite levels in both bone marrows and ear biopsies were lower than in ever-infectious dogs (Wilcoxon rank sum test: z=-5.39; *P*<0.001; z=-5.71; *P*<0.001, respectively) and were also lower than in never-infectious dogs (Wilcoxon rank sum test: z=-3.64; *P*<0.001; z=-4.17; *P*<0.001, respectively) (Figure 22). The sensitivity and specificity of conventional and real-time PCR for identifying ever- and never-infectious dogs according to time post-infection are shown in Figure 22(B). Real-time qPCR of bone marrow and ear biopsy was the most sensitive (75-97% and 60-100%, respectively) but least specific method (15-33% and 0-63%, respectively) for detection of ever-infectious dogs. For detection of ever-infectious animals using bone marrow samples, the maximum sensitivity of kDNA PCR was 79% (25% specificity) at 0-6 months post t0, and maximum sensitivity of ITS-1 PCR was 75% (83% specificity) at 14-20 months post t0. Using ear biopsies, the maximum sensitivity of kDNA PCR was 86% (43% specificity) at 8-12 months post t0, and maximum sensitivity of ITS-1 PCR was 71% (67% specificity) at 8-12 months post t0.

Figure 22. Relationship between parasite burden and time from infection in ever- and never- infectious dogs.

(A) *Leishmania* parasite load measured by real-time qPCR in bone marrow (n=157) and ear biopsy (n=102) samples from ever-infectious and never–infectious dogs, and bone marrow and ear biopsy samples (n=26 each) from non-infectious foxes. Parasite counts are expressed as the median (+/- interquartile range) $\log_{10}$ parasite count per ml DNA extract, normalized to 100ng total DNA ($\log_{10}$ parasites/ml/100ngDNA), by time from first detection of infection (t0) defined as positivity by crude parasite ELISA and /or *in vitro/in vivo* culture. Fox bone marrow (FBM) and fox ear biopsy (FE) parasite counts in samples from ELISA positive foxes are shown for comparison – time t0 was not determined in foxes.

Time points at which tissue parasite burdens were greater in ever-infectious dogs than in never-infectious dogs are indicated (B: bone marrow; E: ear biopsy; **: $P{\leq}0.01$; *:$P{\leq}0.05$; Wilcoxon rank sum test).

(B) Sensitivity (with specificity in parentheses) of conventional PCRs and real-time qPCR for detection of ever-infectious dogs at the indicated time points. Fox samples were all negative by xenodiagnosis, allowing calculation of specificity only.

Figure 22. (A)
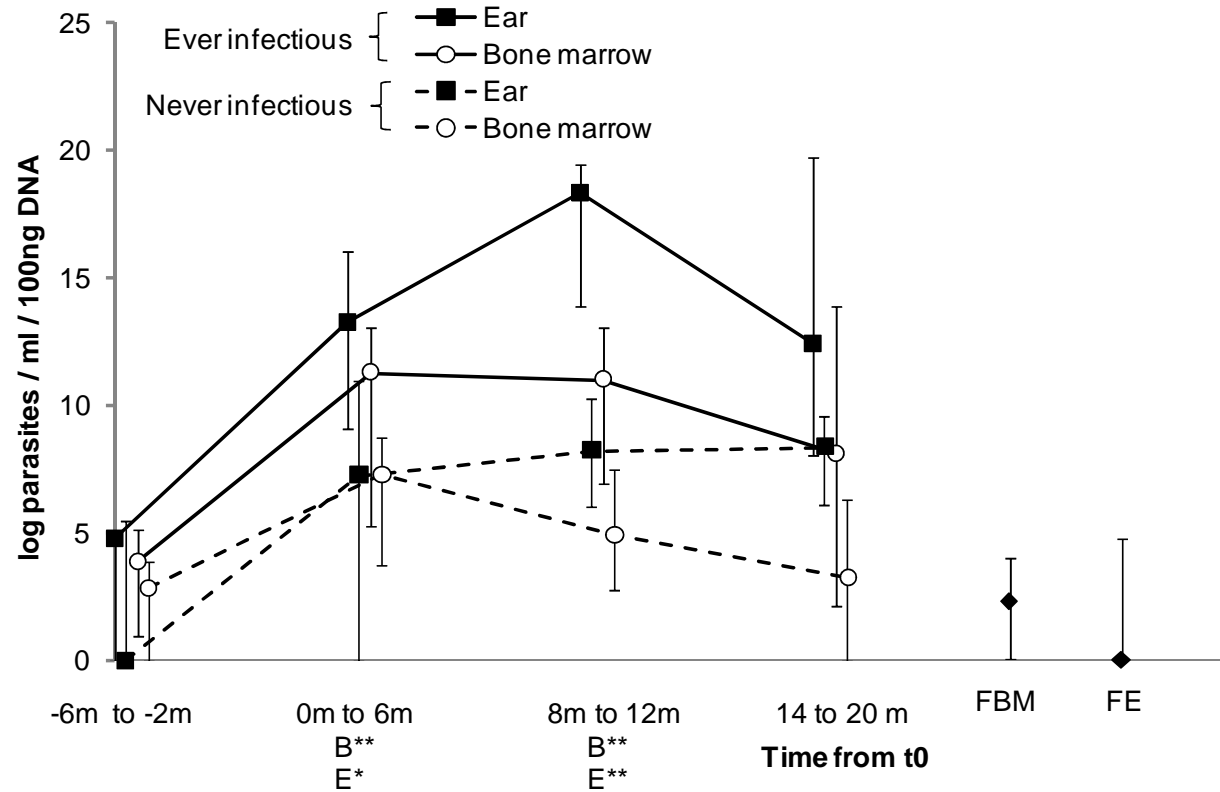
(B)

Bone marrow					
ITS-1	13 (100)	55 (55)	72 (68)	75 (83)	(62)
kDNA	50 (61)	79 (25)	78 (44)	75 (42)	(54)
qPCR	75 (31)	97 (15)	89 (20)	75 (33)	(46)
Ear Biopsy					
ITS-1	0 (100)	50 (82)	71 (67)	42 (75)	(69)
kDNA	0 (100)	63 (64)	86 (43)	75 (50)	(65)
qPCR	60 (63)	88 (37)	100 (19)	100 (0)	(54)

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8.4 Discussion

From the results reported here, the main challenge in adapting conventional or real-time qPCR for use as a proxy measure of the infectiousness of ZVL-infected dogs to sand flies appears to be lack of specificity of these tests in xenodiagnosis-negative dogs. Real-time qPCR was highly sensitive for detection of xenodiagnosis-positive dogs when PCR outcome was considered as a dichotomous result (0 or ≥ 1 parasites/ml/100ng DNA), however conventional nested ITS-1 and kDNA PCR methods showed similar sensitivity to real-time qPCR, as did crude parasite serology. The specificity of real-time qPCR was poor, equivalent to serology and inferior to conventional PCR. However, quantification of parasite burdens allowed stratification of dogs into groups, in which the group with the highest parasite burdens (>10 $\log_{10}$ parasites/ml/100ng DNA) showed the highest probability of being infectious to sand flies, and allowed the selection of a cut-off point, below which the majority of dogs were xenodiagnosis-negative. These findings show interesting parallels with previous analyses of the distribution of anti-parasite antibody titres in infected dogs, which demonstrated the existence of a similar cut-off point in IgG titre below which dogs were mainly non-infectious (Courtenay *et al.*, 2002b). In the present study, the use of optimum cut-off parasite burdens to define xenodiagnosis-positive dogs improved the specificity of bone marrow real-time qPCR, but only to equivalent performance with ITS-1 PCR. In ear biopsy real-time qPCR, use of the optimum cut-off improved specificity to $>90\%$, although this figure was not significantly better than ITS-1 PCR (74% specificity) due to limited sample size. The diagnostic performance of real-time qPCR to predict xenodiagnosis positivity in dogs at the time of sampling, based on ROC analysis, was superior in ear biopsies than in bone marrows. Ear skin biopsies are technically straightforward and rapid to collect under local anaesthesia, though even less invasive sampling methods than those used here could be investigated, for example conjunctival swabs have shown some merit in previous work using conventional PCR to identify infected dogs (Strauss-Ayali *et al.*, 2004; Pilatti *et al.*, 2009), and are under investigation as a sampling technique for real time PCR studies (Francino *et al.*, 2006).

Previous studies, which have used a number of methods (though not real-time qPCR) to detect parasites in canine tissues in relation to infectiousness, have produced conflicting results. For example, in a small cross-sectional study of naturally infected Brazilian dogs, an absence of microscopic evidence of amastigotes in skin samples

from xenodiagnosis-negative animals was in contrast to large numbers of amastigotes in xenodiagnosis-positive animals (Vercosa *et al.*, 2008). Similarly, naturally infected dogs treated with liposomal meglumine antimoniate showed reduced parasite burdens (determined by counting immunolabelled amastigotes) in skin and bone marrow, relative to untreated controls, in association with reduced infectiousness to *Lu. longipalpis* (Ribeiro *et al.*, 2008). Neither real-time nor conventional PCR analysis was performed in either of those two studies. Conversely, and in agreement with our results reported here, conventional PCR carried out on ear and abdomen biopsies of Colombian dogs, and on skin, bone marrow and spleen samples from Brazilian dogs, showed imperfect specificity for detection of infectiousness to *Lu. longipalpis*, with positive PCR results in both infectious and non-infectious animals (Travi *et al.*, 2001; Michalsky *et al.*, 2007). A principal aim of the present study was to determine whether quantification of parasite burdens would enable incrimination of the small proportion of highly infectious dogs which are responsible for the majority of transmission. Where dog culling is the main control measure, this could improve canine welfare by reducing the numbers of dogs culled needlessly. For example in the present study, approximately 20% of all infectious dogs (4/18 in the bone marrow group) were responsible for >75% of all transmission events. However, we found poor correlation between tissue parasite burdens and the proportion of sand flies infected by feeding on individual dogs ($r^2 \leq 0.24$; Figure 18 A & B), meaning that high tissue parasite burdens were of limited use in separating out the most highly infectious dogs. Although the use of the optimum cut-off bone marrow and ear biopsy parasite burdens in real-time qPCR allowed identification of dogs responsible for 99% and 97% of transmission events, respectively, both conventional PCRs identified similarly high proportions of transmission events, though kDNA was superior to ITS-1 in ear biopsies (Figure 21B). It is therefore hard to justify the additional expense of real-time compared to conventional nested PCR for identifying infectious dogs in large scale survey work, since quantification of parasite burdens does not appear to offer any additional diagnostic advantage.

For future implementation of a successful vaccine, it seems intuitively obvious that identification of highly infectious dogs or 'super-spreaders' would improve targeted vaccination strategies. However, in this context it is interesting to consider the effects of vaccinating populations which show heterogeneity in the infectiousness of individuals, as is clearly the case for canine leishmaniasis (Figure 18). Mathematical

modelling has shown that targeted vaccination aimed at super-spreaders does indeed reduce the probability of disease emergence in a susceptible population to a greater degree than random treatment (Yates *et al.*, 2006). However, the effect is only significant when resources (and hence vaccine coverage) are limited. In the model of Yates *et al*., 2006, this was simulated by comparing the reduction of the basic reproduction rate (R_0) by random vaccine allocation (0-100% of the population), with initial targeted vaccination of a sub-population of 5% super-spreaders (which were 10× more infectious than 'normal' individuals), followed by random allocation of remaining vaccination coverage amongst the rest of the population. In that model, the beneficial effect of targeting vaccination at super-spreaders was highest at low overall vaccine coverage rates, and became less marked as coverage of the population increased. Therefore it is recommended that prior to vaccine implementation by public health authorities, cost/benefit analysis should compare the expense and logistic difficulty of attempting to identify and vaccinate super-spreaders, as compared with random vaccine distribution. For such calculations, it is also worth noting that results obtained in the abovementioned mathematical models were identical for a 'perfect' vaccine (i.e. conferring complete protection in vaccinated individuals) applied to 70% of the population, and an imperfect vaccine (which reduced susceptibility by 70%) with 100% population coverage. This emphasises the point that a vaccine against canine leishmaniasis need not be completely protective in order to impact on disease transmission.

All the PCR tests used, both conventional and real-time, showed low specificity due to misclassification of a significant proportion of samples from highly parasite-positive, but xenodiagnosis-negative dogs. Although we have interpreted xenodiagnosis experiments as a 'gold' standard for defining infectiousness, some previous research shows that the proportion of flies infected by an infectious dog can vary from 0% to 100% between consecutive xenodiagnosis experiments even when conducted over relatively short time intervals (mean 6 days (95% C.I.: 3 to 10 days) (Dr. Orin Courtenay, unpublished data). Apparent low specificity of PCR in xenodiagnosis-negative dogs reported here may have resulted from similar wide fluctuations in xenodiagnosis positivity. Such high error margin in degree of infectiousness by examination at a single time point can be reduced by repeated measures from longitudinal sampling. To investigate this possibility, in the current study we also used cumulative positivity from repeated xenodiagnosis experiments to

define ever-infectious and never-infectious dogs. Our results from this analysis suggest that a positive relationship does exist between parasite burdens in bone marrow and ear biopsy samples measured by real-time qPCR, and infectiousness to sandflies. However, the specificity of conventional and real-time qPCR in defining dogs as ever- or never-infectious was not significantly better than their specificity in point estimates of infectiousness. This may indicate that despite PCR evidence of tissue parasitism, a sub-group of infected dogs remained non-infectious to sand flies, or alternatively progressed to infectiousness at some later date, outside the period of follow-up in the current study.

Quantifying the infectiousness of suspected wildlife reservoirs of ZVL is important to allow assessment of their relative contribution to disease transmission by partitioning R_0, as has been done previously in studies of the crab-eating fox in Brazil (Courtenay *et al.*, 2002a). The difficulty of carrying out xenodiagnosis experiments on wild animals would be alleviated by more straightforward PCR-based protocols to estimate infectiousness. As reported here, tissue parasite burdens in infected foxes were lower than in infected dogs (both ever and never-infectious groups). Our data thus provides a plausible parasitological explanation for why these foxes did not contribute to disease transmission. As previously described, anti-*Leishmania* IgG titres in foxes selected for the present study were also lower than in dogs tested here, and foxes were largely asymptomatic, which may reflect differences in exposure or parasite clearance, between the species. The time course of infection in the small number of foxes tested here was not fully characterized, and to validate real-time qPCR as a means of detecting infectiousness in wildlife reservoirs would require larger-scale longitudinal studies with an extended period of follow-up. Moreover, the associations reported here describe the infectiousness of dogs and foxes in Brazil to the locally important vector species, *Lu. longipalpis.* Geographical differences in transmission dynamics of ZVL are likely to exist, for example in the Mediterranean it has been suggested that *Phlebotomus perniciosus* may be a more permissive vector than *Lu. longipalpis*, which can acquire infection from skin with lower parasite burden (Molina *et al.*, 1994). Thus, region-specific studies in both old and new worlds will be required to compare the associations tested here.

In conclusion, for identification of infectiousness to sand flies in ZVL infected dogs, tissue parasite burdens measured by real-time qPCR provided no clear advantage over the dichotomous results of conventional nested PCR which would justify the

expense of the former procedure. In the absence of a 'gold' standard for measuring infectiousness, a moderately sensitive conventional PCR test is likely to identify infectious animals with high sensitivity but low to moderate specificity, which has implications for control programmes which aim to interrupt disease transmission by culling infectious animals. Further longitudinal research is needed to characterize variations in xenodiagnosis results in infected dogs over time, and their relationship with tissue parasite burdens.

Chapter 9: General discussion

DNA/MVA TRYP and LACK prime/boost vaccines are shown in this study to be safe and immunogenic in uninfected, unexposed outbred dogs, with superior antigen-specific levels of the signature type-1 cytokine IFN-γ, and *in vivo* delayed-type hypersensitivity responses, in TRYP compared to LACK vaccinated dogs. The IgG class profile in DNA/MVA TRYP vaccinated dogs was also consistent with protective immunity, based on precedents established in previous canine research. However subsequent comparison of the specificity of monoclonal and polyclonal antibodies reported in Chapter 5 showed inconsistent associations between class levels and parasitological outcome. For future canine vaccine studies, caution is advised in the interpretation of IgG class profiles measured using currently available polyclonal reagents, as a proxy for Th1/Th2 cellular immune response balance.

Characterization of vaccine-induced antibody responses in the target species is an essential component of vaccine development, to ensure that natural exposure to disease can be distinguished from vaccination. The lack of significant CLA and rK39-specific IgG responses in vaccinated animals, in contrast to strong responses reported here in infected animals, show the potential for differentiation of infected from vaccinated animals (DIVA) (van Oirschot, 1999). Legislative requirements for import of vaccinated animals to *Leishmania* non-endemic countries may require the development of a marker vaccine which elicits serological responses against antigens not present in the target pathogen, enabling detection of vaccinated animals by specific serological tests. Modification of the experimental DNA/MVA vaccine used in the present study by insertion of multi-epitope plasmids expressing TRYP in combination with a non-*Leishmania* protein could meet this requirement. From the data reported here, ELISAs incorporating either CLA or rK39 antigens would be appropriate serological tests for measurement of infection incidence in DNA/MVA TRYP or LACK vaccinated animals enrolled in field trials, though CLA ELISA was more sensitive in naturally infected dogs than rK39 in early stage infections, and was also superior to a rapid diagnostic dipstick test incorporating the rK39 antigen. Despite the sensitivity advantage of CLA ELISA, the potential for cross-reactivity with antibodies against other canine pathogens such as *Ehrlichia canis*, which may be co-endemic with *Leishmania infantum*, means that CLA ELISA cannot be recommended as a standalone test to determine infection incidence in vaccine trials, and should be combined with

more specific molecular methods for diagnosis of ZVL.

Molecular methods tested here for detection of *L. infantum* infection were targeted at DNA sequences not present in the experimental DNA/MVA TRYP and LACK vaccines, and were therefore appropriate for measurement of infection incidence in vaccinated animals. For use in large scale field trials where technical facilities are limited, the OligoC-TesT kit was the most straightforward of the PCR protocols tested, with the advantages of standardized pre-formulated reagents which would enable comparison of results between different trials using the same kit. OligoC-TesT showed high sensitivity for detection of infection, which was comparable to or greater than the sensitivity of kDNA and ITS-1 nested PCR, respectively. However, the high price of the kit is a current obstacle to its widespread adoption for large scale survey work.

From the perspective of public health, vaccine-induced sterile immunity against ZVL in the reservoir host is not essential: a vaccine which reduced the infectiousness of dogs to biting sand flies (ideally such that the basic reproductive rate of the disease (R_0) <1), would block or reduce transmission to humans. Investigation of molecular methods, as potential proxy measures to replace time consuming xenodiagnosis, highlighted a number of limitations of real-time and conventional PCR as measures of transmission blocking effect of an experimental canine vaccine. The main challenge was lack of specificity of these tests in xenodiagnosis-negative dogs. Conversely, real-time qPCR was highly sensitive for detection of xenodiagnosis-positive dogs, however conventional nested ITS-1 and kDNA PCR methods showed similar sensitivity to real-time qPCR, as did serology. Despite this, quantification of parasite burdens allowed stratification of dogs into groups with high and low infectiousness based on selection of cut-off parasite burdens. Above the cut-off point, poor correlation between tissue parasite burdens and the proportion of infected sand flies made it difficult to identify the most highly infectious dogs, which could have improved targeted culling strategies aimed at those animals responsible for the majority of transmission events. It is therefore hard to justify the additional expense of real-time compared to conventional nested PCR (4 GBP *vs* 1 GBP per sample, approx.) for identifying infectious dogs in large scale survey work, since quantification of parasite burdens does not appear to offer major diagnostic advantages. For implementation of a successful vaccine against canine leishmaniasis, targeted vaccination strategies aimed at highly infectious dogs would be of most benefit where resources and hence vaccine coverage are limited. A cost/benefit analysis of the potential utility of molecular methods for this purpose

would need to take the above issues into account, in addition to the logistic difficulties of targeted (compared with random) vaccine allocation.

Future research efforts should be directed towards confirmation of vaccine safety and immunogenicity in replicate cohorts of outbred dogs, with dosing-up trials of freeze-dried vaccine formulations as discussed in Chapter 3. Subsequently, community-based field trials will be required to measure vaccine efficacy to reduce canine ZVL infection, disease and infectiousness in a genetically diverse population of dogs exposed to natural infection with *L. infantum*. Outcomes would include clinical disease, seroconversion, molecular detection of parasite DNA in canine tissue samples (and potentially quantification by real-time qPCR, subject to the selection of appropriate cut-off points) as a marker of infectiousness to sandflies, and immunological correlates of these end points (*in vitro* cytokine stimulation assays, and tissue cytokine mRNA expression). Analysis of these outcome measures would quantify differences in the incidence of infection and disease between blinded and randomized vaccine and control groups, and relate these to measured correlates of protective immune responses. Measurement of the longevity of vaccine-induced protective cellular immune responses is critical to determine optimum re-vaccination frequency. In this way, a successful canine vaccine would be shown to provide long-term protection of dogs against *Leishmania* infection and/or disease, and to reduce or eliminate infectiousness of the reservoir host, thereby reducing or preventing transmission to humans.

The end point of vaccine development is the licensing of a commercial product. Potential markets for a canine vaccine against ZVL would be companion animal owners mainly in developed world endemic areas and those who travel with their dogs to such areas, and public health authorities in highly endemic countries where alternative control measures against ZVL are failing, for example in Brazil (Courtenay *et al.*, 2002b). For all markets, a successful experimental vaccine against canine ZVL would need to translate into a cost-effective product in comparison with other available vaccines and disease control interventions. Insecticide-impregnated collars for dogs have proven efficacy in reducing infective sandfly bites, thus ZVL transmission to humans and dogs, and represent a potential competitor to a canine vaccine. However, in the field the persistence of protection from these collars (theoretically 6 months) will also depend on the rate that dogs lose their collars, as briefly discussed in Chapter 1. In Brazilian trials, 17%-34% of collars were lost over 5 months (Oliveira-Lima *et al.*,

2002; Reithinger *et al.*, 2004), compared to 35% in 10 months in Italy (Foglia Manzillo *et al.*, 2006). Thus where transmission is year round, the average dog would require 2 or more collars per year for individual protection and to maintain theoretical herd immunity. The minimum current costs of collars (e.g. c. US$10-15 in Brazil, €17-20 in Europe) are high and conceived as unaffordable by most ZVL endemic communities, whereas vaccine protection of significant duration would avoid problems associated with loss of collars, and loss of protection, and would benefit from decreased inter-intervention interval. Considering logistic constraints on community-wide delivery, the frequency of intervention required for an effective DNA/MVA vaccine would be far lower than that required to maintain dog coverage with collars.

A successful canine vaccine would be a step towards the development and delivery of a future human vaccine against leishmaniasis. With significant potential for future exploitation of DNA/MVA vaccines against leishmaniasis, it is important, even in the initial stages of vaccine development, to be aware of issues surrounding patents and intellectual property (IP). For example, the TRYP antigen used in the current study is subject to claims in patent applications (described alternatively as TSA antigen, as discussed in Chapter 1) which cover the use of the peptide as part of a fusion of more than one antigen, and which cover the peptide as a vaccine component, though other research describing the same antigen as TRYP has been cited as prior art which undermines the novelty of the patent application. It is also clear that there is a complex patent landscape in the area of the DNA/MVA prime boost technology, however, rights to this technology are available for licensing, and would not necessarily impede commercialization of an successful vaccine. The legal issues described above emphasise the need for consideration of matters beyond the purely scientific in the process of vaccine development from the academic to the commercial sectors.

The present study describes DNA/MVA vaccine trials against canine leishmaniasis and validates diagnostic methods for use in future large scale trials to measure vaccine efficacy. The general approaches used here have wide relevance to the development of vaccines against other pathogens, and in other species of animals.

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Appendix I. Trials of experimental vaccines against zoonotic visceral leishmaniasis in dogs

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	*L. infantum* fraction LiF2. 3 intravenous or subcutaneous injections at 1 month intervals, with 5 different adjuvants	Moderate anti-*Leishmania* titres in all vaccinated dogs. Sera blocked *L. infantum* replication.	No exposure	No exposure: immunogenicity trial only	3 months	12 German shepherd dogs.	No side effects recorded	(Ogunkolade *et al.*, 1988)
Purified parasite fraction	*L. infantum* fraction LiF2 with Muramyl Dipeptide adjuvant. 3 subcutaneous injections at 1 month intervals	None recorded	None recorded	Natural exposure	2 years	400 dogs (natural population, randomly assigned to vaccine or control groups)	Infection rate in vaccinated dogs higher than control group in 1st year. No between-group difference in 2nd year.	(Dunan *et al.*, 1989)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Killed whole parasite	Merthiolated sonicated promastigotes of *L. braziliensis* + BCG adjuvant. 600ug by 3 intradermal doses at 21 day intervals.	No detectable anti-*Leishmania* IgG response to vaccination.	Lymphoproliferative response to *Leishmania* antigen in vaccinees. No detectable anti-*Leishmania* IgG in 9/10 vaccinees, high titres in unvaccinated dogs.	Experimental: 2.3×10^6 *L. braziliensis* promastigotes intravenously , 2 months post vaccination	26 months	19 laboratory-reared mongrel dogs	1/10 vaccinated and 9/9 control dogs developed patent infection. Vaccinated dogs developed injection site skin ulcer.	(Mayrink *et al.*, 1996)
Killed whole parasite	Autoclaved *L. infantum* or autoclaved *L. major* with BCG	Positive intradermal test in vaccinated dogs	Positive intradermal test in vaccinated dogs, negative in controls	Experimental: 2.5×10^6 *L. infantum* promastigotes, 90 days post vaccine by intraperitoneal challenge	5 months	16 dogs in four groups	All dogs in unvaccinated groups positive by culture and microscopy, only one vaccinated dog infected.	(Mohebali *et al.*, 1998)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Killed whole parasite	Autoclaved *L. major* promastigotes (ALM) with either saponin or BCG adjuvant. Three doses: second dose after 4 weeks, third dose after 6 weeks.	High anti-*Leishmania* antibody titres in ALM / saponin vaccinated dogs, low lymphocyte proliferation. ALM/BCG dogs showed opposite trends.	No exposure	No exposure: immunogenicity trial only	10 months	18 mixed breed dogs.	No side effects recorded	(Lasri *et al.*, 1999)
Purified parasite fraction	FML enriched purified fraction of *L. donovani* in saline. Three subcutaneous doses 21 days apart,	None recorded	100% vaccinees seropositive to FML ELISA. Positive intradermal test 7 months post vaccine.	Natural exposure to *L. chagasi*	2 years	58 vaccinees 59 controls Domestic mongrel dogs	92% protection. 76% efficacy. 33% control dogs developed clinical or fatal disease. 8% vaccinated dogs showed mild non-fatal disease.	(Da Silva *et al.*, 2000)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	FML enriched purified fraction of *L. donovani*+ QuilA adjuvant. Three subcutaneous doses 21 days apart.	None recorded	100% vaccinees seropositive to FML ELISA. Positive intradermal test 2 months post vaccine.	Natural exposure to *L. chagasi*	3.5 years	44 vaccinees 41 controls Domestic dogs	95% protection. 80% vaccine efficacy 25% fatality rate in control dogs and 5% in vaccinees.	(Borja-Cabrera *et al.*, 2002)
Purified parasite fraction	FML enriched purified fraction of *L. donovani* Three subcutaneous doses 21 days apart.	70% seropositive to FML ELISA.	IgG2 predominated in normal pre-immune and vaccinated animals, whereas IgG1 response greater in infected animals.	Natural exposure to *L. chagasi*	63 days	68 vaccinees, 121 naturally infected controls.	Comparison with infected dogs, hence protective efficacy not recorded.	(de Oliveira Mendes *et al.*, 2003)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
DNA/rVV prime/ boost	Heterologous prime/boost with DNA-LACK and Western Reserve rVV expressing LACK, by subcutaneous injection 15 days apart.	Coincident IL-4 and IFN-γ mRNA peaks 2-4 weeks post vaccination.	Vaccinated dogs expressed IL-4, IFN-γ and IL-12 mRNA (suggestive of mixed Th1/Th2 response), lymphoproliferation response, and a high ratio of anti-LACK IgG2:IgG1.	Experimental: (1×10^8 *L. infantum* promastigotes intravenously), 2 weeks post vaccine.	17 months	20 laboratory Beagles	60% vaccine efficacy for prevention of disease. Reduction of visceral parasite load in partially protected dogs.	(Ramiro *et al.*, 2003)
Recombinant protein	Recombinant multi component chimeric Protein Q plus live BCG 3 intra peritoneal injections at 21 day intervals.	Low positive anti-Protein Q IgG response 21 days after 1st vaccine. Control dogs negative.	Positive intradermal test in 8/ 10 vaccinated dogs and 1/10 control dogs, 21 months post infection. Vaccinees had lower anti-*Leishmania* IgG titres.	Experimental : 5×10^5 *L. infantum* intravenously, 2 months post-vaccination	2 years	20 laboratory beagles	90% protection against infection. Vaccinated dogs asymptomatic, controls diseased. N.B. safety concerns re: live BCG use (Poot *et al.*, 2009).	(Molano *et al.*, 2003)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	FML enriched purified fraction of *L. donovani* + QuilA adjuvant: (as immunotherapy) 3 subcutaneous doses 3-6 weeks apart.	None recorded	All seropositive to FML ELISA at 2 months post infection.	Experimental: $1x10^8$ amastigotes of *L. donovani* intravenously 3 months before vaccination.	1 year	5 mongrel dogs (siblings)	3/5 vaccinees remained asymptomatic, with positive intradermal test, and were parasite negative.	(Borja-Cabrera *et al.*, 2004)
Purified parasite fraction	FML enriched purified fraction of *L. donovani*+ Saponin R adjuvant: 3 subcutaneous doses 21 days apart	None recorded	Increasing anti-FML IgG2 levels and 75%-95% positive intradermal reaction in vaccinated dogs.	Naturally infected pre-vaccination	22 months	21 vaccinees and 46 controls, all asymptomatic FML-ELISA seropositive outbred dogs.	Delay of clinical disease: 90% vaccinated dogs and 37% controls remained asymptomatic at end of trial.	(Borja-Cabrera *et al.*, 2004)
Killed whole parasite	Autoclaved whole *L. major* promastigotes with AlOH adjuvant plus BCG Single dose by intradermal injection.	No anti-*Leishmania* IgG at 4 months post vaccine (before t/m season).	High titre anti-*Leishmania* IgG in 6/162 vaccinated dogs and 17/141 controls at 16 mo post vaccination.	Natural exposure to *L. infantum*	16 months	182 vaccinees. 165 controls. Outbred dogs.	Vaccine efficacy 69.3%, for prevention of seroconversion. Local skin ulcer at vaccine site in 64% of vaccines.	(Mohebali *et al.*, 2004a)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	FML enriched purified fraction of *L. donovani* + Riedel de Hahn saponin adjuvant. 3 subcutaneous doses 21 days apart.	None recorded	100% vaccinated dogs seropositive to FML ELISA	Natural exposure	11 months	32 vaccinees 40 controls Outbred dogs	Controls 25% symptomatic, 57% lymph node PCR positive, 16% blood PCR positive, 25% skin positive. Vaccinees negative by all tests.	(Nogueira *et al.*, 2005)
Purified fraction of promastigote culture supernatant	*L. infantum* excreted/secreted antigens (*Li*ESAp)+ muramyl dipeptide adjuvant. 2 subcutaneous injections 3 weeks apart.	Anti-*Li*ESAp IgG2 and lymphocyte proliferative responses higher in vaccinated dogs than controls. IFN-γ levels higher in cytokine assays of PBMC from vaccinated dogs.	Anti-*Li*ESAp IgG2 and lymphocyte proliferative responses higher in vaccinated dogs than controls.	Experimental: 1×10^8 *L. infantum* promastigotes intravenously, 2 months and 8 months post vaccine	14 months	18 dogs in four groups. Laboratory Beagles.	100% protection against homologous and heterologous challenge Mild local reaction in many dogs to second vaccine dose.	(Lemesre *et al.*, 2005)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
DNA / recombinant protein	DNA/protein prime boost 30 days apart with a mix of Cysteine proteinase type I (CPa) and II (CPb), by intramuscular injection.	Elevated anti-CPa and CPb IgG1 and IgG2 in vaccinated dogs.	Elevated IFN-γ: IL-10 mRNA ratio, lymphoproliferation response and Ag-specific IgG and IgG2 from vaccinated dogs compared to controls. Strong intradermal response in vaccinated dogs.	Experimental: 5x10⁶ *L. infantum* promastigotes intravenously, 3 months post vaccine.	12 months	15 dogs in four groups. Mixed breed dogs.	1/10 of vaccinated dogs bone marrow PCR positive in contrast with 3/4 controls.	(Rafati *et al.*, 2005)
Recombinant protein	Multi subunit recombinant polyprotein MML (TSA, LmSTI1, and LeIF), plus MPL-SE or Adjuprime. 3 subcutaneous injections, 28 days apart, boost at 1 year.	Anti-MML antibody response in 13/15 MPL-SE vaccinated dogs and 8/15 Adjuprime vaccinated dogs.	Weak lymphoproliferative response. Lack of non-specific ConA response in dogs co-infected with *Ehrlichia*.	Natural exposure to *L. infantum*	2 years	3 groups of 15 dogs. Laboratory Beagles	Not protective to clinical disease (? due to concurrent ehrlichiosis). Cumulative incidence 87% (MPL-SE), 100% (Adjuprime), and 100% (control)	(Gradoni *et al.*, 2005)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Recombinant protein and killed whole parasite	Mixture of TSA, LmSTI1, and LeIF proteins, with MPL-SE or Adjuprime adjuvants, or killed *L amazonensis/L. braziliensis* with BCG adjuvant. 3 doses by subcutaneous injection at 4 week intervals.	Antigen –specific antibody response in vaccinated dogs particularly to LmSTI1, maximal after 3rd vaccination then declined for 6 weeks.	MPL-SE vaccinated animals showed IgG2 dominated response to infection. Adjuprime and whole parasite vaccinated groups produced low IgG2 :IgG1 ratios.	Experimental : $1x10^6$*L. chagasi* promastigotes intravenously, 4 months post vaccine.	15 months	7 groups of 5 dogs. Laboratory beagles	Protection and efficacy against clinical disease not reported – immunogenicity trial only.	(Fujiwara *et al.*, 2005)
DNA	Multivalent 10 plasmid DNA vaccine including *L. donovani* Histone proteins, LACK, PSA-2, TSA, STI1, ARP-1, H1. Three injections at 30 day intervals of DNA plasmids by intradermal or intramuscular injection.	Antigen specific lymphocyte proliferative response and increased IFN-γ mRNA level in PBMC of *i/m* vaccinated dogs. No detectable IgG titre	DTH response to viable *L. donovani* promastigotes.-	Experimental: Cutaneous challenge $1x10^6$ *L. donovani* promastigotes	60 days	2 groups of 4 dogs: Foxhounds	Inhibition of parasite replication in draining LN after cutaneous challenge, and *in vitro*. No difference in splenic parasite burden.	(Saldarriaga *et al.*, 2006)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Recombinant protein	Recombinant cysteine proteinases (CPa and CPbs) of *L. infantum* + IL-12, with or without QuilA saponin. 2 subcutaneous injections at 4 week interval.	Elevation of antigen specific anti-CP antibodies in vaccinated dogs.	High anti-CP and anti-*Leishmania* titres in vaccine and control groups. No DTH response in any dogs.	Experimental: 5×10^7 promastigotes of *L. infantum* intravenously, 3 weeks post vaccine.	13 months	3 groups of 5 dogs. Laboratory Beagles.	0% protection – all vaccinated dogs and all controls became clinically and parasite positive by lymph node and bone marrow culture.	(Poot *et al.*, 2006)
Purified fraction of promastigote culture supernatant	*L. infantum* excreted/secreted antigens (*Li*ESAp)+ muramyl dipeptide adjuvant. 2 subcutaneous injections 3 weeks apart, with third dose 12 months later.	None recorded	Increased IgG2 reactivity and enhanced NO mediated anti-leishmanial activity of canine macrophages *in vitro* was seen in vaccinated dogs .	Natural exposure	24 months	Approx 400 IFAT negative outbred domestic dogs in endemic area (randomized to vaccine and control groups)	Lower number of bone marrow PCR positives in vaccinated group than placebo group. (1/165 *cf* 12/175)	(Lemesre at al.,2007)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	FML enriched purified fraction of *L. donovani*+ saponin adjuvant: 3 subcutaneous doses 21 days apart	None recorded	No exposure	No exposure: Safety trial only	14 days	600 outbred dogs	Outcomes of vaccine administration: Local pain (40%) Anorexia (20%) Apathy (24%) Swelling (15.9%) Vomiting (2.4%) Diarrhoea (1.5%)	(Parra *et al.*, 2007)
Recombinant protein	Recombinant *L. infantum* Histone 1 (H1) and *L. donovani* HASPB1, together and separately, with adjuvant (Montanide). 3 intradermal doses at 30 day intervals	High levels of antigen specific IgG detected in all vaccinees. No lymphocyte proliferative response seen in any animals post immunization.	Increase in anti-HASPB1 IgG in vaccinated groups. No lymphocyte proliferative response seen in any animals post challenge.	Experimental: 1×10^8 *L. infantum* promastigotes intravenously, 45 days post vaccine.	16 months	7 groups of 8 dogs. Laboratory beagles.	No clinical symptoms in 5/8 H1, 4/8 HASPB1 and 4/8 H1/HASPB1 dogs, compared with 2/8 control dogs. Adjuvant related local skin inflammation.	Moreno et al., 2007

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction	FML enriched purified fraction of *L. donovani*+ Saponin adjuvant (at double normal adjuvant concentration) :as immunotherapy. 3 subcutaneous doses 21 days apart	None recorded	Vaccine treated dogs showed higher anti-FML IgG titres than controls with higher IgG2 and decreasing IgG1 over time. 75% vaccine dogs showed positive intra dermal test *cf* 50% controls.	Experimental: 2×10^8 *L. chagasi* amastigotes intravenously, 6 months before vaccination	15 months	24 mixed breed dogs, previously infected and randomized to treatment or control groups.	Immuno-therapeutic effect: Lower clinical score in vaccinees compared to controls, with normal CD4+ lymphocyte count *cf* subnormal CD4+ count in controls.	(Santos *et al.*, 2007)
Killed whole parasite	*L. braziliensis* promastigote protein + saponin adjuvant (LBSap). 3 subcutaneous injections at intervals of 4 weeks.	Increased anti-*Leishmania* IgG1 and IgG2 levels, CD4+ and CD8+ cell counts, NO production and lymphocyte proliferation.	No exposure	No exposure: immunogenicity trial only	12 weeks	25 mixed breed dogs in 4 groups	Vaccine side effects: mild local induration at injection site. Protective efficacy not recorded.	(Giunchetti *et al.*, 2007)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
DNA	Multiantigenic plasmid DNA encoding KMPII, TRYP, LACK and GP63 4 injections of vaccine intradermally at 15-day intervals	Sporadic detection of anti-LACK IgG in two dogs. No cellular or humoral response to *Leishmania* antigen in any dogs.	Delay in production of anti-*Leishmania* IgG IgA and IgM in vaccinated dogs compared to controls.	Experimental: 5×10^7 *L. infantum* promastigotes intravenously, 1 month post vaccination	16 months	2 groups of 6 dogs	Similar development of clinical signs in both vaccine and control groups – high amounts of *Leishmania* DNA in visceral organs - no evidence of vaccine induced protection.	(Rodríguez-Cortés *et al.*, 2007)
Killed whole parasite	*L. braziliensis* promastigote protein (LB) +/- saponin (Sap) +/- *L. longipalpis* salivary gland extract (Sal). 3 subcutaneous injections at intervals of 4 weeks	Increased anti-saliva and anti-*L. chagasi* IgG, and increased CD4+ and CD8+ counts, NO production and lymphocyte proliferation in LBSapSal group.	No exposure	No exposure: immunogenicity trial only	12 weeks	25 mixed breed dogs in 4 groups	Vaccine side effects: mild local induration at injection site. Protective efficacy not recorded- immunogenicity trial only.	(Giunchetti *et al.*, 2008)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Recombinant protein antigen	Recombinant A2 antigen Three subcutaneous doses at days 0, 21 and 42 with A2 + saponin	Increased IFN-γ, total IgG and IgG2, and anti-A2 antibodies, with low IL-10 levels in vaccinated animals compared to controls.	Increased IFN-γ and low IL-10 levels in vaccinated animals compared to controls.	Experimental: 5×10^7 *L. chagasi* promastigotes intravenously four weeks after last vaccine dose.	14 months	3 groups of 7 dogs (laboratory beagles).	5 out of 7 vaccinated dogs remained asymptomatic. Whereas 5/7 control dogs developed disease symptoms.	(Fernandes *et al.*, 2008)
DNA/rVV prime/boost	Heterologous prime/boost with DNA-LACK and Western Reserve rVV or modified Vaccinia virus Ankara (MVA) expressing LACK, by subcutaneous injection 15 days apart.	None reported	Both IgG1 and IgG2 increased, though rise in IgG2 was more marked in vaccinated dogs. Total IgG was lower in vaccinated than in unvaccinated infected dogs. Increased IFN-γ mRNA expression in vaccinees.	Experimental: 1×10^8 *L. infantum* metacyclic promastigotes intravenously 2 weeks after last vaccine dose.	10 months	16 dogs in four groups. Laboratory Beagles.	Fewer dogs boosted with MVA rVV showed disease symptoms than Western Reserve strain rVV vaccinees (75% *cf* 50% were asymptomatic, respectively)	(Ramos *et al.*, 2008)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Recombinant protein	Recombinant vaccine Leish-110f (incorporating recombinant antigens TSA, LmSI1 and LeIF) for immunotherapy + adjuvant MPL-SE +/- meglumine antimoniate (Glucantime®)	None reported	Higher *Leishmania*-specific lymphocyte proliferation in groups receiving Leish-110f than in other groups.	Naturally infected prior to vaccination.	6 months	30 symptomatic outbred dogs in 5 groups.	Immuno-therapeutic effect: Animals that received Glucantime®, +/- Leish-110f®/MPL-SE® showed clinical improvement.	(Miret *et al.*, 2008)
Purified parasite fraction	FML enriched purified fraction of *L. donovani*+ saponin adjuvant 3 subcutaneous doses 21 days apart	None reported	Anti-FML IgG detected in 98% vaccinated dogs, and 82% were positive by intradermal test.	Natural exposure	2 years	550 outbred dogs in 2 groups from different endemic areas.	99% vaccinated dogs were asymptomatic compared to 79% unvaccinated dogs. 99% vaccinated dogs survived to 2 years, *cf* 61% unvaccinated dogs.	(Borja-Cabrera *et al.*, 2008)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Purified parasite fraction, and killed whole parasite	Leishmune® or BCG-adjuvanted killed *L. amazonensis* ("Leishvaccine"). Three subcutaneous doses Leishmune or Leishvaccine at 21 day intervals.	Leishmune® - enhanced levels of IFN-γ, NO and anti- *L. chagasi* IgG2. Leishvaccine-Mixed IFN-γ and IL-4 response with enhanced anti-*L. chagasi* IgG1	No exposure	No exposure: immunogenicity trial only	40 days	2 groups of 6 German shepherd dogs.	Protection and efficacy against clinical disease not reported – immunogenicity trial only.	(Araujo *et al.*, 2009)
Recombinant chimerical protein antigen	Recombinant JPCM5_Q protein with adjuvants (muramyl dipeptide, AlOH, Matrix C or killed *P. acnes.*) 2 subcutaneous injections 21 days apart.	IgG specific for Q protein was detected in all vaccinated dogs. AlOH and Matrix C groups showed greater lymphocyte proliferation assay responses than controls.	*Leishmania*- specific IgG increased in all vaccinated and control groups post exposure. DTH responses did not differ between vaccinated and control groups.	Experimental: 5×10^7 stationary phase promastigotes of *L. infantum* JPC strain via intravenous injection, 3-4 weeks after 2nd vaccination.	10 months	43 laboratory beagles.	No protection : all vaccinated dogs and controls were parasite positive and showed clinical disease by end of study.	(Poot *et al.*, 2009)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
Recombinant chimerical protein antigen	Recombinant Q protein, single dose, or 2 doses of 100µg 21 days apart.	IgG2 specific for Q protein increased in vaccinated dogs, though *Leishmania*-specific IgG was not elevated.	All challenged dogs developed anti-*Leishmania* titres post exposure. NO production in lymph node cell cultures from the Q and Q+Q groups was significantly higher than controls.	Experimental: 5×10^5 promastigotes of *L. infantum* via intravenous injection, 60 days after 1st vaccination.	11 months	21 laboratory beagles	Q vaccination reduced clinical severity, and reduced lymph node and skin positivity by culture, qPCR and microscopy. Controls showed gross pathology in organs not seen in vaccinees.	(Carcelen *et al.*, 2009)

Vaccine type	Vaccine protocol	Pre exposure correlates of protection / failure	Post exposure correlates of protection / failure	Exposure to disease	Duration of study	No of animals	Clinical outcome	Reference
DNA/rVV prime/boost	Heterologous prime/boost with DNA-LACK (antibiotic resistance gene free plasmid) and modified Vaccinia virus Ankara (MVA) expressing LACK, by two subcutaneous injections 15 days apart.	Increase in IFN-γ mRNA expression in PBMC pre-challenge.	Lower production of *Leishmania*-specific IgG in vaccinees, and Th1 response indicated by increase in IFN-γ mRNA in PBMC, and corresponding decrease in IL-10 expression. IL-4 higher in non-vaccinated dogs.	Experimental: 1×10^8 *L. infantum* metacyclic promastigotes intravenously 2 weeks after last vaccine dose.	10 months	16 dogs in three groups. Laboratory Beagles.	Reduction in clinical signs and liver parasite burden in vaccinated dogs.	(Ramos *et al.*, 2009)

Appendix II: Primer sequences

Oligonucleotide primer sequences used in the study are summarized below:

Primer	Sequence (5'-3')	Reference
IR1	GCTGTAGGTGAACCTGCAGCAGCTGGATCATT	Cupolillo et al.,1995; Parvizi et al., 2005
IR2	GCGGGTAGTCCTGCCAAACACTCAGGTCTG	Cupolillo et al.,1995; Parvizi et al., 2005
ITS1F	GCAGCTGGATCATTTTCC	Parvizi et al., 2005
ITS2R4	ATATGCAGAAGAGAGGAGGC	Parvizi et al., 2008
LEISH-1	AACTTTTCTGGTCCTCCGGGTAG	Francino et al., 2006
LEISH-2	ACCCCCAGTTTCCCGCC	Francino et al., 2006
Leishprobe	(FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB	Francino et al., 2006
CSB2XF	C/GA/GTA/GCAGAAAC/TCCCGTTCA	Noyes et al., 1998
CSB1XR	ATTTTTCG/CGA/TTTT/CGCAGAACG	Noyes et al., 1998
13Z	ACTGGGGGTTGGTGTAAAATAG	Noyes et al., 1998
LiR	TCGCAGAACGCCCCT	Noyes et al., 1998

Appendix III: Forms for Phase I/II trials

Form A: Information sheet for dog owners (English version)

Form B: Owner / dog details and initial clinical examination

Form C: Recording sheet for baseline clinical examination (before vaccination)

Form D: Daily recording sheet for clinical examination (1-4 days post vaccine)

Form E: Record of veterinary treatments

Form F: Record of Adverse Event

Form A: Information sheet for dog owners (English Version)

Vaccine trial against *Leishmania* in dogs

The University of Crete and two UK research establishments (The University of Warwick and the Cambridge Institute for Medical Research) are testing a potential canine vaccine to protect dogs and humans against leishmaniasis. There are no vaccines widely available against leishmaniasis despite it causing serious illness in both dogs and humans. The novel vaccine that we wish to test has been shown to be protective in laboratory conditions in mice and dogs. Therefore it is an exciting development and we hope it to be the first fully proven canine vaccine against leishmaniasis.

The first phase of the vaccine trial is to test the vaccine in a small number of dogs that are kept under close observation by us in kennels. This requires enrolling young healthy dogs from villages where leishmaniasis is endemic Only on completion of these trials will we be able to proceed to the second phase of the project which is to test the vaccine in a larger number of dogs in endemic villages to evaluate its efficacy in reducing the canine disease. Below are answers to your questions. If you have any doubts please contact:

Maria Antoniou, Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine, Faculty of Medicine, University of Crete, Voutes, Heraklion, Crete 71003, Greece. Telephone: 00 30 2810 394 746.

Your Questions

What is the aim of the first Phase of the trial?
The trial will be carried out in kennels at the University of Crete to show that the vaccine induces the immunological responses known to be important in protecting mice, dogs and humans against leishmaniasis. It will also confirm that the vaccine causes no side effects. Dogs will not be artificially (or naturally) infected with *Leishmania* as this is not necessary nor the intention of the trial.

How safe is the vaccine?

Different components of the vaccine have been previously tested in mice, dogs and primates without any adverse side effects. The vehicle component of the vaccine is widely used in human vaccines and has been rigorously tested for safety. We do not expect any side effects resulting from the vaccine. However, as for the common human vaccines, we cannot say that the risk of side effects is absolutely zero. The most we would expect is a temporary inflammation in the skin at the site of the inoculation for a few days (as occurs in some human vaccines).

What will be required of my dog?

If you agree for your dog to be recruited into the trial, we will first take a blood sample to check that it has not been previously infected with *Leishmania*. If it is negative, then it can be recruited into the trial. The dog will need to stay with us in our kennels for 4 months during which time it will be monitored by our trained veterinarians. At the end of the 4 months your dog will be returned to you without harm.

What will happen to my dog?

If your dog is recruited, we will first ask you to bring the dog to stay in the kennels overnight on a number of occasions for it to become accustomed. The kennels are brand new and built larger than the EEC ethical recommendations for keeping dogs in kennels. Dogs will have an outside run (largely shaded) with a dog house in which to sleep. Food and water adlib will be provided appropriately, and dogs will be checked by our resident trained animal carer, and also checked daily by our veterinarian. It is in our interest to keep your dog as happy and as healthy as possible.

Why does my dog have to stay in the kennel?

Your dog will need to remain with us for approximately 4 months to allow it to fully develop an immunological response to the vaccine. We can only judge the effectiveness of the vaccine if we can follow-up the dog for the full 4 months in a controlled environment, where we can monitor its health carefully and prevent any exposure to infection.

How will the dog be treated?

If not already vaccinated, dogs will be first vaccinated against Distemper, Parvovirus, Hepatitis, Leptospirosis and Parainfluenza virus, and wormed against roundworms, tapeworms and hookworms. It will also receive a full veterinarian clinical examination. After about two weeks, the dog will be inoculated with either the *Leishmania* vaccine or a placebo (for comparison), and a blood sample taken every 2-4 weeks to test for immunological responses. A veterinarian will physically inspect the dog on a daily basis to ensure all is well. The dog will wear an insecticidal collar with proven activity against sandflies to protect it against any potential infection, and we will monitor the area round the kennels using traps to confirm that the area is free of sandflies.

Can any dog be recruited into the trial?

We are looking to recruit dogs that have not been previously exposed to *Leishmania*. This is more likely for dogs >4 months and ≤24 months old. To ensure that the dog has not been exposed to the parasite, we will collect a small blood sample to test for *Leishmania*. Dogs less than 4 months old are excluded from the trial because their immune systems are not fully developed and therefore would give results that are difficult to interpret.

If I agree what are my obligations?

If you agree, then we would expect you to permit the dog to stay with us for the entire 4 months so that the study can provide the results necessary to prove that the vaccine produces the appropriate responses. To avoid excessive disruption and stress which may affect the results of the trial and upset other dogs in the kennels we would ask owners not to visit their dog after the initial introduction period, however you are free to contact us at anytime to enquire regarding the dogs well being. At the end of the 4 months you should accept the return of your dog in good health. You accept that there will be no form of payment for the temporary loan of your dog.

What will happen next?

Informed written consent will be obtained from you at the time of recruitment, and we will contact you concerning arrangements to visit the kennels.

Form B : Owner / dog details and initial clinical examination

<table>
<tr><td>

Owner details

Name……………………………….

Address………………………….

………………………………….

Telephone…..…………………….

Dog details

Dog ID reference number…………

Dog's name……………………………

Breed……………………………

Age (months)
……………………………………..

Sex………………………………

Neutered   YES   NO

</td><td>

Declaration:
I agree to lend my dog to the University of Crete for the purposes described in the Information Sheet which I have fully read and understood, and will comply with the obligations as the owner of the dog as described in the Information Sheet.

Signed……………………………………

Date:……………………………………

</td></tr>
<tr><td>

Previous vaccinations:
                                   Date

Distemper   ……..

Hepatitis   ……..

Parvovirus   ……..

Parainfluenza virus   ……..

Leptospirosis   ……..

Rabies   ……..

Medical history

Condition   Date

</td><td>

Clinical examination

Body weight   ………………….. kg

Condition score (please circle):

      1   2   3   4   5

Thin ———————————→ Fat

Body temperature……………

Are any clinical signs of *Leishmania* present?

Yes / No

Clinical signs……….…………………….

Are any other signs of illness present?

Yes / No

Clinical signs   …………………….
Signature of examiner …………………….

Laboratory Test Results:
ELISA
IFAT
PCR

</td></tr>
</table>

Form C: Recording sheet for clinical examination (Days 0, 28, 42, 70, 98, 126)

Dog ID………………   Examination day: 0   28   42
                                                  70   98   126

Body weight: ………………….. kg

Condition score:

        1   2   3   4   5

Thin   ———————————→ Fat

Body temperature………………………

Pulse: …………………………………..

Resp. rate……………………………….

Capillary refill time…………………..

Mucous membrane colour……………….

Are any clinical signs of *Leishmania* present   Yes / No

Description of clinical
signs…………………………………………………………………….….

…………………………………………………………………………………

Are any other signs of illness present?   Yes / No

Description of clinical
signs……………………………………………………………………………

……………………………………………………………………………………

……………………………………………………………………………………

Form D: Daily recording sheet for clinical exam: Days 1-4 post vaccination

Dog ID:…………………………..　　　　　　**Date of vaccine: …………**

Signature of Examiner…………

DAY 1 post vaccine:	
Temperature:…………………	
Pulse: …………………………Local swelling at injection site	Yes / No
Resp. rate……………………Local pain at injection site	Yes / No
Capillary refill time…………………………Alopecia at injection site	Yes / No
Mucous membrane colour……………………Generalized urticaria	Yes / No
Clinical signs	
observed:…………………………………………………………………………	

DAY 2 post vaccine:	
Temperature:…………………	
Pulse: …………………………Local swelling at injection site	Yes / No
Resp. rate……………………Local pain at injection site	Yes / No
Capillary refill time…………………………Alopecia at injection site	Yes / No
Mucous membrane colour……………………Generalized urticaria	Yes / No
Clinical signs	
observed:…………………………………………………………………………	

DAY 3 post vaccine:	
Temperature:…………………	
Pulse: …………………………Local swelling at injection site	Yes / No
Resp. rate……………………Local pain at injection site	Yes / No
Capillary refill time…………………………Alopecia at injection site	Yes / No
Mucous membrane colour……………………Generalized urticaria	Yes / No
Clinical signs	
observed:…………………………………………………………………………	

DAY 4 post vaccine:	
Temperature:…………………	
Pulse: …………………………Local swelling at injection site	Yes / No
Resp. rate……………………Local pain at injection site	Yes / No
Capillary refill time…………………………Alopecia at injection site	Yes / No
Mucous membrane colour……………………Generalized urticaria	Yes / No
Clinical signs	
observed:…………………………………………………………………………	

Form E: Record of veterinary treatments

Dog ID:…………………………

Date:……………………………

Reason for treatment:……………………………………………………………………

………………………………………………………………………………………………..

Name(s) of Veterinary product(s) used:………………………………………………

………………………………..…………………………………………………………………

………………………………..…………………………………………………………………

How many times was treatment given?…………………………………………………..

How many days was treatment given for?...

Signature of person giving treatment:…………………………………………………..

Form F: Record of Adverse Event

Definition: An Adverse Event is any observation in the trial dogs that is unfavourable and unintended, which occurs after the use of the experimental vaccine, whether or not it is considered to be related to the vaccine.

Dog ID:……………

Letter code of *Leishmania* vaccine administered…………………………………………………….

Date *Leishmania* vaccine administered: ……………………………………………………

Date and time of Adverse event:………………………………………………………………….

Description of Adverse Event……………………………………………………………………….

……………………………………………………………………………………………………

……………………………………………………………………………………………………

……………………………………………………………………………………………………

Duration of Adverse event:………………………..………………………………

Action taken:…………..……………………..…………………………….

Signature of person recording Adverse Event…………………………………………

Date of report…………………………………

Appendix IV: Hellenic Republic General Veterinary

Authority approvals for dog trials (translated)

HELLENIC REPUBLIC
MINISTRY OF RURAL
DEVELOPMENT & FOOD
District of Crete,
Prefecture of Heraklion
Veterinary Authority of Heraklion,
Lochagou Marinelli 17
71201 HERAKLION CRETE
Information: Grigoriou Andreas
               Lambrinidi Sofia
Tel: 0030 2810243481
Fax: 2810 221637

Athens 31/07/2007
Protocol Number: 4381

To : GENERAL VETARINARY AUTHORITY
K.A.F.E.(Veterinary Welfare, Drugs & Applications)
DEPARTMENT A'
Address: Acharnon 2
P.O.box: 10176 Athens
Information: A.Arvaniti
Tel: 0030 2102125736
Fax: 0030 2108231267
Email: ka6u032@minagric.gr

Notification: Laboratory of Clinical
Bacteriology, Parasitology, Zoonoses
& Geographical Medicine
Medical School, University of Crete

SUBJECT: Research work on Leishmaniasis

After the consideration of your official document with ref No 283042/ 19-04-2007 to the Veterinary Authority of Heraklion

After three consecutive visits, by us, to the place where, according to the 829/14-02-07 application and the e-mail dated 15/3/2007 sent by the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses & Geographical Medicine of the Medical School of the University of Crete the experimentation will take place (kennels outside the certified Animal House of PAGNI (University General Hospital of Heraklion) we concluded that:

1) The experimental area has been organized according to our recommendations so that the clauses of articles: 5 (the conditions for the housing and welfare of the dogs is being followed), 9, 12, 16 and the paragraphs 3, 6, 7 of article 17 of the 160/91 (A'64) Greek law (President's Decree) are followed.
2) All measures for safeguarding Public Health have been taken for conducting this experimental work.

The Laboratory of Clinical Bacteriology, Parasitology, Zoonoses & Geographical Medicine has submitted to our Authority (as well as to your Authority) the plan to be followed for the adaptation of the dogs to the experimental area and kennels so that the dogs undergo the less stress possible. We consider this plan satisfactory.

The Laboratory of Clinical Bacteriology, Parasitology, Zoonoses & Geographical Medicine has submitted to our Authority written consent of all dog owners by which it is proved that they are

aware of the experimental procedures and possible side effects. The Laboratory has submitted photocopies of the Health Certificates of all dogs, according to which the dogs are vaccinated and are given all necessary pills and treatment for the eradication of ecto- and endoparasites.

3) We ask you to proceed to the granting of permission for the experimentation according to article 17, paragraph 4 of the 160/91 (A'64) Greek law (President's Decree), as you indicate in your document to our Authority, with ref No 283042/ 19-04-2007.

DIRECTOR OF THE DEPARTMENT OF ANIMAL HEALTH

Grigoriou Andreas

ΑΚΡΙΒΕΣ ΦΩΤΟΑΝΤΙΓΡΑΦΟ

Ηράκλειο ...30/5/08

Η Γραμματέας της ΕΧΟ...

HELLENIC REPUBLIC
MINISTRY OF RURAL
DEVELOPMENT & FOOD
GENERAL VETARINARY AUTHORITY
K.A.F.E.(Veterinary Welfare, Drugs & Applications)
DEPARTMENT A'
Address: Acharnon 2
P.O.box: 10176 Athens
Information: A.Arvaniti
Tel: 0030 2102125736
Fax: 0030 2108231267
Email: ka6u032@minagric.gr

Athens 10/09/2007
Protocol Number: 319083

TO: **Laboratory of Clinical**
Bacteriology, Parasitology, Zoonoses
& Geographical Medicine
**Medical School,**
University of Crete

Notification: Prefecture of Heraklion
Veterinary Authority
Lochagou Marinelli 17
71202 HERAKLION CRETE

SUBJECT: Research work on Leishmaniasis

After consideration of: 1)The official document with ref No Δ/Υ 13-11-2006 of the Veterinary Authority of Lasithi
          2)The official document with ref No 6298/ 28-11-2006 of the Veterinary Authority of Heraklion
          3)Our official document with ref No 261058/ 04-01-2007 to the Veterinary Authorities of Heraklion and Lasithi
          4)The e-mail of Dr. Maria Antoniou dated 15-03-2007
          5)Our official document with ref No 283043/ 19-04-2007 to Dr. Maria Antoniou, Professor of Parasitology
          6)Our official document with ref No 283042/ 19-04-2007 to the Veterinary Authority of Heraklion
          7)The official document with ref No 4381/ 31-07-2007 of the Veterinary Authority of Heraklion

After considering the above official documents related to this subject and according to the article 17, 4th paragraph, of the 160/91 (A'64) Greek law (President's Decree), we accept the part of the research protocol «Novel control strategies for Leishmaniasis» which will be conducted by the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical medicine of the University of Crete, under the scientific supervision of Dr. Maria Antoniou, Assistant Professor of Parasitology and surrogate supervisor Dr. Anna Psaroulaki, Lecturer of Bacteriology, and responsible veterinarian Dr. George Parasiris; the research project will be conducted in the kennels built outside the approved experimental building of PAGNI (University General Hospital of Heraklion) with the understanding that the 160/91 (A'64) Greek law (President's Decree) clauses 5,9,12,16 sections and 3,5,6,7 paragraphs regarding the accommodation and welfare of the dogs will be followed.

This document is promulgated to the Veterinary Authority of the Heraklion Prefecture, which will be making recurrent inspections according to the 6th paragraph of the17th section of 160/1991 Greek law.

ΑΚΡΙΒΕΣ ΦΩΤΟΑΝΤΙΓΡΑΦΟ
Ηράκλειο ....
Η Γραμματέας
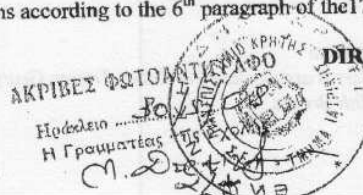
DIRECTOR OF AUTHORITY

HR. PAPADOPOULOS

Ministry of Health

University General
Hospital of Heraklion

proceedings
of meeting 4/31-1-2007

Decision 64

Item 23rd " Authorization to carry out trials of 2 vaccines for leishmaniasis on dogs,
in endemic areas of Crete, and authorization for the construction of 12
kennels, for this purpose, without the requirement of hospital funds in an area
where will be indicated.

First request. According to the document 554/30-1-07 of the Head of the Medical
Committee

The Administration Committee, after the recommendation of the Head of the Medical
Committee, Prof. G. Charissis, and after considering the above document,

Unanimously decides

1. We grant permission for you to carry out trials to study 2 vaccines for
Leishmaniasis, on owned dogs, in the area of the University Hospital animal
house in collaboration with the Universities of Warwick and Cambridge, UK,
under the supervision of the assistant prof. Maria Antoniou.
2. We grant permission for the construction of 12 kennels, according to European
regulations, without the requirement of hospital funds, in the University
Hospital of Heraklion Animal House area, for the needs of the vaccine trials.

The Hospital Manager	The vice president	The Members
Christos Melas	George Belegrakis	Marina Fanioudaki
		Birginia Aligizaki-Nikolaides
The secretary		Pinelopi Ntzilepi
Basiliki Giannadaki		Emmanouil Axladianakis
		Manousos Xristodoulakis

Correct copy of the proceedings

ΑΚΡΙΒΕΣ ΦΩΤΟΑΝΤΙΓΡΑΦΟ
Ηράκλειο
Η Γραμματέας