The epidemiology and control of canine leishmaniasis in Peru and Brazil.

by

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Abstract

The aims of the work presented in this thesis were two-fold: [i] to investigate whether domestic dogs are important reservoir hosts of ACL in a L. [Viannia]-endemic area where domestic transmission to humans has been reported [**Part 1**]; and [ii] to identify the shortcomings of currently practiced leishmaniasis dog control programmes and to evaluate whether topical insecticides could be used to control canine leishmaniasis [**Part 2**].

There is a growing belief that dogs [Canis familiaris] are peridomestic reservoirs of American cutaneous leishmaniasis [ACL], as numerous studies have reported high ACL infection rates in dogs. The work described here is the first longitudinal study of ACL in dogs, and was carried out in 18 Leishmania [Viannia]-endemic villages of the Department of Huánuco, Peru. Over three years [1997-1999] a total 1104 dogs were surveyed, 104 of which prospectively. A polymerase chain reaction [PCR)] protocol to identify L. [Viannia] parasites in dogs was developed [**Chapter 2**], and, together with serology [ELISA and IFAT], used to calculate prevalence and incidence of disease [**Chapter 4**]. The data was used to estimate the basic reproduction number [Ro] [**Chapter 4**] of canine ACL and to demonstrate a correlation between canine and human ACL incidences in the study villages [**Chapter 5**]. Several findings presented in **Chapters 2-5** suggest that dogs are reservoir hosts of [peri-]domestic L. [Viannia] transmission in Huánuco, Peru. First, whereas the prevalence (3.8%) and incidence of clinical disease [4.2%] are low, the prevalence [25.6%] and incidence [29.0% per year] of L. [Viannia] infection in dogs is comparatively high. Second, the average duration of infection [2.2. years] can be as long as the mean life expectancy of an infected dog [2.5 years]. Third, L. [Viannia] parasites in dogs do not remain localised at the site of inoculation but are able to disseminate to both viscera and mucosa. Fourth, the detection of L. [Viannia] parasites by PCR in the blood of a high proportion of both symptomatic [32%] and asymptomatic [7.5%] dogs suggests that infected dogs are potentially infectious to sandfly vectors. Fifth, after controlling for inter-village differences in transmission rates, household dog ownership was shown to be a significant risk factor for human ACL. The results presented here show that if dogs were the main ACL reservoir host and if L. [Viannia] transmission were homogeneous, a dog control strategy (e.g. culling, insecticide-treated dog collars) in the study villages in Huánuco would be very feasible and effective, because the control effort [i.e. coverage] to reduce $R_0 < 1$ would be comparatively small [as low as 47%].

Domestic dogs are established reservoir hosts of ZVL caused by L. infantum. Hence, one of the approaches to reduce the incidence of human ZVL is to target infected dogs. The findings of a comparative study testing topical insecticides and applications to protect

dogs from sandfly bites are presented in **Chapter 7**. It was shown that whilst permethrin and fenthion pour-on lotions had a more immediate effect on sandfly biting rates and mortality, deltamehtrin-impregnated collars [DMC] had a more prolonged protective effect, with the survival rate of bloodfed sandflies reduced by up to 86% after two months of deltamethrin collar application. In the work described in **Chapter 8** the effectiveness of DMC to control canine ZVL was tested in a matched cluster intervention trial. Possible constraints associated with a community-wide implementation of a dog collar ZVL control strategy were also investigated using mathematical modelling. Although ZVL incidence was 32% lower in collared as compared to uncollared dogs after five months collar application, the difference was not significant. However, DMC did significantly reduce the odds (by 50%) of dogs increasing their anti-Leishmania antibody titre. Whether topical insecticides such as DMC will be effective as leishmaniasis control tools will depend on several factors. Firstly, the strategy will be most effective in those endemic areas where domestic dogs are the main ZVL reservoir and the epidemiological significance of wild reservoirs or stray dogs contributing to ZVL transmission is negligible. Secondly, in order to achieve a significant epidemiological impact on ZVL transmission, high dog collar coverage rates are essential. This will not only require the rapid replacement of lost collars, but also the collaring of new dogs recruited into the population; where population turnover rates are high, maintaining high coverage rates will be a greater logistic challenge, which invariably applies to dog populations in tropical, ZVL-endemic countries. Ultimately, the decision to replace the dog culling strategy with community-wide application of DMC will depend on [i] the relative cost of the interventions; and [ii] the practical applicability of DMC in the field [e.g. the willingness of the community to apply DMC and the efficiency with which they replace collars which have detached]. Clearly, the implementation of DMC on dogs is more likely to have the consent of the population at risk than the highly unpopular dog culling policy that continues to be practised in some ZVL-endemic countries.

PhD-related Publications.

2003	**REITHINGER R**, CANALES ESPINOZA J and DAVIES CR. The transmission dynamics of canine American cutaneous leishmaniasis in Huánuco, Peru. Am. J. Trop. Med. Hyg., in press.
2003	**REITHINGER R**, COLEMAN PG, ALEXANDER B, VIEIRA EP, ASSIS G and DAVIES CR. Are insecticide-impregnated dog collars a feasible alternative to dog culling as a strategy for controlling canine visceral leishmaniasis in Brazil? Int. J. Parasitol., in press.
2003	**REITHINGER R,** CANALES ESPINOZA J, COURTENAY O and DAVIES CR. The evaluation of the polymerase chain reaction (PCR) as a diagnostic mass-screening tool to detect Leishmania (Viannia) spp. In domestic dogs (Canis familiaris). J. Clin. Microbiol. 41, 1486-1493.
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2001 **REITHINGER R**, ALEXANDER B and DAVIES CR. A community-based intervention trial to test the effectiveness of deltamethrin-impregnated dog collars (DMICs) to control canine zoonotic visceral leishmaniasis (ZVL) [Abstract for oral presentation at the Research in Progress Meeting of the Royal Society of Tropical Medicine & Hygiene, London, 13.12.2001]. <u>Transactions of the Royal Society of Tropical Medicine & Hygiene</u>. 96, in press

2000 **REITHINGER R**, ALEXANDER B and DAVIES CR. The use of insecticide-impregnated collars to control canine leishmaniasis: a community-based field trial in Brazil [Abstract for oral presentation for the 3rd International Congress of Vector Ecology, Barcelona, 16.-21.9.2001].

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1998 LAMBSON BE, **REITHINGER R**, DAVIES CR and BARKER DC. Molecular diagnostics of infectious diseases: evaluation of different DNA extraction protocols to improve sensitivity and specificity of polymerase chain reaction [Abstract for the Research in Progress Meeting of the Royal Society of Tropical Medicine & Hygiene, London, 23.4.1998]. <u>Transactions of the Royal Society of Tropical Medicine & Hygiene</u> 92, 370.

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1998 DAVIES CR, CANALES J, **REITHINGER R** and LLANOS-CUENTAS. The epidemiology of mucosal leishmaniasis in the Peruvian Andes [Abstract for the British Society for Parasitology Trypanosomiasis and Leishmaniasis Symposium, Arcachon, France, 18.-21.4.1998, p. 77].

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Preface

Think again. A query made in March 1999.

Dear Dr LO,

I work with Douglas Barker in Cambridge and do some PCR on clinical specimens from patients with suspected leishmaniasis. Although we usually use kDNA primers, we also try other primers described in the literature eg. ribosomal, repetitive nuclear sequence, mini-exon, etc.

From reading your papers published in Transactions and JCM in the last couple of years, you appear to favour the small subunit ribosomal primers developed by Van Eys et al. I also use these primers and have found them reliable and not prone to amplify the host DNA non-specificically.

I hope you do not mind, but I would like to ask you a couple of questions about your PCR:

1.   Why do you not increase the sensitivity of the procedure by Southern blotting and hybridisation?

2.   Do you repeat the PCR reaction with the same or different primers to confirm that a positive is really positive and a negative samples remains negative?

I really would appreciate your answers as, although PCR is valuable, at times it frustrates me so.

Yours sincerely, Dr BL

The reply a couple of weeks later.

Dear Dr BL,

On behalf of Dr LO who is absent for a while, I will answer the questions in your E-mail.

1.   We found our PCR in practice sensitive enough and Southern Blotting did hardly improve the number of positive cases. Moreover, apart from the extra labour, cost and time, it mainly led to false positive reactions.

2.   In general never repeat a successful experiment. In our hands, one PCR proved to be sufficiently reliable. However, we run at least 1 negative control per 5 PCR samples. We repeat all tests if one of the negative controls appeared to be positive.

If you pass the stage of frustration about PCR, I am sure you will get bored. I am still puzzling what is worse.

Regards

Dr GS

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Abreviations

A	adenosine
ACL	American cutaneous leishmaniasis
AVL	American visceral leishmaniasis
BCS	buffy coat sample
BMS	bone marrow sample
C	cytosine
CF	complement fixation
C.I.	confidence interval
CL	cutaneous leishmaniasis
d	days
DAT	direct agglutination test
DCL	diffuse cutaneous leishmaniasis
DMC	deltamethrin-impregnated dog collar
DNA	desoxyribonucleic acid
dNTP	denucleotidetriphosphate
DZ	diazino-impreganted dog collar
ELISA	enzyme-linked immunosorbent assay
F	fenthion pour-on lotion
G	guanosine
GBL	guanidine blood lysate
hrs	hours
IFAT	immunofluorescence antibody test
LCL	localised cutaneous leishmaniasis
MCL	mucocutaneous leishmaniasis
min	minutes
MST	Montenegro skin test
PCR	polymerase chain reaction
PM	permethrin pour-on lotion
R_o	basic reproduction number
RH	relative humidity
RNA	ribonucleic acid
s.d.	standard deviation
s.e.	standard error
sec	seconds
T	thymidine
VL	visceral leishmaniasis
WHO	World Health Organisation
ZVL	Zoonotic visceral leishmaniasis

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CHAPTER 1:
General Introduction.

The leishmaniases are a group of zoonotic diseases transmitted to humans and animals by the bite of phlebotomine sandflies. In terms of global burden of disease, the leishmaniases are currently the third most important vector-borne disease [after malaria and lymphatic filariasis], being responsible annually for an estimated 1.81 million Disability Adjusted Life-Years [DALYs] and 57,000 deaths (574). The world-wide annual incidence is estimated at 1.5-2 million clinical cases per year, with 12 million people currently infected and more than 350 million at risk of infection.

Over the past 20 to 30 years there has been a dramatic increase in the number of reported human leishmaniasis cases, a trend that shows no signs of abating. Although partly due to better diagnosis and notification of leishmaniasis cases, this trend is probably due to [i] the adaptation of the transmission cycle to the peridomestic habitat because of urbanisation and deforestation (378, 563, 575); [ii] the failure of vector and reservoir control campaigns (32); [iii] the emergence of *Leishmania* as an opportunistic infection in HIV-infected people (20, 169); and [iv] the increasing evidence of circulating, drug-resistant parasite strains (131, 525) .

Despite the multiple aetiologies [for review see Ref. (298)], the New World leishmaniases can be divided into two broad categories: so-called American zoonotic visceral and American zoonotic cutaneous leishmaniasis [AVL and ACL respectively]. The latter includes localised cutaneous leishmaniasis [LCL], mucocutaneous leishmaniasis [MCL] and diffuse cutaneous leishmaniasis [DCL].

Throughout the Americas, AVL is exclusively caused by *Leishmania [Leishmania] infantum*. It has been isolated in: Argentina, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, El Salvador, Guadeloupe, Guatemala, Honduras, Martinique, Mexico, Nicaragua, Paraguay, Suriname, United States and Venezuela. In contrast, ACL is caused by several *Leishmania* spp.,

including *L. braziliensis,* which is the most widespread *Leishmania* spp. in the New World and also accounts for most cases of ACL. It has been isolated in Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, French Guiana, Guatemala, Honduras, Mexico, Panama, Paraguay, Peru and Venezuela [for review see Refs. (148, 227, 229, 298)]. Over the following sections the clinical presentation, disease pathogenesis, immunology, treatment and prevention of the leishmaniases will be discussed, in particular with reference to *L. [Viannia]* spp. and *L. infantum,* as those are the Leishmaniae of interest in this thesis.

1.1. The American leishmaniases: clinical presentation, disease pathogenesis and immunology.

Clinical presentation. *L. [Viannia]* may cause single or multiple primary lesions [for review see Ref. (565)] [**Plate 1A, 1B, 1D, 2A, 2B** and **2E**], which may be more severe [**Plate 1B**] and long-lasting than lesions caused by other *Leishmania* spp. (246). Lymphatic spread and involvement of lymph glands are frequent (51, 52), and there is a variable tendency for lesions to self-cure [up to 75% of infections, e.g. (122, 123, 240, 266, 348, 475)]. Spontaneous healing usually results in a life-long protection from disease, which may or may not be restricted to the same *Leishmania* sp. (374, 375), and protection is mediated by the host's cellular immunity [see below]. In order to maintain a memory immunity response dependent on continuous antigen presentation, life-long protection against reinfection may involve the persistence of live parasites (2). Thus, recurrence [due to exogeneous reinfection or endogeneous reactivation of persistent infections] may occur (158, 319, 492) [**Plate 1C** and **1E**], and some patients develop a second cutaneous lesion at a different site after their primary lesion has healed. Multiple [i.e. infections with the same *Leishmania* strain (132)] or mixed infections [i.e. infections with a different *Leishmania* strain or species (489, 509)] have also been reported.

Though MCL can be caused by *L. [Viannia] panamensis* (411, 490), *L. [Viannia] guyanensis* (488) and *L. [Leishmania] amazonensis* (53, 483), it is

most commonly associated with *L. [Viannia] braziliensis* (148). Mucosal involvement is the most serious complication in *L. braziliensis* infections and can lead to disfiguring and life-threatening MCL [also called 'Espundia'] in up to 25% of *L. braziliensis* infections (172, 173). MCL is characterised by the ability of the parasite to metastasise to mucous tissues by lymphatic or haemotogenous dissemination [for review see Refs. (345, 565)]. The disease typically begins with nasal inflammation and stuffiness, followed by ulceration of the nasal mucosa and perforation of the septum. In some cases the lips, cheeks, soft palate, pharynx or larynx are involved as well [**Plate 1F, 1G** and **2D**]. Tissue destruction is thought to be due to a hyperergic immune response. MCL never heals spontaneously, is very difficult to treat (65, 206, 346, 347, 462), and secondary bacterial infections are frequent and may be fatal (345).

Almost all cases of AVL are caused by *L. infantum*. In contrast to *L. [Viannia]* spp., *L. infantum* rarely remains localised in the skin in chronic infections and will migrate to the viscera, i.e. spleen, bone marrow and liver. Common clinical AVL symptoms include abdominal swelling, fever, malaise, emaciation and diarrhoea, symptoms that tend to be associated with hepatosplenomegaly, pancytopenia, hypergammaglobulinaemia and hypoalbuminaemia (265, 423). If left untreated, AVL is fatal due to the hyperplasia that leads to liver and renal failure (265, 423). Recent observations, however, indicate that infection with *L. infantum* and other visceralising *Leishmania* spp. remain subclinical in up to 85% of infected individuals, producing comparatively mild oligosymptomatic disease that may spontaneously resolve (37, 40, 41, 269, 307). It also has become clear that sandfly saliva may modulate the course of infection of *L. infantum* and, hence, pathology of disease [see below]. Studies in Central America have shown that different concentrations of maxidilan, a salivary enzyme of the vector *Lutzomyia longipalpis*, may determine whether a *L. infantum* infection remains localised and cutaneous, or disseminates causing classical kala-azar (61, 304, 305, 568).

Disease pathogenesis and immunology. The establishment of the primary *Leishmania* infection is dependent on host susceptibility [e.g. genetic

susceptibility (9, 72-74, 306, 506), acquired immunosuppression (20) and malnutrition (179, 569)], parasite virulence [e.g. parasite strain (230, 279, 590) and species (456)] and dose and/or route of inoculation (577, 590). Evolution of infection and disease is, on the other hand, dependent on a complex set of interactions including signalling properties of the epidermis (377, 528, 529) and the cell-mediated immune response (76, 519).

Over the past years it also has become clear that sandfly saliva may be crucial in establishment of infection and disease pathogenesis. Since the pioneering study by Titus and Ribeiro (535) demonstrated that the course of *Leishmania* infection in mice is significantly enhanced by co-inoculated sandfly salivary gland extract, there have a been a series of studies confirming and extending this finding. These studies show that [i] sandfly saliva increases parasite burden, lesion size and persistence following co-inoculation with *L. major* (59, 364, 386, 533), *L. amazonensis* (533), and *L. braziliensis* (70, 105, 484); [ii] sandfly saliva is vasodilatory and enhances erythema [due to the maxadilan peptide in the sandfly vector *Lu. longipalpis*] (313, 314, 444, 458); and [iii] intraspecific variation in saliva components determines clinical response to *L. infantum* [cutaneous as opposed to visceral] (568).

The immunological basis for these findings is not fully understood, but it appears that proteins in saliva can shift the adaptive immune response from a Th1 to Th2 cell-mediated immune response [e.g. by increasing the production of interleukin [IL]-4 (59, 364) and IL-6 (516), or by inhibiting tumor necrosis factor [TNF]-α (516), interferon [IFN]-γ (364), IL-12 (364) and nitric oxide production (248, 282, 562)]. Immunomodulation may be mediated by action on the pituitary adenylate cyclase-activator polypeptide [PACAP] type 1 receptor (516), or by inhibiting protein phosphatase 1/2A (282, 562). More recently, it has been shown that pre-exposure to sandfly saliva [i.e. with salivary gland sonicate [SGS] (59) or by natural sandfly bites (60)] cancels any enhancing effect from subsequent co-inoculation of SGS with *L. major*. Pre-exposure with SGS [twice at 2 week intervals] causes: 100-fold reduction in parasite load, up to 5-fold reduction in lesion width, an

increase in delayed type hypersensitivity response [i.e. an influx of mononuclear phagocytes and eosinophils] and a reduction in IL-4 production (59, 60). The effect seems to be mediated by anti-saliva antibodies, as pre-incubation of SGS with anti-SGS antibodies has been shown to neutralise the ability of SGS to enhance the severity of *L. major* infections in naïve hosts in the same way as did pre-exposure to SGS [e.g. by reducing IL-4] (59). If this phenomenon acts under natural conditions, it may play a role in explaining variation in host susceptibility to LCL and MCL with age -- as observed in epidemiological surveys of people in ACL-endemic areas (148).

Despite significant differences between the various experimental models and humans in their immune response upon infection with *Leishmania* spp. [for review see Refs. (271, 366), several common features have been identified: [i] disease resolution is mediated by the cell-mediated immune response, not the humoral response; [ii] primary activation of T-cell subsets [mainly CD4+] is important for the development of Th1 and Th2 responses and subsequent course of infection; and [iii] there is strong evidence for a correlation between activation of different T-cell subsets and severity of disease.

Resolution of infection and protection against reinfection is regulated by the expansion of *Leishmania*-specific Th1 type CD4+ T helper cells secreting interferon [IFN] γ, interleukin [IL] 2 and tumor necrosis factor [TNF] β via Ca^{2+} and protein kinase C [PKC]-mediated signalling pathways (58, 109, 208, 410), which will activate macrophages to kill intracellular amastigotes through oxidative and nonoxidative mechanisms (76, 519). IL12 facilitates Th1 responses by stimulating the differentiation of naive [Th0] cells into Th1 cells and by serving as co-stimulus required for maximum IFNγ secretion by antigen-activated Th1 cells and natural killer cells [NK] (496). Moreover, IL12 by itself is required for NK activation (495, 503).

In contrast, susceptibility to *Leishmania* infections is regulated by the expansion of Th2 type CD4+ cells that secrete IL4, IL5, IL6 and IL10, but not IFNγ or IL2, in response to leishmanial antigens (127). IL4 and IL10 suppress the development of murine Th1 responses (254, 280), and deactivation of macrophages occurs actively through intracellular parasites [e.g.

lipophosphoglycan (541)], or indirectly through inhibitory cytokines [e.g. IL4 (267) and transforming growth factor [TGF] β (54, 55)] and other molecules [e.g. prostaglandin E_2 (26) and maxidilan (314)]. In humans with VL, IL10 rather than IL4 is responsible for the suppression of the Th1 response (268, 270).

However, key data exists that cannot be explained in terms of Th1 [i.e. resolution/resistance] and Th2 [i.e. susceptibility] T cell subsets only (18). For example, administration of IL4 [which is thought of regulating the Th2 response] reduced both lesion size and parasitaemia in *L. major*-infected BALB/C mice, and rendered animals resistant to infection (103). This outcome could be explained by the ability of IL4 to synergistically act with IFNγ to activate macrophages to kill *L. major* amastigotes (78). Similarly, the transfer of IFNγ-producing, *L. major*-specific Th1 cells can exacerbate cutaneous leishmaniasis in some circumstances (534). These studies suggest that factors accounting for resistant and susceptible parasites have yet to be identified.

Several studies now have indicated that antigen-presenting Langerhans cells play an important role upon *Leishmania* infection (377, 528): after parasite phagocytosis, Langerhans cells migrate from the site of infection to the draining lymph node, and activate antigen-specific T-cell subsets (75). Activated T-cells migrate via the blood into the lesion where infected macrophages and Langerhans cells that remained in the dermis regulate their effector activity by several mechanisms including cytokine secretion. Langerhans cell differentiation is stimulated by granulocyte-macrophage colony stimulating factor [GM-CSF] and TNFα (107), but inhibited by IL10 (183, 331). Incidentally, both GM-CSF and TNFα are also known to activate macrophages to kill intracellular *Leishmania* amastigotes (267).

There is also some evidence that CD8+ cells may play an important function in the host immune response, either by stimulating IFNγ secretion and activation of macrophages (390), or by a cytotoxic effect of CD8+ cells upon parasitised macrophages (68, 127, 137, 268, 316, 354).

Epidemiological data from patient surveys appear to confirm the Th1/Th2 dichotomy shown in experimental animal models. Briefly, LCL patients, who present limited and ulcerated skin lesions, represent the 'healing form' of the disease, i.e. they display a positive proliferative response to leishmanial antigens: the delayed-type hypersensitivity response [DTH, as measured by the Montenegro skin test] is positive, with induration size correlated to lesion size and occasionally lesion number (29, 147, 573). Patient with recurrent infections have a weaker DTH than patients with sub-clinical infections (83), as do patients with relapses when compared to those with reinfections (492). This emphasizes the role of a Th2-type response in chronic infections. There is some evidence that cytokine profiles vary with time during the course of infection: significant levels of IFNγ are produced upon leishmanial antigen presentation, but during the early phase of infection [<60 days] IFNγ production may be down-regulated, and high levels of IL10 may account for a transient period of high parasite multiplication (461) (459). There is no evidence, however, that patients with low IFNγ production are at risk of developing larger lesions or parasite dissemination. In fact, patients with lower IFNγ may have a better response to therapy with pentavalent antimonials (459). They also tend to produce significant levels of IFNγ, and a limited proportion of patients also produce IL5, though in lower amounts than for DCL and MCL patients. DCL patients display predominantly a Th2-type cytokine response, i.e. DCL patients have a complete anergy to leishmanial antigen, with a negative DTH response and lymphocytes non-responsive to leishmanial antigen. DCL patients have low levels of IFNγ and IL12, but significant serum levels of IL4, IL5 and TNFα. MCL patients display a mixture between Th1 and Th2-type cytokine responses [with high levels of IL2, IL4, IL5 and TNFα] which could explain non-resolution of disease, as the Th2-type response tends to dominate when both type of responses are activated (431, 435). MCL patients tend to have a larger DTH than LCL patients, with comparatively high serum levels of IFNγ and IL2, as well as IL5 and TNFα. Up to date, there are no immunological markers that may help to identify those LCL patients who are at risk of developing MCL. Indeed, studies showing differences in the immune response to different parasite strains or species are scarce. For example, the DTH response to leishmanial is greater in *L. braziliensis*-infected patients than in *L. panamensis*-infected

patients, even after adjusting for time of evolution and lesion type [i.e. LCL or MCL] (491).

1.2. The American leishmaniases: treatment and prevention.

Treatment. The detection and treatment of active cases currently represents the only leishmaniasis control strategy. Although the biochemical basis for their effectiveness remains unknown (330), the mainstay of anti-leishmanial therapy are still pentavalent antimonials [i.e. sodium stibogluconate [Pentostam] and meglumine antimoniate [Glucantime]], at a dosage of 20mg/kg/day for 20-28 days. Amphotericin B [Fungizone] is the second line drug of choice if patients are unresponsive to antimonials, at a recommended dosage of 1mg/kg/day for 20-40 days. The drugs are typically administered intramuscularly, although in areas where MCL is rare, intralesional inoculation is becoming more regular [Llanos-Cuentas, *personal communication*]. In all leishmaniasis-endemic countries, the official policy is to provide free treatment to all patients. This is often not feasible in practice as drugs may be in limited supply in the highly dispersed rural hamlets where ACL or AVL are prevalent. The demand for anti-leishmanial drugs during the 1980s amongst jungle migrants from Cusco, Peru, who were at particularly high risk of MCL, led remarkably to the formation of self-help patient associations. These successfully lobbied for improvements in drug availability and provided a health education service that encouraged patients to seek early treatment (241, 581). In endemic areas, such as in the departments of La Paz and Beni, Bolivia (160), early diagnosis and treatment of patients was greatly facilitated by non-governmental organizations, usually funded by international aid.

In Venezuela, control policy differs radically from the other countries Latin American countries, as the majority of all LCL patients treated by the MOH each year since 1989 have been provided with immunotherapy [i.e. a combination of heat-killed *Leishmania* promastigotes and viable BCG], rather than antimonials; and currently [i.e. in 1999] immunotherapy is applied to

about 83% of all LCL patients in Venezuela. Although trials with LCL patients in Miranda, Venezuela (119), showed that immunotherapy was as effective as standard chemotherapy [as well as being cheaper and with fewer and less severe side effects], no similar trial has been carried out elsewhere; and it remains uncertain whether immunotherapy would be suitable in populations at greater risk of MCL.

The main problems in treating ACL and AVL are that [i] clinical diagnosis can be difficult [e.g. skin lesions with aetiologies other than ACL are frequent, such as tuberculosis, leprosy, and skin carcinomas [**Plate 1H, 2F and 2I**]] (185) as more reliable diagnostic tools are rarely available in rural health posts; and [ii] antimonials and amphotericin B can have serious side effects [e.g. myalgia, pancreatitis, musculoskeletal pains, renal failure, peripheral neuropathy, hepato -and cardiotoxicity] (64), and are relatively ineffective against MCL (190, 205, 206, 320, 344, 346), especially in the latter stages.

Drugs and medical attention due to the side-effects make courses of treatment expensive, and there is an increasing number of reports on patients non-responsive to the drugs either due to the emergence of drug-resistant parasite strains (231-233, 330, 413) or to immunosuppression [as is the case in HIV-infected patients] (20). Also, there are doubts whether -despite clinical cure- available treatments clear the parasites at all (158, 244, 245, 498, 499), which in turn would lead to persistent infections and explain the frequency of relapses reported in several studies (245, 319, 492, 566, 573). The ability of *Leishmania* to persist in the host despite containment or adequate immune response has implications for immunocompromised persons: as is the case for AVL [for review see Refs. (20, 21)], ACL becomes an increasing problem in HIV-infected patients in endemic regions, notably Brazil (81, 124, 138-140, 159, 198, 332, 358, 383, 398, 471), French Guiana (393), Mexico (448), Peru (180) and Venezuela (256, 257), with some studies suggesting that LCL/HIV co-infected patients are more at risk of developing MCL than LCL patients (20, 21).

Finally, the invasiveness of the standard procedure, i.e. a lengthy course of intramuscular inoculations, means that a significant proportion of patients fail

to complete their full course of treament. Hence, most research has focused on the development of alternative dosage schedules or treatments [e.g. pentamidine, allopurinol, dapsone, mefloquine, miltefosine], including immunotherapy (39, 119, 392), thermotherapy (27, 315, 395) and phytotherapy (7, 141, 154, 214, 283, 552). Although the possibility of vaccination as method of leishmaniasis control has been extensively studied [for review see Ref. (249)], no safe and effective ACL or AVL vaccines are currently available and are unlikely for some time.

Despite the number of clinical trials that have tested different dosages, schedules, and drugs against leishmaniasis, comparisons between studies are difficult. Firstly, ACL is characterised by a tendency of lesions to self-cure, which can be as high as 75% (240). Failure to include either negative [placebo] or positive [recommended standard treatment, i.e. pentavalent antimonials] control in the studies (190, 291, 412, 521, 522) makes the interpretation of an effect of either differences in drug, dosage or schedule impossible. This is of particular importance in studies that have used small numbers of patients to evaluate treatment response. Secondly, infecting parasite species and strains are likely to vary in their sensitivities to drugs, and cure rates of ACL patients with moderate [LCL] or severe disease [MCL] are very different (320). Healing rates also depend on host factors, such as localisation and chronicity of lesions, underlying illness or concomitant infection, and acquired resistance to *Leishmania* infection. Thirdly, comparisons between studies are also difficult because the studies vary in experimental protocol [e.g. study design, duration of follow-up] and in particular in their definition of 'clinical cure'. Thus, whereas in one study clinical cure is defined as 'when lesions had >80% re-epithelialised by the first follow-up at 1.5 months' (240), other studies define it as 'complete re-epithelialisation of all lesions at the end of treatment and no reactivation or mucosal involvement during the follow-up' (412).

Leishmaniasis prevention. The principal leishmaniasis prevention strategy available is house spraying with residual insecticides (13, 145, 149, 189, 284, 310, 336, 407), which is practiced infrequently and arbitrarily according to available funds and local political pressures. Anecdotal evidence suggested

that residual insecticide spraying of houses is effective against endophilic and endophagic sandfly vectors [e.g. LCL incidence in Peru dropped dramatically during malaria and bartonellosis house spraying campaigns (144, 263)]. Recently, a randomized control trial in the Peruvian Andes conclusively showed that renewed 6-month spraying inside walls and ceilings with lambda-cyhalothrin reduced ACL incidence by 81% [95% C.I. 20-95] (145). As for other vector-borne diseases, the long-term sustainability of a house-spraying leishmaniasis control programme, whatever its efficacy, must be questioned.

In the absence of a vaccine, alternative control strategies are being explored. Firstly, a number of mostly small-scale trials have been carried out to test the effectiveness of insecticide-impregnated bed nets (16, 166, 408), curtains (16, 166, 408) or clothes (497, 523). Secondly, environmental management (187, 531) combined with the spraying of the peridomestic environment and sandfly resting sites with insecticides (111, 426, 450) has also been studied. Thirdly, the possibility to control sandfly vectors with repellents (12, 44, 94, 118, 130, 497) or biological control agents [e.g. *Bacillus thuringiensis* (151) or *Beauveria bassiana* (454, 567)] has also been investigated.

1.3. Study objectives.

Current thesis is divided into two parts, both of which investigate and discuss the epidemiology and control of zoonotic canine leishmaniasis.

Because several studies have reported high ACL infection rates in dogs there is a growing belief that domestic dogs are ACL reservoir hosts. The **Introduction** in **Part 1** will review current evidence to incriminate dogs as ACL reservoir hosts. **Chapters 2** to **5** will present the findings of a prospective epidemiological study on canine ACL in the Department of Huánuco, Peru, a *L. [Viannia]*-endemic area that has been well described in terms of vector ecology and human disease.

Domestic dogs are established reservoir hosts of *L. infantum*. Hence, one of the approaches to reduce human ZVL has been to cull infected dogs. This strategy has failed to produce the expected reduction in human disease, and the reasons why this has been the case will be discussed in the **Introduction** in **Part 2** of this thesis. **Chapter 6** will assess the potential usefulness of a rapid diagnostic test in canine leishmaniasis mass-screening surveys. Findings will then be presented on studies testing the efficacy of topical insecticides to protect dogs from both sandfly bites [**Chapter 7**] and disease [**Chapter 8**].

Thus, the aims of this thesis were two-fold:

1. To investigate whether domestic dogs are important reservoir hosts of ACL in a *L. [Viannia]*-endemic area where domestic transmission to humans has been reported [**Part 1**].

2. To identify the shortcomings of currently practiced leishmaniasis dog control programmes and to evaluate whether topical insecticides could be used to control canine leishmaniasis [**Part 2**].

Specific objectives were:

A. To test whether a polymerase chain reaction [PCR]-based diagnosis to detect *L. [Viannia]* spp. infection in dogs could be used as a mass-screening tool in epidemiological or control studies [**Chapter 2**];

B. To describe clinically, parasitologically and immunologically the course of *L. [Viannia]* infection in naturally infected [**Chapters 3 and 4**];

C. To test whether dog abundance or dog ownership is an ACL risk factor for human populations in endemic villages [**Chapter 5**];

D. To evaluate the utility of an immunochromatographic dipstick test to detect *L. infantum* infection in dogs [in comparison with ELISA and PCR] as a tool of improving the success of current dog culling strategies [**Chapter 6**];

E. To compare the effect of different topical insecticides and applications for protecting dogs from sandfly vector bites [**Chapter 7**];

F. To test the effectiveness of insecticide-impregnated dog collars to reduce the incidence of canine leishmaniasis in a community-based intervention trial [**Chapter 8**].

PART 1

CHAPTERS 2 - 5

AMERICAN CUTANEOUS LEISHMANIASIS IN DOMESTIC DOGS AND THEIR ROLE OF RESERVOIRS OF HUMAN DISEASE

Canids [e.g. the crab-eating fox *Cerdocyon thous*], and especially dogs [*Canis familiaris*], are the peridomestic reservoir hosts of zoonotic visceral leishmaniasis [ZVL] caused by *L. infantum* (126, 298, 299) [see also **Part 2**]. Because transmission of ACL is increasingly evident in the domestic environment, and because several studies have reported high infection rates of ACL in dogs, there is a growing belief that they are also reservoir hosts for ACL. If dogs are reservoir hosts of ACL, dog control may be a feasible intervention strategy, as it is for the control of ZVL in humans [see **Chapters 6-8**]. Over the following pages, the current evidence to incriminate dogs as ACL reservoir hosts will be critically reviewed.

Originally associated with forested areas (298, 301), the transmission cycle of ACL has now adapted to the domestic environment due to deforestation and urbanisation (225, 378, 563). The transition from sylvatic to domestic transmission cycle was further encouraged by the lack of a specific leishmaniasis control strategy, and the discontinuation of intervention campaigns designed for other vector-borne diseases, e.g. malaria (144, 263). Domestic transmission of *L. braziliensis* has now been demonstrated in Argentina [probably by *Lu. [Nyssomyia] intermedia*] (476-479), Bolivia [by *Lu. [verrucarum group] nuneztovari*] (308), Brazil [by *Lu. [N.] whitmani* or *Lu. [N.] intermedia*] (156, 449), Peru [probably by *Lu. [Helcocyrtomyia] tejadai*] [Davies CR *et al., unpublished data*], Venezuela and Colombia [by species in the *Lu. verrucarum group, e.g. Lu. youngi*] (15, 501). Domestic transmission of *L. panamensis* has been demonstrated in Ecuador, Colombia, Panama and Costa Rica [generally by *Lu. [N.] trapidoi* or *Lu. [Lutzomyia] gomezi*] (114, 264, 382, 389, 588, 589) and *L. peruviana* is transmitted domestically in Peru [generally by *Lu. [H.] peruensis* or *Lu. [H.] ayacuchensis*] (146, 561).

Natural *Leishmania* infections have been detected in a range of non-human hosts, principally marsupials, rodents, edentates and carnivores. However, so far only reservoir hosts for *L. infantum* [*Canis familiaris, Cerdocyon thous*

and *Didelphis marsupialis*], *L. amazonensis* [*Proechimys* spp.], *L. guyanensis* [*Choloepus didactylus*] and *L. panamensis* [*Ch. hoffmani*] have been fully incriminated (33, 229).

Since the historical accounts in the first half of the past century (93, 204, 218, 362, 363, 369, 424, 425, 468), an increasing number of studies have reported ACL infection in dogs, although many failed to confirm the identification of any *Leishmania* parasites. In particular, the status of dogs as reservoir hosts of *L. peruviana* -often reported as conclusive (281, 338, 422, 565)– has been uncertain for decades as Herrer's pioneering studies failed to isolate or characterise any parasites (259, 260). Only recently has *L. peruviana* been isolated and characterised from dogs (322, 400). Circumstantial evidence that dogs might act as reservoir hosts for ACL comes from two observations: [i] *Leishmania* strains isolated sympatrically from dogs and humans are indistinguishable; and [ii] the risk of ACL infection in dogs is correlated with the risk of ACL in humans.

Table 1.1. lists studies that have isolated and identified *Leishmania* parasites from ACL-infected dogs, the geographic distribution of which is shown in **Figure 1.1.** As for human ACL, *L. braziliensis* seems to be the most widespread *Leishmania* sp. causing ACL in dogs in the New World, and accounts for most canine ACL infections with confirmed aetiology. It has been isolated and identified from dogs in Argentina, Bolivia, Brazil, Colombia, Peru and Venezuela. Several other members of the *L. [Viannia]* subgenus have been isolated from dogs: *L. panamensis* in Colombia, Costa Rica, Ecuador, and Panama; *L. peruviana* in Peru; and *L. colombiensis* in Venezuela. *L. panamensis/L. guyanensis, L. braziliensis/L. peruviana* and *L. guyanensis/L. colombiensis* hybrids have been isolated in Ecuador, Peru, and Venezuela, respectively. Other isolates likely to be *L. [Viannia]* spp., but which remain to be identified to species have been reported in Brazil, Peru, and Venezuela. In the *Leishmania* subgenus, *L. mexicana* has been identified in Ecuador, Guatemala, Mexico, and United States; and *L. pifanoi* in Ecuador.

Figure 1.1. Distribution of American cutaneous leishmaniasis in *Canis familiaris.*

Le.(Viannia) spp.

Le.(V.) braziliensis

Le.(V.) peruviana

Le.(V.) panamensis

Le.(V.) guyanensis

Le.(V.) colombiensis

Le.(L.) mexicana

Le.(V.) lainsoni

Le.(V.) guyanensis/Le. (V.) braziliensis hybrids

Le.(V.) peruviana/Le. (V.) braziliensis hybrids
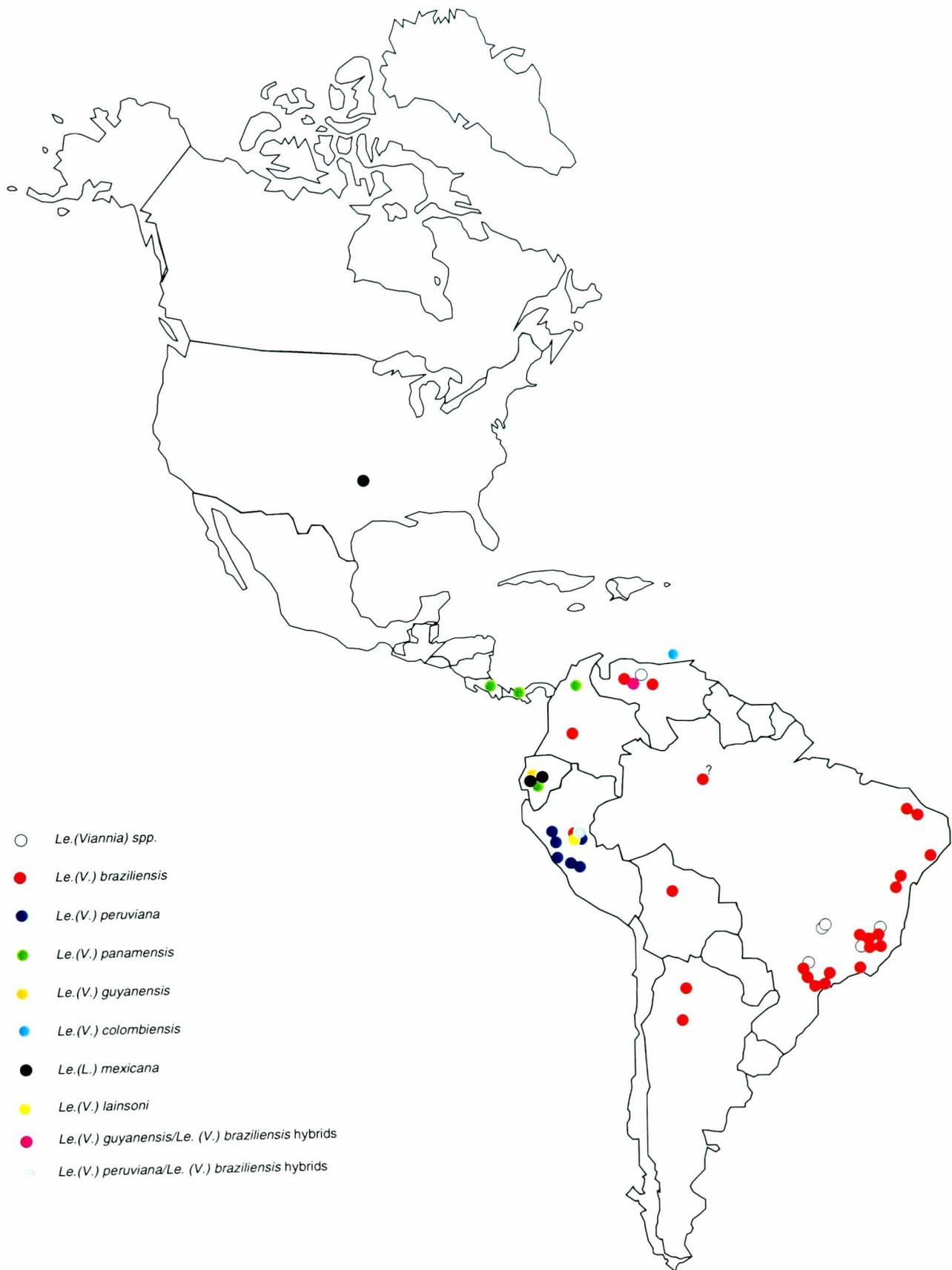
Table 1.1. American cutaneous leishmaniasis in dogs: identified isolates.

| Country	Parasite species	Identification method	Reference
Bolivia			
Alto Beni	LV spp. 1	?	(309)
Brazil			
various¶§	LB 8	MO	(226)
Amazonas✧	LV spp. 1	C, H	(394)
Bahia	LB 9	C, H, ISO, MO, X	(56)
Bahia¶	LB 3	C, H, ISO, MO, X	(133)
Bahia	LB 13	ISO, MO	(470)
Ceára¶	LB 1	ISO	(550)
Ceára¶	LB 2	DNA, ISO	(551)
Ceára¶	LB 1	C, ISO, MO	(549)
Ceára¶	LB 2	C, DNA, ISO	(548)
Ceára	LB 5	C	(155)
Espirito Santo	LB 27	C, H, MO	(191)
Espirito Santo	LB 4	ISO, MO	(192)
Espirito Santo	LB 7	C, H, MO	(504)
Espirito Santo	LB 4	C, H, MO	(504)
Minas Gerais	LV spp. 11	C, H	(170)
Minas Gerais	LV spp. ? 3	C, H	(360)
Minas Gerais¶	LB 5	ISO	(337)
Minas Gerais	LB 4	C, H, ISO	(135)
Minas Gerais	LB 5	C, ISO?	(255)
Minas Gerais✧	LV spp. ? 1	?	(361)
Minas Gerais	LV spp. 1	?	(417)
Minas Gerais✧	LV spp. 1	PCR	(420)
Minas Gerais	LV spp. 3	PCR	(216)
Parana✧	LB 3	C, H, MO	(325)
Parana	LB 5	ISO	(104)
Pernambuco	LV spp. 12	PCR	(86)
Rio de Janeiro¶	LB 4	ISO, ISOEL, RF	(326)
Rio de Janeiro	LB ?	ISO, MO, RF	(128)
Rio de Janeiro	LB 8	C, MO	(409)
Rio de Janeiro✧	LB 28	C, H, MO	(432)
Rio de Janeiro¶	LB 2	C, H, ISO, MO, X	(6)
Rio de Janeiro	LB 4	C, H, ISO, MO	(5)
Rio de Janeiro	LV spp. ?	C, H	(45)
Rio de Janeiro	LV spp. 1	DNA	(399)
Rio de Janeiro	LV spp. 5	C, H	(487)
Rio de Janeiro	LB 12	C, ISO, RF	(46)
Rio de Janeiro	LB 1	?	(272)
São Paulo	LB 2	ISO, MO	(586)
São Paulo	LA	PCR	(536)
Colombia			
various¶	LB 3	ISO	(120)
Valle del Cauca¶	LP 1	ISO	(120)
Ecuador			
Azuay	LPIF 2	C, ISO	(252)
Azuay	LM 1	C, ISO, MO	(253)
Esmeraldas	LP/LG	C, ISO	(43)
Pichincha	LP 1	C, ISO	(262)

Table 1.1. American cutaneous leishmaniasis in dogs: identified isolates (continued).

Country	Parasite species	Identification method	Reference
Panama			
Central Panama	LP 9	C, H, ISO	(167)
Peru			
Ancash	LV spp. 3	C, ISO, PCR	(322)
Huánuco	LPR 1, LB/LPR 10	C, ISO, PCR	(400)
Lima	LV spp. 2	C, ISO, PCR	(322)
United States of A.			
Oklahoma	LM 1	ISO	(292)
Venezuela			
-¶	LV spp. 3	DNA, PCR	(245)
Cojedes	LV spp. 3	C, H, X	(3)
Cojedes	LB 2	C, H, ISO, MO	(5)
Lara	LV spp. 9	C, H	(80)
Lara¶	LB/LG 12	ISO	(134)
Nueva Esparta	LC 1	ISO	(161)
Vale Hondo¶§	LB 1	MO	(226)

NOTE. The studies reporting canine ACL of known etiology are listed by country and state. Unless otherwise stated (¶,✧), studies were carried on random dog samples. Also given are methods of parasite isolation and identification, and parasite species (number of *Leishmania* isolates identified). Abbreviations are C: culture; DNA: DNA probes; H: hamster inoculation; ISO: isoenzyme electrophoresis; ISOEL: isoelectric focusing; LA: *L. amazonensis*; LB: *L. braziliensis*; LB/LG: *L. braziliensis/L. guyanensis* hybrid; LB/LPR: *L. braziliensis/L. peruviana* hybrid; LC: *L. colombiensis*; LG: *L. guyanensis*; LM: *L. mexicana*; LP: *L. panamensis*; LP/LG: *L. panamensis/L. guyanensis* hybrid; LPR: *L. peruviana*; LPIF: *L. pifanoi*; LV: *L. (Viannia)* spp.; MO: monoclonal antibodies; PCR: polymerase chain reaction; RF: restriction enzyme electrophoresis; X: xenodiagnosis. ¶, characterization and identification of parasite isolates only; ✧, non-random dog sample, i.e. dogs with cutaneous lesions and/or scars; §, sample was part of a larger study assessing the distribution of *Leishmania* spp. in the New World. Out of 227 isolates identified as *L. braziliensis* by monoclonal antibodies, 53 came from dogs, mainly from suburban areas of Brazil.

Of course, the identification of parasites from dogs does not distinguish whether dogs are accidental or reservoir hosts. Similarly, the reported coincidence between households with ACL patients and the presence of infected dogs (192) reflects the fact that humans and dogs are likely to be exposed in the same way to the sandfly vector, but is not evidence for dogs being a reservoir of disease. Furthermore, most of the studies reporting ACL infections in dogs fail to report comparable data on the *Leishmania* infection rates of other animals found in the domestic environment [see **Chapter 5**]. For example, *L. braziliensis* has been isolated or detected in cats [*Felis*

domesticus] (419), equines [*Equus caballus* and *Equus asinus*] (3, 4, 49, 79, 195), rodents [e.g. *Akodon arviculoides* and *Oryzomys* spp.], and opossums [*Didelphis marsupialis*] (228); and both *L. panamensis* and *L. peruviana* have been isolated from opossums and rodents [e.g. *Heteromys desmarestianus, Akodon* sp. and *Phylottis andinum*] (229).

Direct evidence for a reservoir role for dogs depends on the measurement of three parameters, which together determine the probability that a domestic dog population plays a significant role in ACL epidemiology (286): [i] the prevalence of ACL infections in dogs; [ii] the biting rate of sandfly vectors on dogs; and [iii] the infectiousness of ACL-infected dogs to sandfly vectors. Each of these parameters is examined below. But in general, the reported measurements of prevalence are difficult to interpret, measurements of dog biting rate and dog infectiousness are scant, and the threshold values required for all three parameters in order to incriminate dogs as ACL reservoir hosts need to be determined by mathematical modelling.

Measurements of prevalence. Isolation and identification of parasites provides a conclusive [i.e. there are no 'false positives'], but insensitive test for *Leishmania* infection in dogs, [i.e. there are many 'false negatives'], and measurements of prevalence typically depend on less specific diagnostic tools. These include clinical diagnosis [the detection of characteristic lesions or scars], parasitological diagnosis [usually by microscopic inspection of impression smears from lesions], and immunological diagnosis [detection of a serological or cell-mediated immune response]. Difficulties associated with each diagnostic test are discussed in **Chapter 2**.

Table 1.2. lists reported studies that provide estimates of the prevalence of ACL in dogs surveyed in different endemic areas (453). These estimates depend on the diagnostic test used, and the studies are divided accordingly. A small number of studies have used more than one diagnostic test, therefore appearing more than once in the table, and permit direct comparisons of the 'sensitivity' [note: the studies listed in **Table 1.2.** give ACL prevalence; strictly speaking the sensitivity can not be estimated as a diagnostic gold standard does not exist] of the diagnostic tests used. For

example, in eight out of nine direct comparisons the enzyme-linked immunosorbent assay [ELISA] appeared to be more sensitive than the immunofluorescence antibody test [IFAT]. Indirect comparisons of 'sensitivity' can also be made by comparing the results for all reported surveys in **Table 1.2.** For example, the proportion of dogs with clinical symptoms ranges from nil to 0.409 [amongst the 47 surveys reported] with a median of 0.082 [SE±0.017]. In contrast, the median proportion of dogs with a positive parasitological diagnosis is only 0.049±0.024 [n=19; range: 0-0.364] by microscopic detection of amastigotes in impression smears or aspirates, or 0.072±0.037 [n=5; range: 0-0.197] by using the polymerase chain reaction directly on skin samples. Reported seroprevalences tend to be highest using ELISA, with a median of 0.167±0.043 [n=16; range: 0.063-0.589], compared to median of 0.080±0.027 [n=37; range: 0-0.636], and 0.000 [n=3] using IFAT or the complement fixation test [CF], respectively. The only study to use the direct agglutination tests [DAT] for diagnosing ACL in dogs recorded a prevalence of 0.382. The median reported prevalence using the Montenegro skin test [MST] is 0.286±0.066 [n=18; range: 0-0.856].

However, these reported estimates of ACL prevalence in dog populations [**Table 1.2.**] must be treated with caution because many are based on non-random [e.g. clinically positive] sampling of dog populations (309, 325, 359, 361, 394, 420, 432, 588), mainly designed to provide parasite isolates for species identification and characterisation (120, 133, 134, 162, 170, 226, 245, 337, 470, 548-551).

Table 1.2. American cutaneous leishmaniasis in dogs: reported infection rates.

Prevalence	Country	Reference
Clinical diagnosis		
0.409 (9/22)^{LV}	Lara (VZL)	(80)
0.375 (18/48)	Rio de Janeiro (BR)	(355)
0.375 (18/48)^{LV}	Lima (PR)	(259)
0.322 (165/513)^{LV}	Lima (PR)	(259)
0.320 (8/25)	Rio de Janeiro (BR)	(409)
0.315 (85/270)	Rio de Janeiro	(46)
0.308 (8/26)	Espirito Santo (BR)	(504)
0.300 (15/50)^{LV}	Rio de Janeiro (BR)	(399)
0.283 (17/60)^{LV}	Rio de Janeiro (BR)	(47)
0.259 (21/81)	Rio de Janeiro (BR)	(5)
0.250 (4/16)	Rio de Janeiro (BR)	(500)
0.247 (46/186)	Espirito Santo (BR)	(191)
0.208 (33/159)^{LV}	Rio de Janeiro (BR)	(45)
0.195 (8/41)^{LV}	Espirito Santo (BR)	(196)
0.159 (7/44)	Espirito Santo (BR)	(504)
0.152 (5/33)	Paraná (BR)	(30)
0.143(5/35)	Amazonas (BR)	(25)
0.140 (6/43)^{LV}	Cojedes (VZL)	(3)
0.133 (13/98)	Bahia (BR)	(57)
0.130 (7/54)^{LV}	Espirito Santo (BR)	(194)
0.127 (7/55)^{LB}	Rio de Janeiro (BR)	(272)
0.125 (7/56)	Espirito Santo (BR)	(192)
0.111 (73/659)$^{LB,LPR,LB/LPR}$	Huánuco (PR)	¥
0.082 (52/631)^{LV}	Minas Gerais (BR)	(417)
0.078 (8/103)	Cojedes (VZL)	(5)
0.076 (27/355)^{LV}	Espirito Santo (BR)	(170)
0.067 (2/30)	São Paulo (BR)	(586)
0.066 (6/91)	Minas Gerais (BR)	(527)
0.062 (7/113)	Minas Gerais (BR)	(418)
0.058 (2/34)^{LP}	Pichincha (ECR)	(167)
0.057 (5/88)	Minas Gerais (BR)	(255)
0.047 (7/148)^{LV}	Minas Gerais (BR)	(216)
0.045 (6/132)	Paraná (BR)	(510)
0.045 (5/112)	Pará (BR)	(486)
0.043 (1/23)	Rio de Janeiro (BR)	(396)
0.042 (9/214)^{LV}	Rio de Janeiro (BR)	(487)
0.041 (12/293)^{LV}	Lima (PR)	(259)
0.033 (11/333)^{LP}	Central Panama (PAN)	(262)
0.031 (8/261)	Rio de Janeiro (BR)	(335)
0.029 (2/70)	Amazonas (BR)	(242)
0.023 (5/215)	Rio de Janeiro (BR)	(48)
0.010 (7/697)	Minas Gerais (BR)	(135)
0.002 (1/643)^{LV}	Ancash (PR)	§
0.000 (0/310)	Rio de Janeiro (BR)	(334)
0.000 (0/139)	Pernambuco (BR)	(88)
0.000 (0/65)^{LPIF}	Azuay (ECR)	(252)
0.000 (0/5)$^{LM?}$	Trepoda (DOM)	(277)

0.082±0.017 (median±S.E.)
[n=47; range:0-0.409]

Table 1.2. American cutaneous leishmaniasis in dogs: reported infection rates.

Prevalence	Country	Reference
Parasite detection		
(i) PCR		
0.197 (12/61)[LV]	Pernambuco (BR)	(86)
0.119 (8/67)[LV]	Ancash (PR)	(322)
0.072 (7/97)[LV]	Lima (PR)	(322)
0.000 (0/72)[LV]	Piura (PR)	(322)
0.000 (0/39)[LV]	Lima (PR)	(322)
0.072±0.037 (median±S.E.)		
[n=5; range: 0-0.197]		
(ii) Biopsy smears		
0.364 (8/22)[LV]	Lara (VZL)	(80)
0.291 (45/158)[LV]	Lima (PR)	(322)
0.173 (54/312)	Espirito Santo (BR)	(193)
0.172 (32/186)	Espirito Santo (BR)	(191)
0.163 (8/49)	Espirito Santo (BR)	(193)
0.125 (8/64)[LP]	Central Panama (PAN)	(262)
0.125 (7/56)	Espirito Santo (BR)	(192)
0.067 (2/30)[LM]	Azuay (ECR)	(253)
0.057 (5/88)	Minas Gerais (BR)	(255)
0.049 (2/41)[LV]	Piura (PR)	§
0.047 (7/148)[LV]	Minas Gerais (BR)	(216)
0.034 (1/29)[LV]	Lima (PR)	§
0.032 (3/93)	Bahia (BR)	(56)
0.030 (24/800)[LV]	Minas Gerais (BR)	(360)
0.000 (0/35)	Amazonas (BR)	(25)
0.000 (0/33)[LV]	Ancash (PR)	§
0.000 (0/23)	Rio de Janeiro (BR)	(396)
0.000 (0/12)[LV]	Lima (PR)	§
0.000 (0/10)[LV]	Lima (PR)	§
0.049±0.024 (median±S.E.)		
[n=19; range: 0-0.364]		
(iii) Histology		
0.043 (1/23)[LV?]	Rio de Janeiro (BR)	(396)
Serology		
(i) CF		
0.000 (0/697)	Minas Gerais (BR)	(135)
0.000 (0/355)[LV]	Minas Gerais (BR)	(170)
0.000 (0/170)[LV?]	Minas Gerais (BR)	(82)
0.000 (median)		
[n=3]		
(ii) DAT		
0.382 (13/34)[LP]	Pichincha (ECR)	(167)

Table 1.2. American cutaneous leishmaniasis in dogs: reported infection rates.

Prevalence	Country	Reference
(iii) ELISA		
0.589 (10/17)^{LM}	Chimborazo (ECR)	(384)
0.550 (11/20)^{LP,LG}	Manabi (ECR)	(384)
0.412 (40/97)	Pará	(486)
0.390 (62/159)^{LV}	Rio de Janeiro (BR)	(45)
0.328 (19/58)^{LM}	Chimborazo (ECR)	(372)
0.233 (21/90)^{LV}	Ancash (PR)	(243)
0.216 (11/51)^{LV}	Ancash(PR)	§
0.180 (35/194)^{LV}	Rio de Janeiro (BR)	(487)
0.153 (11/72)	São Paulo (BR)	(367)
0.147 (5/34)^{LP}	Pichincha (ECR)	(167)
0.141 (26/184)^{LB}	Paraná (BR)	(104)
0.102 (22/215)	Rio de Janeiro (BR)	(48)
0.080 (8/100)^{LB}	Bahía (BR)	(203)
0.078 (4/55)^{LB}	Rio de Janeiro (BR)	(272)
0.069 (18/261)	Rio de Janeiro (BR)	(335)
0.063 (10/159)	Paraná (BR)	(104)

0.167±0.043 (median±S.E.)
[n=16; range:0.063±0.589]

Prevalence	Country	Reference
Serology		
(iv) IFAT		
0.636 (21/33)	Paraná (BR)	(30)
0.632 (86/136)	Ceará (BR)	(155)
0.433 (26/60)^{LV}	Rio de Janeiro (BR)	(47)
0.378 (17/45)	Rio de Janeiro (BR)	(50)
0.302 (48/159)^{LV}	Rio de Janeiro (BR)	(45)
0.265 (9/34)^{LP}	Pichincha (ECR)	(167)
0.260 (13/50)^{LV}	Rio de Janeiro (BR)	(399)
0.244 (66/270)	Rio de Janeiro (BR)	(46)
0.229 (11/48)	Rio de Janeiro (BR)	(355)
0.203 (30/148)^{LV}	Minas Gerais (BR)	(216)
0.188 (3/16)	Rio de Janeiro (BR)	(500)
0.187 (26/139)	Pernambuco (BR)	(88)
0.182 (24/132)	Parana (BR)	(510)
0.182 (27/148)	Minas Gerais (BR)	(217)
0.169 (35/207)^{LV}	Rio de Janeiro (BR)	(487)
0.119 (37/310)	Rio de Janeiro (BR)	(334)
0.086 (32/373)	Rio de Janeiro (BR)	(128)
0.083 (6/72)	São Paulo (BR)	(367)
0.080 (7/88)	São Paulo (BR)	(215)
0.071 (4/56)	São Paulo (BR)	(537)
0.069 (7/102)	Pará (BR)	(486)
0.048 (5/104)^{LB}	Bahia (BR)	(203)
0.039 (35/905)	Goiás (BR)	(381)
0.033 (7/215)	Rio de Janeiro (BR)	(48)
0.032 (20/617)^{LV}	Minas Gerais (BR)	(417)
0.029 (1/35)	Amazonas (BR)	(25)
0.026 (10/389)	São Paulo (BR)	(275)
0.018 (73/4110)	Rio de Janeiro (BR)	(402)
0.018 (1/55)^{LB}	Rio de Janeiro (BR)	(272)
0.014 (10/697)	Huilla (CO)	¥
0.063 (26/407)	Minas Gerais (BR)	(135)
0.004 (1/261)	Rio de Janeiro (BR)	(335)

Table 1.2. American cutaneous leishmaniasis in dogs: reported infection rates.

Prevalence	Country	Reference
Serology		
(iv) IFAT (continued)		
0.003 (1/327)	Goiás (BR)	(340)
0.000 (0/973)	São Paulo (BR)	(493)
0.000 (0/170)$^{LV?}$	Minas Gerais (BR)	(82)
0.000 (0/77)	São Paulo (BR)	(537)
0.000 (0/10)	Distrito Federal (BR)	(547)
0.000 (0/10)	Distrito Federal (BR)	(401)
0.076±0.027 (median±S.E.)		
[n=38; range: 0-0.636]		
MST		
0.856 (83/97)	Pará	(486)
0.813 (13/16)	Rio de Janeiro (BR)	(500)
0.667 (40/60)^{LV}	Rio de Janeiro (BR)	(47)
0.644 (29/45)	Rio de Janeiro (BR)	(50)
0.478 (76/159)^{LV}	Rio de Janeiro (BR)	(45)
0.409 (9/22)^{LV}	Lara (VZL)	(80)
0.389 (105/270)	Rio de Janeiro (BR)	(46)
0.375 (18/48)	Rio de Janeiro (BR)	(355)
0.307 (27/88)	Minas Gerais (BR)	(255)
0.264 (24/91)	Minas Gerais (BR)	(527)
0.200 (10/50)^{LV}	Rio de Janeiro (BR)	(399)
0.102 (21/205)^{LV}	Rio de Janeiro (BR)	(487)
0.088 (19/215)	Rio de Janeiro (BR)	(48)
0.071 (4/56)	São Paulo (BR)	(537)
0.043 (1/23)	Rio de Janeiro (BR)	(396)
0.009 (1/107)	Minas Gerais (BR)	(418)
0.004 (1/261)	Rio de Janeiro (BR)	(335)
0.000 (0/77)	São Paulo (BR)	(537)
0.286±0.066 (median±S.E.)		
[n=12; range: 0-0.667]		

Table 1.2. NOTE. The studies reporting canine ACL prevalence rates are listed by country and state, and diagnostic method used. All studies were carried out on random dog samples in *L. braziliensis*-endemic areas (as determined by other isolate characterization studies). Also are given the median±S.E. of all reported studies using a particular diagnostic method, thereby allowing indirect comparison of sensitivity between the methods. Abbreviations: BR, Brazil; CF, complement fixation test; DAT, direct agglutination test; DOM, Dominican Republic; ECR, Ecuador; ELISA, enzyme-linked immunosorbent assay; IFAT, immunofluorescence antibody test; LA, *L. amazonensis;* LB, *L. braziliensis;* LB/LPR, *L. braziliensis/L. peruviana* hybrid; LG, *L. guyanensis;* LM, *L. mexicana;* LP, *L. panamensis;* LPR, *L. peruviana;* LPIF, *L. pifanoi;* LV: *L. (Viannia)* spp.; MST, Montenegro skin test; PAN, Panama; PCR, polymerase chain reaction; PR, Peru, VZL, Venezuela. ¥ Nicholls, *unpublished.*

The biting rate of sandfly vectors on dogs. There appear to be no studies that directly measure the biting rate of ACL vectors on dogs. However, the relative attractiveness of dogs has been measured for a number of ACL vectors, such as *Lu. whitmani* (101). The proportion of blood meals taken from dogs [the dog blood index] has been measured for some populations of ACL sandfly vectors, such as *Lu. peruensis* (404). This index, usually estimated by the detection of dog antigens [by ELISA or the precipitin test] in wild-caught, bloodfed sandflies (404), will depend on the relative abundance of dogs [in comparison with other potential bloodmeal sources], sandfly host preferences, and the accessibility of dogs to sandflies (380, 388, 404, 443). Accessibility is determined by factors such as sandfly dispersal (10, 17, 387, 442), and dog ecology. This may explain why, for example, ZVL prevalence has been found to be higher in 'hunting' than in 'urban/domestic' dogs (126, 587). Finally, the probability of collecting a sandfly with dog blood will additionally depend on the location of the trap, and, hence, an objective measurement of the dog blood index of a sandfly population is therefore rarely attainable (11).

The infectiousness of dogs to sandflies. Although it is axiomatic that ACL-infected dogs can only be reservoir hosts if they are infectious to sandflies, none of the studies listed in **Tables 1.1.** and **1.2.** measured this parameter. Only two small xenodiagnosis studies have been reported and they are outlined in detail in **Chapter 5**. However, no generalisations can be made from these two studies, because a low number of replicates was used and because sandflies were fed artificially on active ACL lesions. Also, the *L. braziliensis* infection rate of flies fed on dogs was less than the one reported for flies feeding on human ACL patients or opposums [**Chapter 5**].

The course of ACL infection in dogs. The studies reviewed here imply that dogs are readily infected with ACL, but −as opposed to canine ZVL (1, 96-99, 115, 177, 178, 290, 352, 353, 427, 429, 439-441, 513, 553)- little is known about the clinical, parasitological and immunological course of canine ACL infections [see **Chapter 3**]. Combined with xenodiagnosis studies, this information could identify correlates of infectiousness, enabling putative dog control programs to maximise their effectiveness by focusing on those dogs

that are infectious (176) [see **Chapter 6** and **8**]. The course of infection will also determine the potential reservoir role of dogs; i.e. the role will be maximised if infectiousness is relatively permanent, rather than if ACL infected dogs tend to recover from infection and infectiousness. If infectiousness is transient, dogs will act as reservoirs only if there is a sufficient recruitment rate of susceptible dogs into the population. This rate in turn will depend on dog demography and on the rate at which infected dogs recover their susceptibility upon healing.

Excluding studies where the infecting parasite was not identified (350), descriptions of the course of *L. braziliensis* infection in dogs come from only four experimental studies (135, 212, 213, 434). In the first study (135), lesions developed in 3/4 inoculated dogs after 4, 7 or 8 months, and seroconversion occurred within 2-4 months after appearance of lesions [i.e. giving a serological pre-patent period of 8-11 months]; the fourth dog seroconverted within four months but clinical symptoms [a mucosal ulcer] were not detected for another 12 months. In the second study (434), 3/6 inoculated dogs developed nodules by 22 days, and 5/6 had ulcerated lesions two months post-inoculation; 6/6 seroconverted by day 36 and 5/6 had a positive MST response five months post-inoculation. In the third study (212), all 10 inoculated dogs had a positive MST response within four months post-inoculation. In the last study (213), 18/24 inoculated dogs seroconverted within four months post-inoculation and all developed lesions. These results should be treated with caution, as there is considerable variation between the studies protocol, sample sizes were small, and the immune response to parasite inoculation may differ from the response to natural infections. However, they are within the same range of pre-patent periods [3-25 months] reported for dogs infected with *L. infantum* (1, 96-99, 177, 178, 352, 353, 427, 429, 439-441).

The only information on the natural serorecovery rates of dogs infected with ACL comes from a single field study that showed that 3/14 *L. braziliensis*-infected dogs healed spontaneously 2-3 months after parasite demonstration, with complete clinical and serorecovery (432) [**Chapter 4**]. However, lesions and positive IFAT reappeared in all three dogs during the 11 months follow-

up. It has been suggested that dog immunity to ACL is dependent on host genetic susceptibility as is the case for humans, because some dog breeds seem to differ in their response to infection (513, 517). The proportion and epidemiological significance of ACL-resistant dogs in natural populations is unclear, and it remains to be established whether they may be infectious to the sandfly vector. Other factors likely to affect a dog's immune response include host malnutrition (179), and parasite variation [i.e. strain or species] (279, 456). For example, the course of L. braziliensis infection in dogs can be compared with the results of three reported studies on experimental infections of dogs with L. peruviana (260, 322, 351): L. peruviana infections seem to be more benign than L. braziliensis infections, frequently causing asymptomatic infections, with parasitological and clinical pre-patent periods of at least 2 and 2.5 months, respectively. There appear to be no reported studies on the immunological or parasitological course of L. panamensis infection in dogs, but positive parasite diagnosis of naturally infected dogs are typically associated with clinical symptoms, e.g. ulcers or depigmentation (262). Lesions caused by L. panamensis can persist for at least 45 months, although self-healing within 7 or 11 months has also been observed (262).

Dogs as risk factors for ACL. Finally, the most convincing evidence for incriminating dogs as reservoir hosts of ACL should come from studies demonstrating that either dog ownership or dog abundance are risk factors for ACL in humans. The epidemiological evidence to date is inconsistent as two case-control studies of ACL in domestic transmission settings have shown a positive association between dog ownership and human ACL in Argentina and Costa Rica, but no association was detected in two other studies in Argentina and Peru [outlined in detail in **Chapter 5**]. Direct incriminatory evidence would also come from a dog control trial. Were dogs reservoirs of ACL, dog control programs should be effective in reducing not only canine incidence but human incidence as well. However, so far there is no evidence that targeting dogs with ACL is effective.

Over the next four chapters, findings of a prospective study investigating the epidemiology of canine ACL in a well-studied area of human ACL transmission will be presented. Specifically, the merits of PCR as a mass-screening tool [as

compared to clinical and serological diagnosis] in epidemiological studies will be tested and discussed [**Chapter 2**]. The findings presented in **Chapter 2** are then placed into an epidemiological context in **Chapter 3** and **4**, where the data will be used to describe the course of infection and disease of ACL in dogs. The findings will also be used to discuss their meaning for human disease transmission in terms of ACL prevention and control. Using data available on human ACL and dog ownership patterns, we also investigated whether dogs represent a significant risk factor for human ACL [**Chapter 5**].

CHAPTER 2:
The use of the polymerase chain reaction [PCR] as a diagnostic tool in mass-screening surveys to detect Leishmania [Viannia] spp. infection in domestic dogs [Canis familiaris].

Abstract

Several studies have suggested that the PCR could be used in epidemiological mass-screening surveys to detect Leishmania [Viannia] spp. infection in human and animal hosts. Dogs from a L. braziliensis and L. peruviana-endemic area were screened for ACL infection using established PCR-based and ELISA protocols. PCR detected L. [Viannia] infection in a total 90/1066 [8.4%] dogs: 32/368 [8.7%], 65/769 [8.5%] and 7/42 [16.7%] were PCR positive on whole blood, buffy coat and bone marrow aspirates, respectively. ELISA detected infection in 221/1059 [20.9%] of tested dogs. The high prevalence of L. [Viannia] parasites detected by PCR in blood of both asymptomatic [7.5%] and symptomatic [32%] dogs provides further circumstantial evidence for their suspected role as reservoir hosts of ACL, and suggests that haematogenous dissemination of parasites may be a more common pathological phenomenon than previously acknowledged. However, the low sensitivity of PCR [as compared to ELISA] indicates that PCR cannot be used for mass-screening in ACL epidemiological studies. Unless more sensitive PCR protocols were to be developed, its use will probably be restricted to diagnosis of active [canine and human] cases and to the parasitological monitoring of patients after chemotherapy.

2.1. Introduction.

In order to carry out epidemiological surveys to consider putative canine leishmaniasis control strategies, sensitive and specific tests for identifying ACL-infected dogs are paramount. Serological tests are the standard tools for identifying *Leishmania*-infected dogs during epidemiological mass-screening surveys, because clinical and parasitological diagnoses [eg. biopsy smears, parasite culture] are characteristically insensitive, and because ACL infections in dogs are frequently asymptomatic [**Part 1: Introduction**]. However, difficulties associated with the interpretation of cross-sectional seroprevalence data are that [i] serology is prone to non-specific cross-reactions ['false positives', e.g. to *Trypanosoma cruzi* or *L. infantum* infection (112, 113, 556)]; [ii] there may be a delay between infection and seroconversion (177, 178, 439, 440); [iii] a fraction of infected dogs may never seroconvert [e.g. due to innate resistance] (178, 437); and [iv] seroconversion may not be permanent [e.g. due to development of humoral or cell-mediated immune response] (178). Thus, sensitivity and/or specificity of serological tests with respect to infection can vary quite considerably, and may underestimate true prevalence and incidence of disease, and hence the scale of the control problem.

It has been suggested that PCR-based methods for *Leishmania* diagnosis may provide the best gold standard for determining the presence and identity of leishmanial infections not only when diagnosing active cases, but also for monitoring parasitological cure of patients after chemotherapy and as a mass-screening tool to detect *Leishmania* infections in vertebrate hosts or sandfly vectors (421, 511). Various PCR protocols have been reported for the detection of ACL-causing *Leishmania* in humans using either purified DNA [from cultured parasites (152, 153)] or clinical specimens [including lesion and scar biopsies (36, 62, 153, 251, 327, 328, 371, 433, 447, 465, 485, 498, 499, 515, 583), or blood (164, 184, 244, 245, 430)] and have consistently proven to be more sensitive than other diagnostic methods, including *in vitro* culture of biopsies, biopsy smears and hamster inoculation [**Table 2.1.**]. However, at present only one large study has been reported

where PCR was used as a diagnostic mass-screening tool to detect ACL in humans or putative animal reservoirs (322). *L. [Viannia]* parasites were detected in skin aspirates or biopsies from 15/276 [5.4%] dogs, 4/153 [2.6%] *Akodon* spp., 2/72 [2.8%] *Didelphis albiventris,* 1/499 [0.2%] *Phyllotis andinum,* 0/178 *Mus musculus,* 0/8 *Oryzomys* spp. and 0/8 *Rattus rattus.* A second diagnostic test for comparative data on infection rates was not carried out, and hence no conclusions about the PCR assay's sensitivity and specificity can be made.

In the work presented in this chapter the utility of PCR as a diagnostic mass-screening tool in epidemiological studies was evaluated. Specifically, the sensitivity and specificity of established PCR protocols on canine blood and bone marrow were compared to an ELISA for detecting *L. [Viannia]* spp. infection in dogs.

2.2. Materials & Methods.

Sample population and dog sampling. Dogs came from 18 villages in the Department of Huánuco, a *L. braziliensis* and *L. peruviana*-endemic area in Peru [10°00' S, 76°15' W] [**Map 1** and **Plates 3A-H**]. Dogs were surveyed between April to November 1997, June to September 1998 and between April to June 1999 either by house-to-house visits or in the village-square during rabies vaccination campaigns carried out by the local Ministry of Health [see **Chapter 4** for description of the study site]. None of the dogs belonged to a recognisable breed and all were guard or hunting dogs. Interviews with owners were carried out to provide information about the number of dogs kept, their age and residence time in the valley. The demography of the canine study population and canine ACL epidemiology will be addressed in the following chapter [**Chapter 4**].

Table 2.1. Published studies using PCR to detect American cutaneous leishmaniasis in humans.

Disease	PCR primers*	PCR	Culture	BMS	HIS	MST	Serology	Ref.
CL^{LV}	4	14/18^{LB}	13/18	12/18	-	16/18	9/13	(327)
CL^{LV}	4	15/24^{LB}	7/20	0/24	-	-	-	(327)
CL^{LV}	2	14/19^{LB}	11/19	5/19	2/8	15/17	-	(153)
CL^{LV,LMEX}	1	226/233^{LB}	100/233	147/233	-	233/233	-	(465)
CL^{LV}	5	58/60^{LB}	-	-	-	-	-	(538)
CL^{LV}	4	18/25	14/25	10/24	6/25	-	-	(100)
CL		14/24	-	13/16	-	-	-	(250)
CL/DCL^{LV}	4	11/17^{LB}	8/13	8/13	-	-	-	(542)
CL^{LV,LMEX}	1, 4	20/24^{LB,SC}	1/24	16/24	-	-	-	(62)
CL^{LV}	5	19/19^{LB}	9/9	9/17	7/19	20/20	-	(498)
CL^{LV,LMEX}	3	20/32^{LB}	-	-	-	-	-	(251)
CL^{LV,LMEX}	2, 6	18/26^{LB}	9/26	-	-	-	-	(258)
CL	4	21/30^{LB}	-	6/30	-	-	-	(455)
CL	5	23/35^{LB}	-	22/35	-	-	-	(343)
CL^{LV,LMEX}	2, 4	9/23^{LB}	10/23	4/24	5/23	3/5	2/24	(36)
CL^{LV}	7	13/13^{LB,SC,LA}	11/12	7/13	-	-	-	(357)
CL^{LV}	5	178/184^{LB}	86/144	76/176	65/175	184/184	-	(433)
CL^{LV}	2	12/14	-	-	-	14/14	-	(485)
CL^{lv}	2, 5	53/99	-	-	-	-	-	(464)
CL^{LV}	2	10/26^{LB}	21/26	-	-	26/26	26/26	(150)
CL	2	21/40	56/115	18/37	-	76/92	-	(447)
CL^{LG}	5	35/35	35/35	35/35	-	35/35	-	(469)
CL^{LV}	2	193/255^{LB}	141/255	119/255	-	-	-	(572)
CL^{LV}	2	20/44^{LB}	10/44	2/44	-	-	-	(572)
MCL^{LV}	4	10/12^{LB}	5/12	5/12^{LB}	-	11/11	6/6	(327)
MCL^{LV}	4	7/9^{LB}	2/4	2/9^{LB}	-	-	-	(327)
MCL^{LV}	4	2/2^{LB}	-	-	-	-	-	(542)
MCL^{LV}	5	24/34^{LB}	5/17	4/13	7/30	24/24	-	(433)
MCL^{LV}	4	30/36^{LB}	-	-	-	-	-	(433)
Total CL		1035/1319 (78%)	542/1041 (52%)	509/1037 (49%)	85/250 (34%)	622/644 (97%)	37/63 (59%)	
Total MCL		73/93 (78%)	12/33 (36%)	11/34 (32%)	7/30 (23%)	35/35 (100%)	6/6 (100%)	

NOTE. BMS, lesion biopsy smear; CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; HIS, histology; LA, lesion aspirate; LB, lesion biopsy; LG, *Leishmania guyanensis*, LMEX, *L. mexicana;* LV, *L. (Viannia)* spp.; MCL, mucocutaneous leishmaniasis; MST, Montenegro skin test; SC, scar biopsy. *PCR primers. 1, 13A/B *Leishmania* spp.-specific primers (463); 2, B1/2, *L. (Viannia)* spp.-specific primers (152); 3, LB3C, LM3A, LC3L, LV5A (251); 4, MP1L/MP3H, *L. (Viannia)* spp.-specific primers (327); 5, OCT120: *Leishmania* spp. primers (197); 6, M1/2 *L. mexicana*-specific primers (184); 7, V1/2, I1/2, P1/2, G1/2 and B1/2 (357).

Dogs were examined for clinical signs of ACL, i.e. cutaneous lesions or scars [**Plate 4C-H**]. Impression smears were made of dermal scrapings and/or lesion biopsies from dogs with active cutaneous lesions, GIEMSA-stained and examined microscopically [light microscope, oil immersion, x100 objective] for *Leishmania* amastigotes. Blood, 2-10ml was taken from a total 1104 [1997: 553; 1998: 289; 1999: 262] dogs by venepuncture and aliquoted into sterile, EDTA-coated 10ml polypropylene tubes [**Plate 4A**]. The samples were processed 4-10hrs after collection, one of the aliquots was centrifuged at 800g for 20min and the buffy coat layer removed and stored at -20°C [i.e. buffy coat sample, BCS]; the second blood aliquot [2-3ml] was mixed with an equal volume of 6M guanidine HCl/0.2M EDTA [pH8.0] and stored at 4°C [i.e. guanidine/blood lysates, GBL] (34, 35, 91, 92, 278, 578, 579). Bone marrow was aspirated from the iliac crest from a subset of dogs [n=46] using a mixture of medetomidine [Domitor®, SmithKline Beecham, UK] and ketamine hydrochloride [Vetalar®, Parke-Davis Veterinary, UK] as anaesthetic, and stored at -20°C [i.e. bone marrow samples, BMS] [**Plate 4B**]. BCS, GBL and BMS were all prepared in 1997, as at that particular stage a PCR protocol for *L. [Viannia]* detection in dogs had to be developed which, amongst others, tested whether PCR sensitivity was associated with sample origin [i.e. either blood or bone marrow] or DNA extraction protocol [see below]. In 1998 and 1999, only BCS were prepared from dogs.

DNA extraction. *Sensitivity titration assay.* Choice of DNA extraction protocol and primers to be used for mass-screening of field samples was based on a series of sensitivity titration assays [STA]. One hundred-fold dilutions of 10^8 water-lysed *L. braziliensis* [MHOM/BR/75/M2903] were added to 200μl aliquots of guanidine dog blood lysate from a single dog, yielding a concentration range from $0.01-10^6$ parasites per spiked sample. Water was added to a separate aliquot for a negative control. DNA was extracted according to standard protocols using either phenol/chloroform [PC], Chelex100® resin [Biorad, UK] or DNeasy® DNA extraction kit [Qiagen, UK]. GBL were heated for 10min in boiling water to denature the concatenated minicircle DNA molecules which constitute most of the *Leishmania* kinetoplast DNA [kDNA] network, and allowed to cool to room temperature. After one PC extraction, DNA was back-extracted with TE [10mM Tris-HCl, 1mM EDTA,

pH8.0], then chloroform extracted and ethanol-precipitated, resuspended in 50μl TE, and stored at 4°C. Chelex and DNeasy DNA extractions were carried out according to Walsh *et al.* (564) and the manufacturer's protocol, respectively. To increase DNA yield of the Chelex extracted samples, 300μl of the extract's supernatant was ethanol-precipitated and re-suspended in 30μl TE.

Field samples. BCS and BMS were mixed with an equal volume of DNA extraction buffer [10mM TrisHCl pH8.0, 0.1M EDTA pH8.0, 0.5% SDS], proteinase K was added to a final concentration of 50μg/ml, and samples were incubated for 5hrs at 50°C. Aliquots, 200μl of GBL, BCS and BMS were taken and DNA was PC extracted as before.

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┌─────────────────────────────────────────────────────────────────┐
│                                                                   │
│              Box 1. Primers used in the PCR assays.               │
│                                                                   │
│  B1/B2. Primers are specific for all Leishmania (Viannia) spp.    │
│  kDNA but L. lainsoni. Amplification yields a full-sized kDNA     │
│  minicircle (750bp) (152).                                        │
│  B1:    5'-GGG GTT GGT GTA ATA TAG TGG-3'                         │
│  B2:    5'-CTA ATT GTG CAC GGG GGA GG-3'                          │
│  B3:    5'-TTG AAC GGG GTT TCT GTA TG-3'                          │
│                                                                   │
│                                                                   │
│  MP1L/MP3H. Primers are specific for L. (Viannia) spp. kDNA.      │
│  Amplification yields a 75bp fragment (327).                      │
│  MP1L: 5'-TAC TCC CCG ACA TGC CTC TG-3'                           │
│  MP3H: 5'-GAA CGG GGT TTC TGT ATG C-3'                            │
│                                                                   │
│                                                                   │
│  R221/R332. Primers are specific for all Leishmania spp. small    │
│  subunit ribosomal RNA genomic DNA. Amplification yields a        │
│  603bp fragment (546).                                            │
│  R221: 5'-GGT TCC TTT CCT GAT TTA CG-3'                           │
│  R332: 5'-GGC CGG TAA AGG CCG AAT AG-3'                           │
│                                                                   │
│                                                                   │
│  Min11B/Min22. Primers are specific for L. (Viannia) spp.         │
│  subtelomeric DNA. Amplification yields a 491bp fragment (207).   │
│  Min11B:     5'-GGA TCG CTG GGA ACA ATC-3'                        │
│  Min22:      5'-CAT GAA TGG CTT TCG TTT CAG-3'                    │
│                                                                   │
│                                                                   │
│  PO3/PO5. Primers amplify acidic ribosomal phosphoprotein        │
│  genomic DNA. Amplification yields a 469bp fragment (474).        │
│  PO3:   5'-GGA GAA GGG GGA GAT GTT-3'                             │
│  PO5:   5'-TCA TTG TGG GAG CAG ACA-3'                             │
│                                                                   │
└─────────────────────────────────────────────────────────────────┘
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Polymerase chain reaction [PCR]. *Sensitivity titration assay (STA).*
Spiked samples and the original culture water-lysate dilutions were amplified
using four different PCR assays [3 replicates], each one using a different set
of primer pairs: B1/B2, MP1L/MP3H, Min11B/Min22 and R221/R332 [**Box 1**].
Briefly, 1μl [2-5ng] of DNA was amplified on a Biometra Thermocycler
[Biometra, UK] in a total reaction volume of 25μl overlayed with 30μl of
mineral oil [Sigma, UK]. **Table 2.2.** summarises reaction conditions.
Amplification products were analysed by electrophoresis on 1.5% agarose
gels in 1xTAE [40mM Tris, 40mM acetic acid, 1mM EDTA, pH 8.3] buffer

Table 2.2. PCR-hybridisation reaction conditions.

	B1/B2	M1L/M3HL	Min11/Min22	R221/R332	PO3/PO5
PCR Product	whole kDNA minicircle (750bp)	kDNA minicircle fragment (75bp)	subtelomeric repeat (491bp)	ribosomal DNA repeat (603bp)	genomic DNA repeat (469bp)
Reaction Mixture					
PCR Buffer	10mM TrisHCl (pH8.3) 50mM KCl 1.5mM MgCl$_2$ 0.01% gelatin	10mM TrisHCl (pH8.3) 50mM KCl 2.0mM MgCl$_2$ 0.01% gelatin	10mM TrisHCl (pH8.3) 50mM KCl 1.5mM MgCl$_2$ 0.01% gelatin	10mM TrisHCl (pH8.3) 50mM KCl 1.5mM MgCl$_2$ 0.01% gelatin	10mM TrisHCl (pH8.3) 50mM KCl 1.5mM MgCl$_2$ 0.01% gelatin
dNTP	0.2mM each	0.2mM each	0.2mM each	0.2mM each	0.2mM each
Primer	50pmol	50pmol	50pmol	50pmol	45pmol
Taq polymerase	1 U	1 U	0.5 U	0.5 U	1 U
Cycles					
Initial Denaturation	95°C/6min	94°C/3min	95°C/30sec	95°C/5min	95°C/5min
Initial Annealing	64.5°C/2min	54°C/2min	57°C/45sec	60°C/30sec	55°C/30sec
Extension	72°C/1min	72°C/1min	72°C/1min	72°C/30sec	72°C/30sec
Denaturation	95°C/30sec	95°C/1min	95°C/30sec	95°C/30sec	93°C/30sec
Annealing	60.5°C/1min	54°C/2min	57°C/45sec	60°C/30sec	55°C/30sec
No. cycles	35	35	35	35	34
Final extension	72°C/10min	72°C/10min	72°C/10min	72°C/10min	72°C/10min
Hybridisation					
Probe	B3 oligonucleotide endlabelling (γ^{32}-P)	Positive control random primer (α^{32}-P)	Positive control random primer (α^{32}-P)	Positive control random primer (α^{32}-P)	NA
Sensitivity of PCR†					
Gel electrophoresis					
(i) Pure culture	0.1	10^5	0.001*	10	-
(ii) PC	0.8	8000	0.008	0.8	+
(iii) DNeasy	1.9	1.9 x 10^4	0.019	1.9	+
(iv) Chelex100 resin	-	-	-	-	-
(v) Chelex/ethanol	6.7 x 10^4	-	-	6.7 x 10^4	+
Hybridisation					
(i) Pure culture	0.001*	10^3	0.001*	0.1	NA
(ii) PC	0.008	80	0.008	0.8	NA
(iii) DNeasy	0.00019*	190	0.00019*	0.019	NA
(iv) Chelex100 resin	-	-	-	-	NA
(v) Chelex/ethanol	6.7 x 10^4	-	6.7 x 10^4	6.7 x 10^4	NA

NOTE. PCR and hybridisation were carried out as described in Materials & Methods. †, sensitivity of the PCR STA is given as the minimum number of parasites in the PCR sample required for successful amplification; *, most dilute sample tested for the assay; -, no amplification product see on the gel; +, amplification product seen on the gel; NA, does not apply; PC, phenol/chloroform extraction.

(482). To evaluate sample degradation or PCR inhibition, sample DNA was also amplified for a canine housekeeping gene, acidic ribosomal phosphoprotein fragment, using PO3/PO5 primers [**Box1**]. When samples did not yield amplification, they were extracted again, until positive amplification was obtained [**Plate 5B**]. Each amplification cycle included negative [no DNA, DNA from uninfected dog] and positive [water-lysates of cultures obtained from Huánuco dog isolates] controls. PCR-grade H$_2$O was used throughout. To avoid cross-contamination, separate areas were used for DNA extraction, PCR sample preparation and amplification.

Hybridisation. Agarose gels were processed according to standard procedures, i.e. in denaturation [1.5M NaCl, 0.5M NaOH] and neutralisation [1.5M NaCl, 1M Tris, pH7.4] buffer for 20min each, Southern blotted onto a nylon membrane [Boehringer Mannheim, UK], and DNA was fixed to the membrane by UV-crosslinking (482). Membranes were prehybridised at 42°C and hybridised with either a [α^{32}P]-dATP or [γ^{32}P]-ATP-labelled probe for 8-12hrs [**Table 2.2.**], then washed at 42°C or 65°C for 2x15min in 2xSSC/0.1% SDS and in 0.1xSSC/0.1%SDS, before being exposed for autoradiography 36hrs and 72hrs at -70°C (482).

Field samples. Based on the results of the STA, all field samples were amplified using a PO3/PO5 [**Plate 5B**] and B1/B2 primer pairs. Hybridisation was carried out as described above using a [γ^{32}P]-ATP-labelled B3 oligonucleotide primer probe [**Box 1**].

Enzyme-linked immunosorbent antibody tests [ELISA]. Log-phased *L. braziliensis* promastigotes [MHOM/BR/76/M2903] were harvested at a concentration of ca. $1x10^7$ cells/ml, centrifuged at 5000rpm, washed [x3] in PBS [0.14M NaCl, 1.5mM KH_2PO_4, 8mM $Na_2HPO_4.12H_2O$, 2.7mM KCl, pH7.2] and frozen at -20°C. Cells were then freeze/thawed [x3] and sonicated for 15min at 250Amp in a Soniprep sonicator [Soniprep, UK]. Antigen [10^5 promastigotes/well] was added to polysterene microtitre plates [Immunolon® 2, Thermo LabSystems, UK] in 100µl of carbonate coating buffer [15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH9.6] and incubated overnight at 4°C. Plates were washed [x3] with PBS and blocked with 100µl/well 2% milk powder in coating buffer for two hours at 37°C. They then were washed [x3] with PBS, and serum samples were added at 1/100, 1/400 and 1/800 dilutions in 100µl incubation buffer [PBS/0.05% Tween20+2% milk powder], and again incubated for two hours at 37°C. After washing [x6] with PBS/0.05% Tween20, peroxidase-conjugated, affinity-purified rabbit anti-dog IgG [Sigma, UK] was added at 1/1500 in 100µl incubation buffer, and plates were incubated for two hours at 37°C. Plates were washed [x6] with PBS/0.05% Tween20 before 100µl of substrate solution [O-phenylenediamine dihydrochloride in phosphate-citrate buffer

[55mM citric acid, 50mM $Na_2HPO_4.12H_2O$], pH5.5] was added. The reaction was stopped with 50µl 2M H_2SO_4 and plates were read at 490nm in an ELISA plate reader.

ELISA Standardisation. The method used follows Quinnell *et al.* (440). Briefly, on each plate a positive control serum was titrated two-fold from 1/20 to 1/327680. The positive control serum was assigned an arbitrary number of units/ml, 81920/ml, which was defined as the reciprocal of the highest dilution at which absorbance was greater than the mean + 3 s.d. of background [i.e. no antibody] wells. Absorbance was calculated as observed absorbance minus mean background absorbance. A standard line was fitted over the range 1/80 to 1/81920 to the positive control absorbance values using a log-logit transformation (440) Absorbances of the three test sera dilutions were expressed as log anti-*Leishmania* antibody units [LAU]/ml using the standard line, from which the test sample's geometric mean number of LAU/ml was calculated. Where the dilution curve for any test serum was noticeably non-parallel to the standard, test sera were repeated at dilutions of 1/100, 1/720 and 1/4320.

Negative and positive controls. Three groups of uninfected dog sera were used as negative controls for all diagnostic tests. The sera came from [i] dogs of various ages and breeds which had attended a veterinary clinic in Lima [Peru; n=18]; [ii] mongrel dogs from Belém [Brazil; n=13]; and [iii] dogs of various ages and breeds which had attended a veterinary clinic in Cambridge [UK; n=13]. Though *L. Viannia* has been isolated from dogs both in Peru and Brazil, Lima and Belém are not *L. [Viannia]*- endemic areas. The positive standard control serum as well as 15 other positive control sera came from lesion and biopsy smear-positive dogs surveyed in the study.

2.3. Results.

PCR sensitivity titration assay. Table 2.2. summarises the sensitivity of the PCR assays according to DNA extraction protocol and primer pairs used. Briefly, the Min11B/Min22 primer pairs were 10^6 to 10^8-fold more sensitive than the MP1L/MP3H primer pair, and 10^2 to 10^4-fold more sensitive than both B1/B2 and R221/R332 primer pairs in amplifying DNA from culture dilutions, PC or DNeasy-extracted samples [**Table 2.2.**]. None of the Chelex-only extracted samples could be amplified. PCR using PC extracted samples was 2- and $> 10^4$-fold more sensitive than reactions using DNeasy or Chelex-ethanol extracted samples respectively. Hybridisation generally increased the assay's sensitivity by 10^2-fold, but up to 10^4-fold for DNeasy extracted samples amplified with the B1/B2 primer pair [**Plate 5A**]. All but the Chelex only-extracted samples were successfully amplified with PO3/PO5. PC was used as the DNA extraction protocol for field samples as it was almost as good as the DNeasy kit in extracting parasite DNA from blood [**Table 2.2.**], but at a significantly lower economic cost. The B1/B2 primer pair was chosen for mass-screening because [i] with hybridisation it yielded the greatest sensitivity, along with the Min11B/Min22 primer pair [**Table 2.2.**]; [ii] it did yield fewer PCR product artifacts [unlike Min11B/Min22 and MP1L/MP3H]; and [iii] it has previously been tested on clinical field samples [though not blood] (153). PC combined with B1/B2 primers and B3 probe could detect parasitemias of one *L. [Viannia]* parasite/400ul canine blood.

Field samples: clinical diagnosis. Of surveyed dogs, 21/1104 [1.9%] had active cutaneous lesions, and a further 21/1104 [1.9%] had scars and/or ulcers. 17/20 [85%] dogs with active lesions were biopsy smear positive.

Field samples: reproducibility of PCR assay. As described in Materials and Methods, all samples were tested using a PCR-hybridisation protocol, with PCR amplification products being visualised by gel electrophoresis [PCR] prior to hybridisation to a *L. [Viannia]*-specific probe [HYB]. To assess the reproducibility of the used PCR-hybridisation protocol, all field samples were tested twice; when diagnostic outcomes differed, the samples were tested a

third time, the diagnostic outcome being definitive. The diagnostic outcome was defined as samples being either positive by PCR and/or HYB or negative by both PCR and HYB.

Hence, GBL were classed into four different groups [**Plate 5C** and **5D**]: they were [i] PCR+ if an amplification product could be seen on the agarose gel; [ii] PCR- if an amplification product could not be seen on the gel; [iii] HYB+ if an amplification product hybridised to the B3 probe; and [iv] HYB- if an amplification product did not hybridise to the B3 probe.

BCS and BMS were classed as either positive [PCRHYB+] or negative [PCRHYB-] by PCR-hybridisation, as the prior distinction as done for GBL could not be made due to the frequency of PCR artifacts [non-specific bands, see above]] on the agarose gel [see below and **Plate 5E** and **5F**].

Guanidine blood lysates [GBL]. A total of 794 PCR assays were carried out on 368 tested GBL samples [**Table 2.3.**]. 368 GBL samples were tested at least twice, 310/368 [84%] of the samples had the same diagnostic outcomes in both replicates, 58/368 [16%] of the samples had to be tested a third time because the outcomes of the two PCR-hybridisation assays differed [**Table 2.3.**].

353/368 [96%] of GBL were PCR- in two replicates, of which 73 of the 706 replicate samples [10%] were HYB+. Of the 15 GBL that were PCR+ in at least one replicate, 7 [47%] were consistently PCR positive with all 14 sample replicates being HYB+; of the 8 GBL that were inconsistent [i.e. were PCR+ in one replicate, PCR- in the other], 15/16 [94%] of the replicates were HYB+ [**Table 2.3.**]. Hence, there is good reason to believe that those samples with inconsistent PCR results were true positives.

288/368 [78%] GBL were consistently HYB-, all of which were PCR-. 22/368 [6%] GBL were consistently positive, with 21/44 [48%] of the replicate samples being PCR+. Of the remaining 58 samples that were inconsistent, 57 [98%] were PCR- in both replicates. The one exception was PCR-HYB- when re-tested [see above]. Of the 57 PCR negative samples with inconsistent

hybridisation result, only 10 [18%] were HYB+ when tested a third time [whilst remaining PCR-]. Thus, there is some evidence that inconsistent hybridisation results can include a proportion of false positives.

Hybridisation with the B3 probe detected all 22 B1/2 amplification products visible by gel electrophoresis [PCR+HYB+], and was positive for a further 90/772 [12%] GBL where B1/2 amplification products were not visible by gel electrophoresis [PCR-HYB+] [**Table 2.3.**], i.e. hybridisation increased the proportion positive by almost five-fold.

Buffy coat samples [BCS]. A total 769 BCS were tested at least twice by PCR-hybridisation [**Plate 5**]. 701/769 [91%] of the BCS had the same results in the first two PCR replicates, with 39/701 [6%] testing PCRHYB+ and 662/701 [94%] BCS testing PCRHYB-. Of the 68 samples that had to be tested a third time, 26 [38%] tested PCRHYB+ and 42 [62%] tested PCRHYB-.

Bone marrow samples [BMS]. A total 42 BMS were tested at least twice by PCR-hybridisation [**Plate 5**]. 37/42 [88%] of BMS had the same results in both replicates, with 5/38 [16%] and 32/38 [84%] testing PCRHYB+ and PCRHYB-, respectively. Of the remainder 2 [40%] tested PCRHYB+ and 3 [60%] PCRHYB- in the third replicate.

Table 2.3. Reproducibility of PCR-hybridisation protocol: guanidine blood lysates.

	First PCR assay			
	PCR+/HYB+	PCR+/HYB-	PCR-/HYB+	PCR-/HYB-
Second PCR assay				
PCR+/HYB+	7	-	2	1[b]
PCR+/HYB-	-	-	-	-
PCR-/HYB+	5	-	8	24[c]
PCR-/HYB-	-	-	33[a]	288

NOTE. Field samples were amplified with B1/B2 primer pair and products then hybridised with $[\gamma^{32}P]$-labelled B3 probe as described in Material & Methods. [a], 4 and 29 samples were PCR-/HYB+ and PCR-/HYB- in the third PCR assay, respectively;[b], the sample was PCR-/HYB- in the third PCR assay; [c], 6 and 18 samples samples were PCR-/HYB+ and PCR-/HYB- in the third PCR assay, respectively; [d], samples were only amplified once.

Comparison of the reproducibility and sensitivity of PCR for GBL, BCS and BMS.

No difference between the reproducibility [i.e. in the proportion of samples that were consistent] of the PCR-hybridisation protocol on GBL, BCS or BMS was observed [Chi square test, Yates-corrected, $\chi^2 = 0.72$, df=2, p=0.7]. Samples were considered PCR-hybridisation positive if out of a maximum three replicates, two replicates were PCR+ HYB+ or PCR-HYB+ [for GBL samples only], or HYB+ [for BCS and BMS]. Additionally GBL samples were considered positive if one replicate was PCR+ HYB+ and one replicate PCR-HYB+ . Samples were considered PCR-hybridisation negative if out of a maximum of three replicates, two were negative after hybridisation. Using the above definitions, 32/368 [8.7%], 65/769 [8.5%] and 7/42 [16.7%] were PCR-hybridisation positive on whole blood [GBL], buffy coat [BCS] and bone marrow [BMS] [**Table 2.4.**], respectively. Although there was an observed difference in the proportion positive of the PCR-hybridisation protocol between GBL, BCS and BMS, this difference was not significant [Chi square test, Yates-corrected, $\chi^2 = 3.35$, df=2, p=0.19]. When more than one sample was assayed from dogs, there were highly significant associations between the results: for example, amongst those 42 dogs with bone marrow samples, all four positive by GBL [and 4/4 positive by BCS] were also positive by BMS.

Table 2.4. Detection of *L. (Viannia)* spp. in field dogs using PCR-hybridisation and ELISA.

	Whole blood (GBL)	Buffy coat (BCS)	Bone marrow (BMS)	ELISA
1997				
Dogs with active lesions	4/11 (36.4%)	3/7 (42.9%)	0/4 (0.0%)	9/10 (90.0%)
Dogs with scars	2/5 (40.0%)	2/5 (40.0%)	0/1 (0.0%)	6/8 (75.0%)
Dogs without lesions or scars	26/352 (7.4%)	16/209 (7.7%)	7/37 (18.9%)	100/497 (20.1%)
Dogs BMS taken	4/33 (12.1%)	4/41 (9.8%)	7/42 (16.7%)	13/39 (33.3%)
Dogs BMS not taken	5/38 (13.2%)	6/38 (15.8%)	-	8/30 (26.7%)
Dogs BMS not taken, BCS ND	23/297 (7.7%)	-	-	65/282 (23.0%)
Dogs BMS not taken, GBL ND	-	11/142 (7.7%)	-	18/130 (13.8%)
Dogs BMS not taken, BCS/GBL ND	-	-	-	11/34 (32.4%)
Total 1997	32/368 (8.7%)	21/221 (9.5%)	7/42 (16.7%)	115/515 (22.3%)
1998				
Dogs with active lesions	-	1/8 (12.5%)	-	6/8 (75.0%)
Dogs with scars	-	3/9 (33.3%)	-	3/8 (37.5%)
Dogs without lesions or scars	-	15/270 (5.6%)	-	39/268 (14.6%)
Total 1998	-	19/287 (6.6%)	-	48/286 (16.8%)
1999				
Dogs with active lesions	-	0/2	-	0/2 (0.0%)
Dogs with scars	-	3/4 (75.0%)	-	1/4 (25.0%)
Dogs without lesions or scars	-	22/255 (8.6%)	-	57/252 (22.6%)
Total 1999	-	25/261 (9.6%)	-	58/258 (22.5%)
1997-1999				
Dogs with active lesions	4/11 (36.4%)	4/17 (23.5%)	0/4 (0.0%)	15/20 (75.0%)
Dogs with scars	2/5 (40%)	8/18 (44.4%)	0/1 (0.0%)	10/20 (50.0%)
Dogs without lesions or scars	26/352 (7.4%)	53/734 (7.2%)	7/42 (16.7%)	196/1019 (19.2%)
Total 1997-1999	32/368 (8.5%)	65/769 (8.5%)	7/42 (16.7%)	221/1059 (20.9%)

NOTE. Field samples were amplified with B1/B2 primer pair and products then hybridised with [γ^{32}P]-labelled B3 probe; the ELISA was carried out as described in Material & Methods using the 4.16 LAU/ml cut-off. BCS, buffy coat sample; BMS, bone marrow sample; GBL, guanidine blood lysate; ND, not done.

Field samples: definition of PCR-hybridisation positivity. A dog was considered PCR-hybridisation positive when one of the PCR-hybridisation assays on either GBL, BCS or BMS was positive [**Table 2.4.**]. Thus, using B1/B2 primers and the B3 probe, the PCR-hybridisation assay detected *L. [Viannia]* parasites in 8.4% [90/1066] of tested dogs, with 32% [13/41] of the clinically symptomatic and 7.5% [77/1025] of the clinically asymptomatic dogs testing PCR-hybridisation positive [Chi square test, Yates-corrected, χ^2=26.8, p<0.001].

Enzyme-linked imunosorbent assay [ELISA]. *Negative controls.* The mean antibody levels for the three groups of negative controls were 5 584 [s.d.= 3 150] [Lima], 2 602 [s.d.= 1 707] [Belém] and 4 803 [s.d.= 4 728] [Cambridge] units/ml [**Figure 2.1.**]. Whereas the mean log anti-*Leishmania* antibody units [LAU]/ml of Lima and Belém [ANOVA, $F_{1,29}= 17.01$, $p < 0.001$] and Cambridge and Belém [ANOVA, $F_{1,24}= 4.87$, $p= 0.04$] dogs was significantly different, the mean LAU/ml of Lima and Cambridge dogs was not [ANOVA, $F_{1,29}=1.58$, $p=0.22$] [**Figure 2.1.A** inset].

Positive controls and symptomatic dogs. The mean antibody levels for positive control [i.e lesion biopsy smear positive] and scar positive dog sera were 1 243 388 [s.d.= 3 635 762] units/ml and 154 825 [s.d.= 419 317] units/ml, respectively, with the mean LAU/ml significantly different between the two groups [ANOVA, $F_{1,33}=7.91$, $p=0.01$] [**Figure 2.1.A** inset].

Total field samples and definition of ELISA cut-off. A total of 1059 field dogs were tested by ELISA, the frequency distribution of which is represented in **Figure 2.1.A.** Two different methods were used to analyse the data.

Firstly, the standard definition of positive cut-off [CO], i.e. the mean level of antibody units/ml of negative controls + 3 s.d. was used (177, 178, 437, 440). The mean level of antibody units/ml of all negative controls was 4 472 [s.d.= 3299] units/ml, hence the CO for positivity was 14 369 units/ml [i.e. 4.16 LAU/ml]. Using this CO, 221/1059 [20.9%] dogs tested positive, 25/40 [62.5%] symptomatic and 196/1019 [19.2%] asymptomatic dogs [Yates-corrected Chi square test: $\chi^2=36.1$, $p<0.001$] [**Table 2.4.**].

However, this CO is conservative and it is likely that it will underestimate the true proportion of infected dogs (177, 178, 440). Hence, a second method to determine the positive CO was used, which took into account that the frequency distribution of log antibody units/ml is bimodal. Using maximum likelihood, two log-lognormal distributions were fitted to the observed data, varying mean and standard deviation [**Figure 2.1.B.**]. Both distributions yield cumulative probability functions, where the positive CO is the point a which the distribution intersect [**Figure 2.1.B.** inset], i.e. at 4.1 log antibody

units/ml. Using this CO, 230/1059 [21.7%] dogs were positive, 25/40 [62.5%] symptomatic and 205/1019 [20.1%] asymptomatic dogs. This proportion of infected dogs is close to the best estimate of 249 [23.5%] dogs predicted by the fit of the log-lognormal distributions [i.e. the area under the log-lognormal distribution fitted to the right-hand tail of the ELISA LAU/ml distribution].

Field samples. PCR-hybridisation and ELISA. **Table 2.5.** summarises the ELISA and PCR-hybridisation results of field samples, negative and positive controls, depending on which ELISA cut-off was used. The frequency distribution of LAU/ml relative PCR-hybridisation positivity is represented in **Figure 2.2.** None of the 44 negative control samples were positive by PCR-hybridisation or ELISA [CO 4.16], but 3 [one dog from Lima and two from Cambridge] dogs were positive using CO 4.1 [**Table 2.5.**]. Thus, specificity of PCR and ELISA$_{CO4.16}$ was 100%, whereas the specificity of ELISA$_{CO4.1}$ was 95%. 5/15 and 13/15 positive control samples were positive by PCR and ELISA [no matter which CO was used], i.e. sensitivity for PCR and ELISA was 33% and 87%, respectively [**Table 2.5.**]. The proportion of samples PCR positive was shown to be associated with LAU/ml [Linear regression analysis after arc-sine transformation of the data, r=0.54, d.f.=50, p<0.001] [**Figure 2.2.A.**], the frequency distributions of PCR positive and negative samples is shown in **Figure 2.2.B.**

Based on the results of both PCR and ELISA tests dogs were diagnosed as positive when they were either positive by PCR or ELISA$_{CO4.16}$, or both. The reason why we decided to choose $CO_{4.16}$ as the positive cut-off is because of the higher specificity when using $CO_{4.16}$. Hence, out of 1022 samples that were tested by both PCR and ELISA, 262/1022 [25.6%] were diagnosed as positive [**Table 2.5.**]: 30/39 [76.9%] clinically symptomatic and 232/983 [23.6%] clinically asymptomatic dogs.

Figure 2.1. Log anti-Leishmania antibody units/ml frequency distributions of field samples.

A
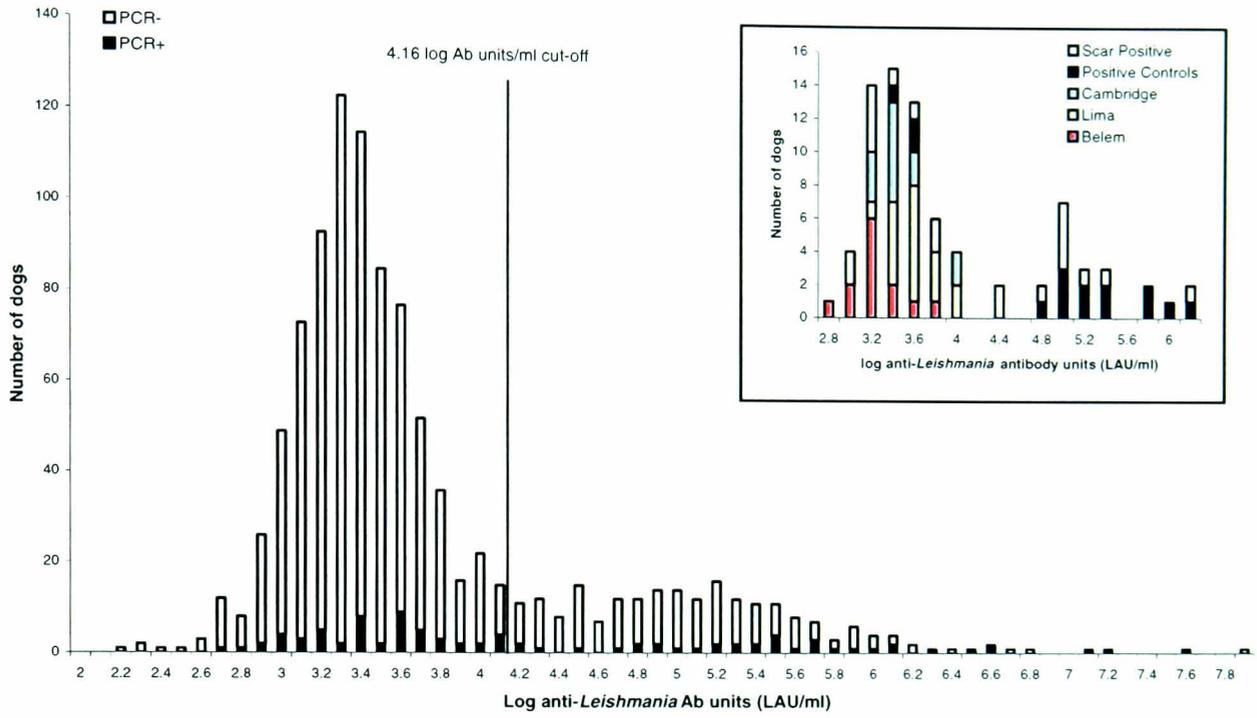
B
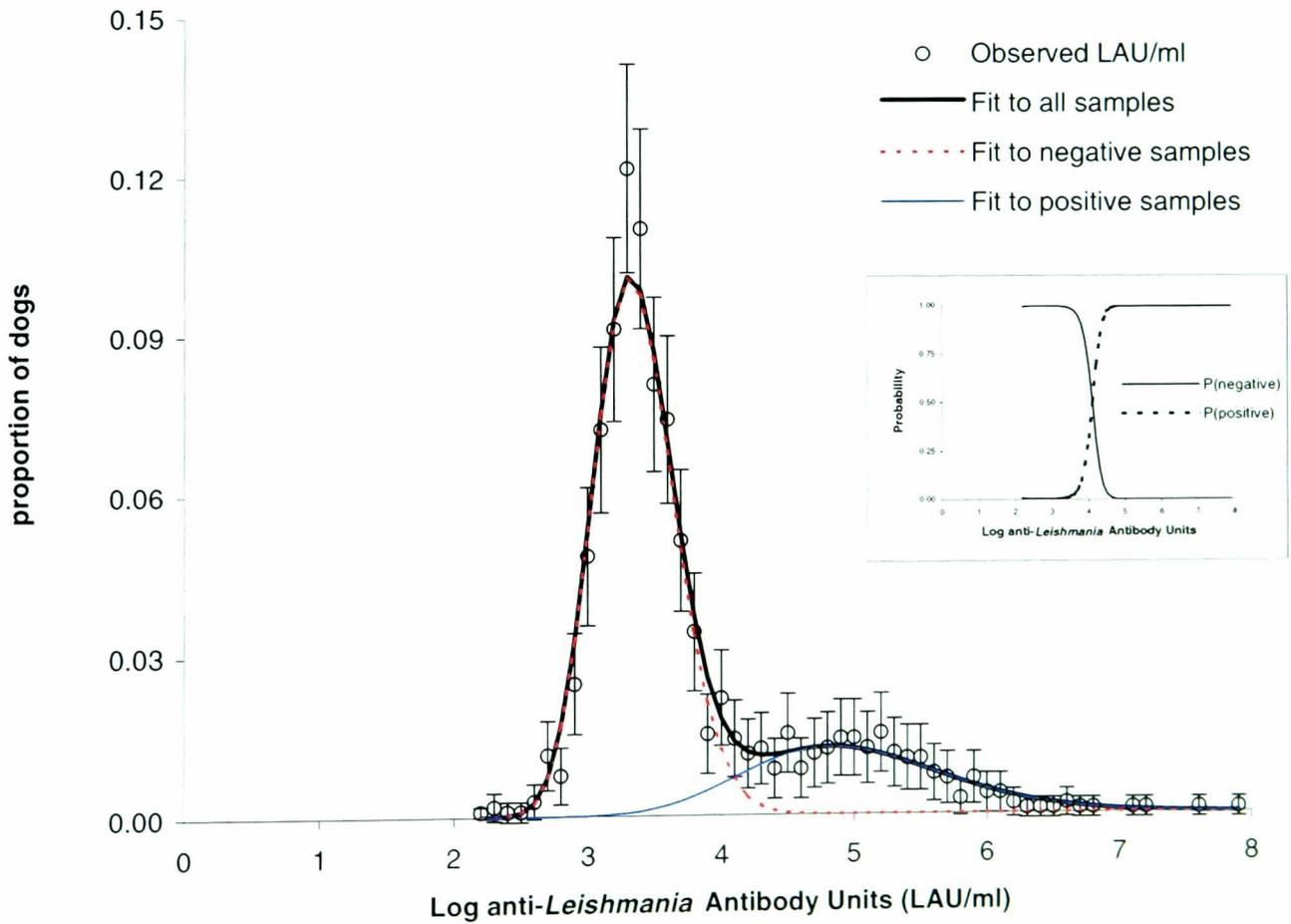
Figure 2.2. Relationship between PCR-hybridisation and ELISA.

A

4.16 log Ab units/ml cut-off

Proportion of dogs PCR positive

log anti-*Leishmania* Ab units (LAU/ml)

B

4.16 log Ab units/ml cut-off

PCR+
PCR-

Proportion total dogs tested

log anti-*Leishmania* Ab units (LAU/ml)
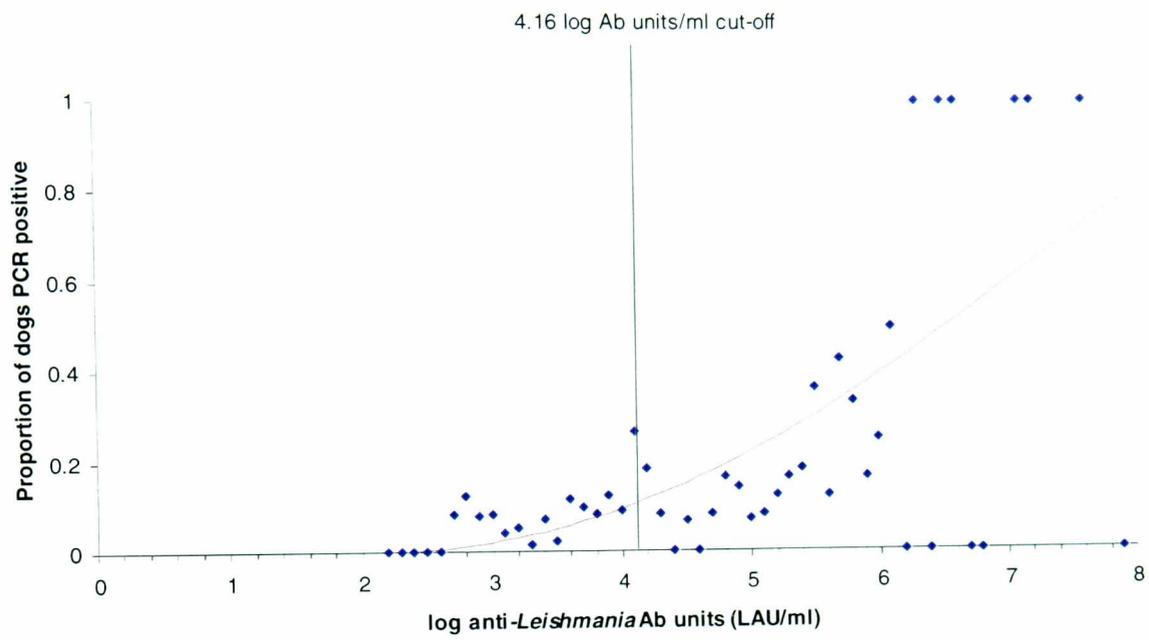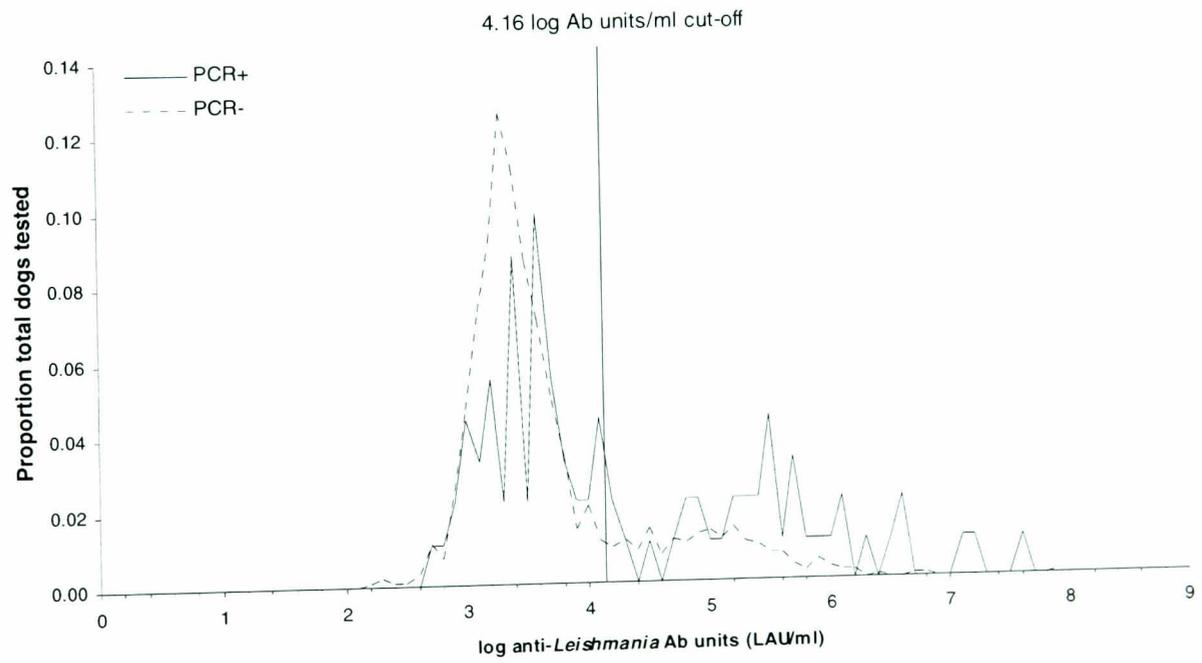
Table 2.5. Comparative diagnosis of *L. [Viannia]* spp. in dog blood.

	Negative Controls[a] [n=44]		Positive Controls[b] [n=16]		Other [n=1006]	
	PCR+	PCR-	PCR+	PCR-	PCR+	PCR-
ELISA+	-	- [3]	4 [4]	9 [9]	33 [35]	163 [171]
ELISA-	-	44 [41]	1 [1]	2 [2]	52 [50]	758 [750]

NOTE. [a] PCR on negative Belem and Cambridge controls were carried out on sera, not buffy coat;[b] dogs with active lesions that were biopsy smear positive. Of 21 dogs with active lesions [see **Table 2.4.**], 17 were biopsy smear positive, and 16 were tested by both ELISA and PCR. Numbers represented are based on $ELISA_{CO4.16}$ and $ELISA_{CO4.1}$ [in brackets].

2.4. Discussion.

In this chapter we assessed the utility of PCR as a diagnostic mass-screening tool for epidemiological surveys and compared its sensitivity and specificity to ELISA, a commonly used test to detect *L. [Viannia]* in dogs. We chose ELISA rather than IFAT as comparative test, because previous studies had shown ELISA to be more sensitive and specific than IFAT for detection of ACL in dogs [**Part 1: Introduction**].

PCR protocol and sensitivity titration assay. PCR assays rarely justify choice of DNA extraction protocol and PCR primers (323), but both were shown here to impact significantly on assay sensitivity [an observation that was recently confirmed (294, 296)]. Furthermore, most reported STAs have been based on either pure *Leishmania* parasite culture lysates or on standard amounts of background host DNA added to known quantities of parasite DNA (152, 207, 327, 546). Both fail to mimic the situation encountered in the field: the concentration of background host and parasite DNA will vary considerably with biopsy sample, thereby influencing the outcome of the PCR assay, as will other factors related to the host's medical condition [eg. haematocrit] (116). The present STA demonstrates that DNA from a fraction of *Leishmania* parasite can be amplified by PCR in the presence of host canine background DNA, but generally less readily than pure parasite culture lysates [**Table 2.2.**]. Hybridisation with a ^{32}P-labelled probe usually

increased the sensitivity of the assay by 10^2 to 10^4-fold [**Table 2.2**]. Contrary to previous reports [**Table 2.1.**] the M1L/M3HL primer pair performed rather poorly. Though the target DNA to be amplified was the smallest, M1L/M3HL was 10^4 to 10^6 less sensitive than other primer pairs used. A particular problem associated with using M1L/M3HL was the difficult visual separation of amplification product and primer dimers on standard agarose gels [and subsequently on the probed filters]. Though organic solvents are known to persist in DNA extracts and can inhibit the PCR reaction, PC extraction was comparable to the DNeasy kit in preparing samples for PCR. Commercial DNA extraction kits [eg. DNeasy] may have the advantage of speed and reduced safety hazard (323), but they are expensive when compared to PC extraction, and as shown here no more efficient. Quicker and easier DNA extraction techniques using Chelex were not as successful [10^3 to 10^4-fold less sensitive] as the DNeasy kit or PC extraction procedures when preparing samples for PCR. The reason why none of the Chelex only-extracted samples yielded amplification of the target DNA may be due to the presence of a PCR inhibitor not removed by the extraction method, or remaining Chelex particles. Though ideal for screening large numbers of samples, because of the minimal manipulations required and the reduced risk of specimen-to-specimen contamination (564), this extraction protocol appears unsuitable for DNA extraction when using clinical specimens containing very small numbers of parasites or large numbers of potential PCR inhibitors, eg. heme. In contrast, *L. [Viannia]* spp. DNA has been successfully extracted from lesion scrapings using Chelex resin (36, 62, 251). The advantage of using guanidine HCl is that blood samples can be stored at 4°C [and possibly at room temperature] (34, 35, 90, 91, 578, 579), which is useful in the field where there is often no access to freezers. As for *T. cruzi* (34, 35, 90, 91, 578, 579), *Leishmania* DNA in guanidine HCl remained undegraded for months, and we successfully amplified *Leishmania* DNA originating from samples stored at 4°C for 1½ years [recently it was reported that *Leishmania* DNA in guanidine can be successfully amplified from samples stored for up to 3 years (294)]. However, it should be noted that guanidine HCl is a salt that could inhibit PCR amplification, so dilutions of extracted DNA may be required for successful amplification.

PCR as a diagnostic mass-screening tool. The classic diagnostic techniques for ACL diagnosis have a number of limitations. Microscopic examination of skin scrapings or lesion biopsy touch imprints, though rapid and low-cost, has limited sensitivity, particularly in chronic lesions [e.g. MCL] (571). Whilst *in vitro* culture techniques are slightly more sensitive than microscopic examination of lesion biopsy smears or histological samples, they are labour-intensive and costly and are susceptible to microbiological contamination. They also are hampered by the particular growth requirements of different *Leishmania* strains and as some strains grow better than others *in vitro*, the dominant strains can be inadvertently selected for when culturing mixed infections (28). MST detects specific cutaneous delayed-type hypersensitivity, but cannot distinguish between active and past infections (571). Also, logistical problems would be associated with its application in epidemiological dog surveys [e.g. dogs would have to be kennelled or monitored for 48 hours prior reading the MST induration size]. PCR has been shown to be particularly useful for the diagnosis of *L. [Viannia]* infection, as parasite numbers in clinical samples are typically sparse (62, 153, 244, 245, 433, 498).

Although a positive PCR was associated with a higher antibody titre [LAU/ml [**Figure 2.2.**], the ELISA test was much more 'sensitive' than PCR in detecting *Leishmania* infection in symptomatic [Chi square test, Yates-corrected, $\chi^2 = 6.5$, p= 0.1] and asymptomatic [Chi square test, Yates-corrected, $\chi^2 = 58.9$, p< 0.001] field dogs. Considering the 100% specificity of the ELISA$_{CO4.16}$ test and the absence of any potential cross-reacting parasites in the study area, it is very likely that the 163 ELISA positive but PCR negative dogs were true positives [see below]. Based on negative and positive control samples, the positive predictive value [PPV] for PCR and ELISA$_{CO4.16}$ was 100%, and 81% for ELISA$_{CO4.1}$. The negative predictive value [NPV] was 80% for PCR and 94% for ELISA, respectively. If one estimates the PPV from the PCR GBL replicate data, then the PPV for PCR+/HYB+ is similar, i.e. 95% [out of the 22 PCR assays that were PCR+/HYB+ at least once, 21 were diagnosed as positive, **Table 2.3.**]. However, the PPV for PCR-/HYB+ is only 31% [out of 80 samples that were PCR-/HYB+ at least once, 25 were diagnosed as positive]. The reason why this was the case is that for

these samples the PCR method was performing at its highest, possible sensitivity. Whether future PCR protocols and new PCR primers will be able to reduce that false negative rate remains to be established. Replicate results of established PCR protocols are rarely reported (295, 439), but are of importance when interpreting epidemiological data, because they show that the used diagnostic test is either not reliable [i.e. there is contamination] or performing at its highest level of sensitivity. Considering that, in our case, a positive PCR result means that a dog is potentially infectious, the lack of replicates may have a significant impact on the outcome on dog intervention [e.g. culling] campaigns.

A PCR-based assay on blood is advantageous as samples can be obtained less invasively from the patient [human or dog] and are easy to process. We also show that the choice of biopsy material does not appear to be as critical as for studies using PCR to diagnose human ACL patients [skin snips, lesion aspirates], as the sensitivity of the PCR on GBL, BCS or BMS was not significantly different. Studies using PCR to detect *L. infantum* in dogs reported similar observations to the ones made here, showing that sensitivity was higher in clinically symptomatic than asymptomatic dogs (31, 67, 356, 451, 473, 507, 518, 595). Only in two studies did the sensitivity of PCR vary according to biopsy origin (356, 518). The reason why we could not demonstrate such an association is probably due to the pathogenesis of canine *L. [Viannia]* infections, with parasites remaining localised at the site of infection [see below].

Table 2.6. Use of PCR as a mass-screening tool in epidemiological studies of ACL.

Disease	Animal Species	PCR	PCR primers*	Parasite isolates [N]	Reference
CL^{LV}	Bradypus variegatus	$3/7^B$	OCT120	-	¥
	Didelphis marsupialis	$0/3^B$		-	
CL^{LV}	D. marsupialis	$1/5^{SS}$	B1/2	-	(14)
	Microreus demerarae	$2/3^{SS}$		-	
	Microryzomys minutus	$1/2^{SS}$		-	
	Melanomys caliginosus	$3/14^{SS}$		-	
	Rattus rattus	$2/4^{SS}$		-	
	Rhipidomys latimanus	$0/4^{SS}$		-	
	Silvilagus braziliensis	$1/1^{SS}$		-	
CL^{LV}	Marmosa spp.	$1/?^{SB}$	?	-	§
	Rattus alexandrinus	$1/?^{SB}$		-	
	Rattus frugivorus	$1/?^{SB}$		-	
CL^{LV}	Akodon spp.	$4/153^{SS}$	MP1L/MP3H	-	(322)
	Canis familiaris	$15/276^{SS}$		L. peruviana [3]	
	D. albiventris	$2/72^{SS}$		L. peruviana [1]	
	Mus musculus	$0/178$		-	
	Oryzomys spp.	$0/8$		-	
	Phyllotis andinum	$1/499$		L. peruviana [2]	
	Rattus rattus	$0/8$		-	
CL^{LV}	Akodon arviculoides	$0/1^{B,SS}$	?	-	¶
	Akodon cursor	$0/2^{B,SS}$		-	
	Nectomys squamipes o.	$0/1^{B,SS}$		-	
	Oligoryzomys nigripes	$0/21^{B,SS}$		-	
	Oryzmoys raytticeps	$0/1^{B,SS}$		-	
	Proechimys iheringi	$0/2^{B,SS}$		-	
	Rattus rattus	$0/2^{B,SS}$		-	
	Rhipidomys mostacalis	$1/5^{B,SS}$		-	
$CL^{LV,LMEX}$	Akodon spp.	$1/14^B$ [LMEX]	L1/2	L. braziliensis [1]	(530)
	Conepatus chinga rex	$1/2^B$ [LMEX]		-	
	D. marsupialis	$0/12^B$		-	
	Microreus cinerea	$0/2^B$		-	
	Oligoryzomys spp.	$2/8^B$ [LMEX]		-	
	Oryzmoys spp.	$0/1^B$		-	
	Rhipidomys lencodactylus	$0/2^B$		-	

NOTE. B, blood; LMEX, L. mexicana; LV, L. [Viannia] spp.; SB, spleen biopsy; SS, skin snip. *PCR primers. B1/2, L. [Viannia] spp.-specific primers (152); L1/2: Trypanosomatidae-specific primers; MP1L/MP3H, L. [Viannia] spp.-specific primers (327); OCT120: Leishmania spp. primers (197); ¥, Pirmez et al., unpublished; §, Brandão-Fillho et al., unpublished; ¶, Soares et al., unpublished.

Only one large (>50 tested samples) study has used PCR as a mass-screening tool in epidemiological studies of ACL and reported lower ACL infection rates in dogs than the ones reported here (322). Similarly, other small studies using PCR to detect ACL in putative animal reservoirs [**Table 2.6.**] failed to observe high ACL infection rates. Incidentally, in all studies a second, comparative diagnostic test such as serology was not carried out. An established experimental animal model for *L. [Viannia]* infection would provide some answers to above questions regarding diagnostic test sensitivity and specificity as PCR could be carried out under controlled conditions – however, such an experimental model does currently not exist.

Epidemiological considerations. The high prevalence shown in both asymptomatic and symptomatic dogs provides further evidence for their suspected role as [peridomestic] reservoir hosts of ACL [**Part 1: Introduction**]; and the detection of *L. [Viannia]* DNA in canine blood implies that infected dogs should be infectious to bloodfeeding sandfly vectors. However, xenodiagnosis studies will be required to prove this. Although *L. [Viannia]* DNA was detected in the blood and bone marrow of a relatively high proportion of tested dogs, indicating that metastasis by haematogenous dissemination may be a more common phenomenon than previously acknowledged (19, 570), blood samples from the majority of dogs with active [and biopsy smear positive] lesions were PCR negative. This is probably because *L. [Viannia]* parasites are first localised at the site of infection in the dermis, with haematogenous dissemination occurring after an undefined interval [if at all] (570). An interesting observation is the 52 PCR positive but ELISA$_{CO4.16}$ negative asymptomatic field dogs. Because the PCR was 100% specific, it is unlikely that they were false positives. They may have been false ELISA negatives, but considering that their mean level of LAU/ml [i.e. 3.61] was much lower than the 4.18 log antibody units/ml cut-off this seems improbable. One possible explanation is that these dogs represent dogs that are innately resistant [e.g. studies on *L. infantum* in dogs have shown that up to 50% of asymptomatic dogs have a anti-*Leishmania* cellular immune response (1, 97, 98, 288, 518)]. Another explanation is that dogs have developed cellular immunity after self-cure, as it now becomes increasingly evident that persistence of parasites is required to maintain cell-mediated

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immunity in the long-term (570). Of note is that despite the high prevalence of infection only 4% of surveyed dogs had clinical symptoms of ACL, which is considerably lower than the figures observed in other studies [**Part 1: Introduction**]. A possible explanation could be that the circulating *L. [Viannia]* parasites are of low virulence [i.e. a small proportion of infection causes clinical disease] but high pathogenicity [i.e. a high proportion of parasite inoculations cause infections] (570); which is characteristic of *L. peruviana*. Surprisingly little known is known about the clinical and parasitological course of *L. [Viannia]* in dogs [as in humans and other mammalian hosts] and the role of cellular immunity during infection [**Part 1: Introduction**]. It also remains to be established whether *L. [Viannia]* resistant dogs could have an important role in ACL disease transmission.

Conclusion. This is the first large-scale study to test the feasibility of using PCR to detect *L. [Viannia]* spp. parasites in host blood and to test whether PCR could be used as a diagnostic, mass-screening tool in epidemiological studies. As is the case for ZVL (31, 67, 356, 451, 473, 507, 518, 595), PCR on blood alone does not appear to be the elusive 'gold standard' for diagnosing ACL infections in dogs [or humans]. Unless a more sensitive PCR protocol [e.g. PCR-ELISA] is developed to detect asymptomatic ACL infections, the use of PCR will be restricted to the diagnosis of active cases [e.g. hospitals in Europe and elsewhere increasingly rely on PCR for *Leishmania* diagnosis in human patients (71, 89, 515, 583)] and in particular MCL cases where common diagnostic tests [e.g. parasite culture, biopsy smears, histolopathology] are less sensitive [**Table 2.1.**]. The use of PCR on blood will, however, have an important epidemiological application in studies to monitor the clinical and chemotherapeutic follow-up of patients with ACL (164, 245, 498). Detection of disseminating *Leishmania* parasites in patient blood would indicate that they are at risk of developing mucocutaneous lesions, the treatment of which is more complicated than the treatment of the single lesions characteristic of ACL (570). Also, PCR combined with specific DNA probing and sequencing should help to identify and characterise those strains that are drug resistant and that cause the different clinical pathologies associated with ACL.

Thus, although there are concerns about the specificity of serological tests and although they are unable to distinguish past from present infections, they will remain the main diagnostic tool for epidemiological *Leishmania* mass-screening surveys. The main advantages of serological tests are that large numbers of samples can be processed readily, inexpensively, with comparatively low technical expertise required. Significantly, the recent identification of specific recombinant *Leishmania* antigens [e.g. the *L. donovani* and *L. infantum*-specific rK39, see **Chapter 6**] suggests that serological tests may become more specific in the future. Several studies have shown that serological tests based on recombinant antigens [e.g. rK39-ELISA] are able to distinguish not only between present and past infections, but between symptomatic and asymptomatic infections as well. Surprisingly, serological tests based on known *L. [Viannia]* spp.-specific recombinant antigens (379) have yet to be tested as a diagnostic tool for either active cases or in epidemiological surveys.

CHAPTER 3:
Leishmania [Viannia] spp. dissemination and tissue tropism in naturally infected dogs [Canis familiaris].

Abstract

Although domestic dogs are suspected reservoir hosts of ACL, descriptions of the course of L. [Viannia] spp. infection in dogs are scarce and most information on the natural course of L. [Viannia] spp. infection is restricted to studies carried out on sloths. The presented observations provide the first evidence for L. [Viannia] spp. dissemination and tissue tropism in the domestic dog. Using PCR and histology, parasites were detected in the conjunctiva and lymph nodes of both dogs studied and the detection of parasites in the blood indicates that parasite dissemination to those organs may have been haematogeneous. Leishmania [Viannia] spp. parasites were additionally detected in the lung and ovary of one of the dogs.

3.1. Introduction.

In a minority of ACL infections, L. [Viannia] parasites disseminate from the primary cutaneous lesion to viscera and mucosa, causing MCL [**General Introduction**]. The mechanisms underlying L. [Viannia] dissemination and tissue tropism are unknown, but it appears that it is dependent upon [i] host susceptibility and parasite virulence (73, 306, 506); [ii] persistence of live parasites in host tissues (164, 244, 245, 498); and [iii] stimulation or enhancement by trauma (63, 539, 583). Successful detection or isolation of L. [Viannia] parasites from human (84, 164, 244, 245, 349), rodent (86, 322), sloth (261) and canine (245) [**Chapter 2**] blood suggests that dissemination may be haematogenous.

Previous reports on the course of L. [Viannia] infection and dissemination are almost exclusively from experimental studies using golden hamsters [Mesocricetus auratus] or mice [Mus spp.], which are not natural hosts (271). Whereas the L. [Viannia]-hamster model mirrors human disease, the self-curing, non-disseminating L. [Viannia]-mouse model does not (271). Hence, it is difficult to draw any conclusions from the murine studies. Also, most mouse and hamster studies have used inoculates containing high numbers of parasites, injected either intra-venously, -muscularly or –peritoneally, a protocol which does not mimic natural infection by sandfly bite. The course of natural L. [Viannia] infections is likely to be affected by sandfly saliva whose role in modulating ACL infection [and perhaps dissemination] is unclear (70, 106, 174, 317, 484) [**General Introduction**].

Descriptions of the course of canine of L. [Viannia] infection come only from a handful of experimental studies, which exclusively report the macroscopic, clinical evolution of infection [**Part 1: Introduction**]. Information on the natural course of canine L. [Viannia] infection comes from a single study of three parasitologically-proven dogs (434), in which L. braziliensis could not be detected in spleen, liver or lymph nodes [either by in vitro culture, Giemsa-stained imprints or histopathology].

Reports on *L. [Viannia]* tissue dissemination are almost exclusively restricted to studies carried out on sloths. Herrer & Christensen (261) isolated *L. panamensis* from 56/497 skin, 46/451 spleen, 26/450 liver, 14/382 bone marrow, and 4/267 lung biopsies of *Choleopus hoffmani.* Lainson (300) reported the isolation of *L. guyanensis* from viscera [i.e. spleen, liver, lungs] and skin from one *Choleopus didactylus,* from the viscera only of one *Tamandua tetradactylus* and one *Didelphis marsupialis,* and from skin only of one *Proechimys guyannensis.*

The objective of the study were to determine the extent of *L. [Viannia]* dissemination and tissue tropism in two naturally infected dogs which were given to the Ministry of Health in Huánuco because the owners wanted the dogs to be killed.

3.2. Materials & Methods.

Dogs and sampling. Using the polymerase chain reaction [PCR] and histology, *L. [Viannia]* dissemination was investigated in two naturally infected dogs, dog 1 [D1] came from Limapampa [906], dog 2 [D2] came from La Esperanza [921] [**Map 1** and **Chapter 2**].

Dog 1 [D1] was a 2-year-old female mongrel [weight 8.5kg] presenting an active cutaneous ulcer [L1] [size: 6x4cm; duration: 4 months] on the snout. No other clinico-pathological symptoms indicative of ACL were observed. D1 had a *Toxocara canis* infection.

Dog 2 [D2] was a 6-year-old female mongrel [weight 11.3kg] presenting two active cutaneous lesions [L1 and L2]. L1 [size: 7x5cm; duration: 12 months] was a mucocutaneous lesion on the snout that had destroyed the septum and respiratory cavity [**Plate 4H**]. D2 was suffering from breathing difficulties due to the lesion. L2 [size: 6x3cm, duration: 5 months] was an active cutaneous ulcer on the left side of the neck. Other clinical symptoms included onychogryphosis, brittle and dry hair, conjunctivitis, and flea-induced

dermatitis on the underside of the abdomen. Macroscopic pathological signs included hepatosplenomegaly, enlarged right cervical and mandibular lymph nodes; enlarged and hemorrhagic mesenteric lymph nodes; and *T. canis* and *Espinocerca lupis* infection of the oesophagus.

Blood aliquots [5ml] were taken from the jugular vein following euthanasia using sodium pentobarbitone [Euthanol®, MTC Pharmaceuticals, Cambridge, Canada] upon approval of their owners. Blood was processed as previously described [**Chapter 2**], and stored as either buffy coat sample [BCS] at -20°C, or guanidine/blood lysate [GBL] at 4°C. The dogs were necropsied and tissue samples were taken and placed in 1ml of PBS or 10% formalin for PCR and histology respectively. All samples stored in PBS were frozen at -20°C.

PCR and hybridisation. For PCR, DNA from BCS, GBL and biopsies was extracted using the DNeasy® extraction kit [Qiagen, Crawley, Great Britain], and amplified using three different sets of primers: B1/B2, Min11B/Min22 and R221/R332 [**Box 1**]. Amplification products were hybridised with either a [α^{32}P]-dATP or [γ^{32}P]-ATP-labeled probe, before being exposed for autoradiography at -70°C [**Chapter 2**].

Enzyme-linked immunosorbent assay [ELISA]. The ELISA was carried out as described in **Chapter 2**, i.e. using *L. braziliensis* [MHOM/BR/85/LB300] promastigotes as antigen and peroxidase-conjugated IgG as antibody.

Table 3.1. PCR and histology of different tissue biopsies and blood.

	D1				D2			
	PCR B1/B2	PCR Min11B/22	PCR R221/332	HIS	PCR B1/B2	PCR Min11B/22	PCR R221/332	HIS
L1	+	+	+	ND	+	+	+	+
L2	NA	NA	NA	NA	-	-	-	-
Conjunctiva (left eye)	ND	ND	ND	ND	-	-	-	+
Conjunctiva (right eye)	+	+	+	-	+	+	+	+
Mandibular lymph n. (left)	+	+	+	+	+	+	-	-
Mandibular lymph n. (right)	+	-	+	+	ND	ND	ND	ND
Cervical lymph n. (left)	-	-	-	-	ND	ND	ND	ND
Cervical lymph n. (right)	-	-	+	+	ND	ND	ND	ND
Mammary lymph n. (left)	-	-	-	+	-	-	-	ND
Mammary lymph n. (right)	-	-	-	+	-	-	-	ND
Maxillary lymph n. (left)	+	+	-	+	ND	ND	ND	ND
Maxillary lymph n. (right)	+	-	-	-	ND	ND	ND	ND
Mesenteric lymph n.	+	+	+	+	ND	ND	ND	ND
Popliteal lymph n. (left)	-	-	-	-	-	-	-	-
Popliteal lymph n. (right)	-	-	+	-	-	+	-	-
Heart	-	-	-	ND	-	-	-	-
Liver	+	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-
Lung	ND	ND	ND	ND	-	+	+	+
Spleen	-	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-	ND
Ovary	ND	ND	ND	ND	+	-	+	+
Whole blood (BCS)	+	+	+	ND	+	+	+	ND
Guanidine/blood (GBL)	+	-	+	ND	+	+	+	ND

PCR and histology were carried out as described in Materials and Methods. NOTE. n, node; NA, does not apply; ND, not done. For B1/B2, Min11B/22 and R221/332 PCR primer sequences see **Chapter 2**.

Histology. For histological examination, biopsy samples were processed and embedded in paraffin according to standard procedure (42). Sections [2-3μm thick] were cut on a microtome, stained with haematoxylin and eosin, and assessed for histopathological changes or presence of amastigotes.

3.3. Results.

Both dogs had high anti-*Leishmania* log antibody units/ml, with D1 and D2 having 5.8 and 5.5 log antibody units/ml [the positive cut-off being 4.16 log antibody units/ml, **Chapter 2**], respectively. The results of PCR and histology with respective biopsies and blood are represented in **Table 3.1**. Although three PCR protocols of different sensitivities and specificities were used to

maximise parasite detection, on three occasions histology was positive when all three PCRs were negative. Parasites were detected in a single lesion on both dogs confirming that both had ACL. L2 of D2 was parasitologically negative, both by PCR and histology, suggesting that the lesion had a non-*Leishmania* aetiology. Most tissue sections from both dogs showed signs of advanced fibrosis and marked infiltration of cells, such as lymphocytes, macrophages, polymorphs and fibroblasts; but the abundance of *Leishmania* amastigotes seen was generally low. In D1, most sampled tissues had clear evidence of advanced fibrosis. Amastigotes were observed in mandibular, cervical, mammary and mesenteric lymph node tissue sections, but not in sections from other tissues. In D2, advanced fibrosis was observed, but no parasites, in lymph node tissue sections. Areas of cellular infiltration were seen in lung tissue sections, particularly near larger vessels and airways, with small numbers of amastigotes present. Small foci of cellular infiltrate were also detected in conjunctiva tissue sections, and the area behind the membrane was inflamed with amastigotes present. The ovary had marked cellular infiltrate, particularly in the connective tissue, with amastigotes seen throughout. Liver and spleen biopsies from both dogs were degenerate, which may be due to a delay before fixation.

3.4. Discussion.

Whereas canine ZVL is a systemic infection and clinical signs are manifold (115, 290, 513), the course of canine ACL infection appears to be analogous to human ACL, where parasites remain localised at the dermal site of infection, with potential haematogenous or lymphatic dissemination occurring after an undefined interval (570). However, as shown in **Chapter 2**, parasite dissemination may not be a rare event as *L. [Viannia]* parasites were detected by PCR in the blood of 7.5% of asymptomatic dogs. Clinical symptoms associated with canine ACL infections are cutaneous ulcers or nodules, though not in the frequencies as reported for ZVL, because most ACL infections appear to be sub-clinical [**Chapter 2**].

Observations presented here provide the first evidence for *L. [Viannia]* tissue dissemination in the domestic dog. Parasites were detected in the conjunctiva and various lymph nodes of both dogs studied, and the detection of parasites in the blood indicates that parasite dissemination to those organs may have been haematogenous. Parasites were additionally detected in the lung and ovary of D2.

This is the first report of *L. [Viannia]* parasites detected in conjunctiva and ovary of a vertebrate host, and the first to detect *L. [Viannia]* parasites in the lung of dogs. The detection of parasites in the conjunctiva and lung is frequently associated with canine *L. infantum* infections (115, 290, 513), and *L. panamensis* parasites have been detected in the lungs of sloths (261). Detection of *Leishmania* parasites in the ovary or testis has been reported in natural dog infections caused by *L. infantum* (513), and the detection of *L. infantum* in dog semen could explain both canine and human cases of congenital transmission (77, 157, 181, 182, 222, 324, 365, 368, 373, 403, 445, 505, 584). Surprisingly, we did not detect parasites in liver or spleen, organs that generally are associated with *Leishmania* infection (570).

Conclusion. The detection of *L. [Viannia]* parasites in blood and conjunctiva implies that dogs are infectious to sandfly vectors, although confirmation requires xenodiagnosis studies. It is also likely that, as for canine ZVL (200, 513) and ACL in rodents (322) or sloths (261), *L. [Viannia]* parasites may disseminate to an infected dog's skin, thereby enhancing their infectiousness to sandfly vectors and their importance as reservoir host for human disease.

CHAPTER 4:
The epidemiology of canine American cutaneous leishmaniasis in Huánuco: transmission rates estimated from cross-sectional and prospective data.

Abstract

A prospective survey to study the epidemiology of American cutaneous leishmaniasis [ACL] in domestic dogs was carried out in 18 villages in the Department of Huánuco, Peru. Over three years 1022 dogs were surveyed, with cumulative village ACL prevalence ranging between 0% to 100%. The incidence of L. [Viannia] infection was estimated to be 0.285/year [95% C.I. 0.160-0.410] and 0.291/year [95% C.I. 0.195-0.387] using cross-sectional data and data from 108 dogs that were surveyed repeatedly over three years, respectively. The recovery rate was estimated to be 0.456/year [95% C.I. 0.050-0.862] and 0.520/year [95% C.I. 0.302-0.738], respectively. The findings were used to estimate the basic reproduction number $R_0 \approx 1.9$; implications for control are discussed.

4.1. Introduction.

Although several studies have tried to implicate dogs as domestic reservoirs of *L. [Viannia]* spp. [**Part 1: Introduction**], it is surprising that the number of studies reporting incidence and recovery rates in naturally infected dogs are extremely scarce. As shown in **Chapter 3**, description of the course of *L. [Viannia]* infection are restricted to four experimental studies, from which it is difficult to draw any conclusions due to differences in experimental protocol and small sample size. Also, the course of infection following experimental parasite inoculation is likely to differ from the course of infection due to natural infection via sandfly bite. The only information on the natural [sero-] recovery rates of dogs infected with *L. [Viannia]* parasites comes from a couple of field studies. In a small study in Brazil 3/14 *L. braziliensis*-infected dogs were shown to heal spontaneously 2-3 months after parasite demonstration, with complete clinical and sero-recovery; lesions and positive IFAT results re-appeared in all three dogs during the 11-month study follow-up (432). Recently, a prospective survey in a *L. braziliensis*-endemic area in Argentina reported yearly incidence rates of 12% [6/52] and 19% [10/52] for clinical disease [i.e. appearance of ACL lesions] and infection [i.e. as diagnosed by ELISA], respectively; no information on serorecovery rates was reported (341).

Comparatively vast is the literature on studies trying to describe the transmission dynamics of canine ZVL. Several cross-sectional and prospective studies, mainly in Europe and South America, particularly Brazil, have shown that [i] dogs are highly susceptible to *L. infantum* with high infection rates observed throughout the *L. infantum*-endemic range (125, 220) with a basic reproduction number [R_0] between 1.06 and 11 (23, 177, 440, 587); [ii] susceptibility to and persistence of *L. infantum* infection is dependent on host genetic background [e.g. pure breed dogs are more susceptible than mongrel dogs (513, 517)] and dog ecology [e.g. rural or hunting dogs have a higher risk of ZVL infection than urban or pet dogs, respectively (126)]; [iii] once infected, dogs tend to remain parasitologically positive for life and, hence, infectious to the sandfly vector (125).

The objectives of the analyses presented here were [i] to describe the natural course of *L. [Viannia]* infection in dogs parasitologically and immunologically estimating both incidence and recovery rates using data from both cross-sectional and prospective surveys; and [ii] to assess the epidemiological role of dogs in the *L. [Viannia]* transmission cycle in Huánuco, Peru.

4.2. Materials and Methods.

Study site. As described in the previous chapter, all surveyed dogs came from 18 villages in the Department of Huánuco, Peru [10°50'S, 76°10'W]: Pomacucho [Code 901], Conchumayo [902], Quechualoma [903], Chinobamba [904], Vilcabamba [905], Limapampa [906], Chullay [907], Coz [908], Huancapallac [909], Virroy [914], Cochachinche [915], Parcoy [916], Atahuayón [917], Mauca [918], Cochatamba [919], El Rancho [920], La Esperanza [921] and San Rafael [922] [**Map 1** in **Chapter 2**]. All villages lie at an altitude of ca. 2000-3500m above sea level, in a mountainous and relatively dry area, and each have between 150-650 inhabitants [**Plates 3A-H**]. The main economic activity is agriculture [the main crops being potatoes and maize] and people often own domestic animals [guinea pigs, chickens, goats, donkeys, dogs and cats].

Since 1987, there have been about 500-1000 annual reported ACL cases in the Department of Huánuco. A prospective epidemiological study of 2374 people carried out by UPCH [Universidad Peruana Cayetano Heredia, Lima, Peru] and LSHTM [London School of Hygiene & Tropical Medicine] from 1994 to 1998 showed that village cumulative ACL prevalence rates [as measured by a positive response to the Montenegro skin test] range from 10%-56% [Davies and Llanos-Cuentas, *unpublished*]. Though not gender related, risk of infection was shown to increase with age, and 23% and 3% of surveyed individuals had cutaneous and mucotaneous lesions or scars, respectively. Previous studies carried out by UPCH/LSHTM also showed that [i] there is

transmission of ACL in the domestic environment, caused by the bite of *Lu.* *[Helcocyrtomyia] tejadai* [incriminated on the grounds of its abundance, man-biting habits and the PCR-based detection of *L. [Viannia]* parasites in the gut] [Roncal & Davies, *unpublished*]; [ii] households often own one or more dogs [see **Chapter 5**]; and [iii] there are ACL infections in dogs, as *L. peruviana* [n=1] and *L. braziliensis/L. peruviana* hybrids [n=10] were isolated from 2% [11/563] of randomly sampled dogs (400). There is no *L. infantum* or *T. cruzi* transmission in the study area. Transmission season of ACL is generally after the raining season, from April to November.

Dog sampling and diagnosis. Dogs were surveyed either by house-to-house visits or gathered in the village square during rabies vaccination campaigns carried out by the local Ministry of Health. None of the dogs belonged to a recognisable breed and all were guard or hunting dogs. Interviews with owners were carried out to provide information about the number of dogs kept, their age and residence time in the valley. In follow-up visits, owners were also asked whether previously surveyed dogs were still alive or had died. Dogs were surveyed at approximately yearly intervals between 1997 and 1999, and ACL was diagnosed clinically, parasitologically [PCR] or immunologically [ELISA] as described before, with dogs diagnosed as ACL positive when positive by either PCR or ELISA, or both ['infection'] [**Chapter 2**].

Data analysis. *Cross-sectional surveys.* The instantaneous per capita conversion [i.e. force of infection, λ] and recovery [ρ] rates were estimated from age-prevalence data where the proportion of animals positive at age *a*, *P[a]*, is given by (576):

[E1] $$P(a) = \frac{\lambda}{(\lambda + \rho)} \cdot (1 - e^{-(\lambda + \rho)a})$$

The equation assumes that [i] λ and ρ are age independent and constant over time; [ii] that individuals in the host population are homogeneously exposed; [iii] that individuals that are infected become instantaneously positive; and [iv] that the association between age and prevalence is observed at

80

equilibrium. λ and ρ can then be estimated by maximum likelihood using observed age-prevalence data (576).

Prospective surveys. The instantaneous incidence rate among the susceptible dog population was estimated from the frequency of conversions from a negative to a positive PCR or ELISA diagnosis during the intervals between 1997, 1998 and 1999 surveys. Both λ and ρ can be derived from the prospective survey data by solving the simultaneous equations (87):

$$[E2] \quad P(c) = \frac{\lambda}{(\lambda + \rho)} \cdot (1 - e^{-(\lambda + \rho)t})$$

$$[E3] \quad P(r) = \frac{\rho}{(\lambda + \rho)} \cdot (1 - e^{-(\lambda + \rho)t})$$

Where *P[c]* is the proportion of individuals converting from a negative to a positive diagnosis in time *t*, and *P[r]* is the proportion of individuals converting from a positive to a negative diagnosis.

4.3. Results.

Dog demography. During the three years of the study 1114 dogs were surveyed, of which 1022 [1997: 481, 1998: 284, 1999: 257] were sampled and diagnosed by both ELISA and PCR [**Chapter 2**]. 138/1022 [14%] of samples came from dogs that were sampled more than once [see below]. Of the 1022 dogs included in the analyses, 370 [36%] [1997: 161, 1998: 110, 1999: 99] and 630 [62%] [1997: 303, 1998: 171, 1999: 156] were female and male, respectively; 22 dogs were not sexed [**Table 4.1.**]. Mean dog age during the study period was 30 months [range: 1-180] [1997: 31 months [3-180], 1998: 29 months [4-120], 1999: 28 months [1-180]]; the age frequency distribution is represented in **Figure 4.1.** The life expectancy [*L*] of dogs was calculated from the cohort of dogs followed-up between 1997 to 1999 according to the following equation (125):

$$[E4] \quad \delta = -ln(1 - \frac{n_{t+m}}{N_t}) \cdot \frac{12}{(m/n_{t+m})}$$

$$[E5] \quad L = \frac{1}{\delta}$$

where δ is the mortality rate per year, n_{t+m} is the number of dogs that died m months after time t [i.e. the first time dogs entered the cohort], N_t is the total number of dogs at time t, and m the total time in dog-months of cohort dogs that died between times t and $t+m$. Follow-up data was available for 333 dogs, 122 of which died between any two surveys after a total 2032 dog-months. The total mortality rate was 0.329/year [95% C.I. 0.227-0.452]; hence, the life expectancy was 3.04 years [95% C.I. 2.21-3.61, range between study valleys: 1.81-5.36]. A higher mortality rate was observed in ACL-infected dogs [0.399/year, 95% C.I. 0.301-0.497, n=47] than in non-infected dogs [0.298/year, 95% C.I. 0.237-0.357, n=75]; thus, the life expectancy of an ACL-infected dog was 2.51 years [95% C.I. 2.01-3.32], compared to 3.36 years [95% C.I. 2.80-4.22] for a non-infected dog. However, this difference was not significant [Chi-square, Yates-corrected, χ^2 = 2.35, p=0.13].

Cross-sectional data. In **Chapter 2** it was shown that 26% [262/1022] of sampled dogs were infected with *L. [Viannia]*, yearly village ACL prevalence and cumulative ACL prevalence are shown in **Table 4.1.**

For comparative analyses, villages were grouped into five different valleys [V]: V1 [901, 902], V2 [903, 904, 905], V3 [906, 909], V4 [907, 908] and V5 [914-919] [**Map 1**]. The proportion of infected dogs was significantly different between valleys for the total three year study period [Chi square, Yates-corrected, χ^2=20.13, df=17, p<0.001], in 1997 [χ^2=10.85, df=4, p=0.028] and 1998 [χ^2=10.14, df=3, p=0.017], but not in 1999 [χ^2=3.25, df=2, p=0.2]. Though a lower proportion of infected dogs was observed in all study villages in 1998 compared to 1997 or 1999, this difference was not significant [χ^2=3.57, df=2, p=0.17]. In any one sampling year, prevalence of infection was highest in V4 and lowest in V3, with observed cumulative prevalence of infection being as high as 0.44±0.07 [proportion infected ±

s.e.] [1997] and 0.07±0.03 [1998], respectively. The highest observed canine ACL prevalence observed during the three years of the study was in village 908 in 1997, where 21/38 [0.55±0.08] dogs were ACL positive. No ACL-infected dogs were found on two occasions only, in villages 909 [in 1998] and 922 [in 1997] [**Table 4.1.** and **4.2.**].

Using maximum likelihood, the instantaneous incidence [λ ± s.e.] and recovery [ρ ± s.e.] rates were estimated from the cumulative ACL prevalence data, according to the diagnostic criteria used, i.e. dogs diagnosed as positive by PCR, ELISA or both [**Table 4.1., 4.2.** and **Figure 4.2.**]. The estimated instantaneous incidence rate for 'infection' [i.e. dogs positive by either ELISA or PCR, or both] $\lambda_{infection}$ was 0.285±0.064/year and the recovery rate for infection $\rho_{infection}$ was 0.456±0.207/year. Best estimates of $\lambda_{infection}$ and $\rho_{infection}$ varied between valleys and were as high as 0.83±0.34/year and 0.97±0.44/year, respectively, in V4 [**Table 4.2.**].

Table 4.1. Yearly and cumulative ACL prevalence in study villages.

Study village	901	902	903	904	905	906	907	908	909
1997									
Number of dogs	43	36	65	30	37	38	14	38	38
[females, males]	[14, 29]	[10, 16][a]	[17, 48]	[12, 17][a]	[12, 25]	[21, 17]	[7, 7]	[21, 17]	[13, 25]
Mean dog age	28	27	32	27	25	16	17	35	29
[months] [range]	[5-84]	[5-72]	[3-144]	[9-120]	[5-60]	[4-36]	[4-72]	[3-180]	[6-144]
PI [infection]	0.23	0.39	0.29	0.33	0.08	0.13	0.14	0.55	0.29
PI [PCR]	0.09	0.14	0.09	0.07	0.03	0.11	0.00	0.24	0.08
PI [ELISA]	0.14	0.31	0.25	0.30	0.05	0.05	0.14	0.47	0.24
1998									
Number of dogs	44	36	38	51	38	28	9	27	13
[females, males]	[24, 20]	[13, 22]	[14, 22][a]	[17, 34]	[12, 25][a]	[14, 14]	[4, 5]	[11, 16]	[1, 12]
Mean dog age	31	26	31	31	31	26	31	29	26
[months] [range]	[12-96]	[8-72]	[12-120]	[4-84]	[6-120]	[4-108]	[5-96]	[5-96]	[5-72]
PI [infection]	0.11	0.28	0.24	0.35	0.08	0.11	0.33	0.33	0.00
PI [PCR]	0.05	0.17	0.05	0.08	0.05	0.00	0.00	0.07	0.00
PI [ELISA]	0.07	0.17	0.18	0.29	0.05	0.11	0.33	0.26	0.00
1999									
Number of dogs	55	48	43	62	-	-	-	49	-
[females, males]	[25, 28][A]	[21, 27]	[15, 28]	[18, 44]				[20, 29]	
Mean dog age	31	30	24	29	-	-	-	28	-
[months] [range]	[6-72]	[6-180]	[5-132]	[1-96]				[4-108]	
PI [infection]	0.35	0.19	0.12	0.31	-	-	-	0.37	-
PI [PCR]	0.09	0.10	0.02	0.15	-	-	-	0.10	-
PI [ELISA]	0.33	0.13	0.09	0.27	-	-	-	0.33	-
1997 - 1999									
Number of dogs	142	120	146	143	75	66	23	114	51
[females, males]	[63, 77][a]	[44, 66][a]	[46, 98][a]	[47, 95][a]	[24, 50][a]	[35, 31]	[11, 12]	[52, 62]	[14, 37]
Mean dog age	30	29	29	29	28	20	24	31	29
[months] [range]	[5-96]	[5-180]	[3-144]	[1-120]	[5-120]	[4-108]	[4-96]	[3-180]	[5-144]
PI [infection] ± s.e.	0.24±0.07	0.28±0.07	0.23±0.05	0.33±0.09	0.08±0.04	0.12±0.05	0.20±0.11	0.43±0.13	0.30±0.10
PI [PCR] ± s.e.	0.08±0.04	0.13±0.06	0.06±0.03	0.10±0.06	0.04±0.03	0.06±0.04	0.00	0.14±0.06	0.06±0.04
PI [ELISA] ± s.e.	0.19±0.06	0.19±0.07	0.19±0.05	0.29±0.08	0.05±0.04	0.08±0.04	0.22±0.11	0.37±0.08	0.18±0.06

| Study village	914	915	916	917	918	919	920	921	922
1997									
Number of dogs	14	20	19	11	9	57	4	2	6
[females, males]	[4, 10]	[5, 15]	[6, 13]	[9, 2]	[8, 1]	[47, 10]	[1, 3]	[2, 0]	[a]
Mean dog age	30	26	39	30	46	45	69	-	-
[months] [range]	[5-108]	[8-60]	[6-144]	[8-48]	[7-180]	[5-168]	[36-84]		
PI [infection]	0.43	0.31	0.37	0.45	0.11	0.11	0.75	1.00	0.00
PI [PCR]	0.07	0.10	0.11	0.09	0.11	0.07	0.00	1.00	0.00
PI [ELISA]	0.36	0.20	0.37	0.45	0.11	0.04	0.75	1.00	0.00

NOTE. The proportion of infected dogs [PI] is tabled according which diagnostic test was used [PCR or ELISA, or both, i.e. 'infection'], see **Chapter 2** for details. PI, proportion infected; [a] some dogs not sexed.

Table 4.2. Yearly and cumulative infection rates in study valleys.

Study valley	V1	V2	V3	V4	V5	Total
1997						
Number of dogs	79	132	76	52	130	481
[females, males]	[24, 45][a]	[41, 90][a]	[34, 42]	[28, 24]	[79, 51]	[209, 255][a]
Mean dog age	27	29	22	30	38	31
[months] [range]	[5-84]	[3-144]	[4-144]	[3-180]	[5-180]	3-180]
PI [infection]	0.30	0.24	0.21	0.44	0.24	0.27
PI[PCR]	0.11	0.07	0.09	0.17	0.08	0.10
PI [ELISA]	0.22	0.20	0.14	0.38	0.28	0.22
1998						
Number of dogs	80	127	41	36	-	284
[females, males]	[37, 43]	[43, 81][a]	[15, 26]	[15, 21]		[110, 171][a]
Mean dog age	29	31	26	29	-	29
[months] [range]	[8-96]	[4-120]	[4-108]	[5-96]	-	[4-120]
PI [infection]	019	0.24	0.07	0.36	-	0.21
PI [PCR]	0.10	0.06	0.00	0.06	-	0.06
PI [ELISA]	0.11	0.19	0.07	0.31	-	0.17
1999						
Number of dogs	103	105	-	49	-	257
[females, males]	[46, 55][a]	[33, 72]		[20, 29]	-	[99, 156][a]
Mean dog age	30	27	-	28	-	28
[months] [range]	[6-180]	[1-132]		[4-108]		[1-180]
PI [infection]	0.27	0.23	-	0.37	-	0.27
PI [PCR]	0.10	0.10	-	0.10	-	0.10
PI [ELISA]	0.23	0.19	-	0.33	-	0.24
1997 - 1999						
Number of dogs	262	364	117	137	130	1022
[females, males]	[107, 143][a]	[117, 243][a]	[49, 68]	[63, 74]	[79, 51]	[418, 582][a]
Mean dog age	29	29	24	29	38	30
[months] [range]	[5-180]	[1-144]	[4-144]	[3-180]	[5-180]	[1-180]
PI [infection] $\pm$ s.e.	0.26$\pm$0.05	0.24$\pm$0.04	0.16$\pm$0.04	0.39$\pm$0.07	0.24$\pm$0.04	0.26$\pm$0.02
PI [PCR] $\pm$ s.e.	0.10$\pm$0.03	0.07$\pm$0.02	0.06$\pm$0.03	0.12$\pm$0.04	0.08$\pm$0.02	0.09$\pm$0.01
PI [ELISA] $\pm$ s.e.	0.19$\pm$0.04	0.19$\pm$0.03	0.12$\pm$0.04	0.34$\pm$0.07	0.18$\pm$0.03	0.21$\pm$0.02
$\lambda \pm$ s.d.	0.35$\pm$0.12	0.19$\pm$0.04	0.12$\pm$0.04	0.84$\pm$0.34	-[b]	0.29$\pm$0.04
$\rho \pm$ s.d.	0.76$\pm$0.22	0.17$\pm$0.05	-[b]	0.97$\pm$0.44	-[b]	0.46$\pm$0.06
L [years]	5.36	3.51	1.81	1.81	-[b]	3.04
R_0	2.88	1.67	1.22	2.52	-[b]	1.88

NOTE. Annual incidence [λ] and recovery [ρ] rates for 'infection' were estimated by maximum likelihood from cross-sectional data as described in Materials & Methods. The basic reproduction number R_0 [1+L/A] was estimated using incidence rates estimated by maximum likelihood. PI, proportion infected; [a] remaining dogs were not sexed; [b] could not be estimated.

Figure 4.1. Age frequency distribution
of the sampled dog population.
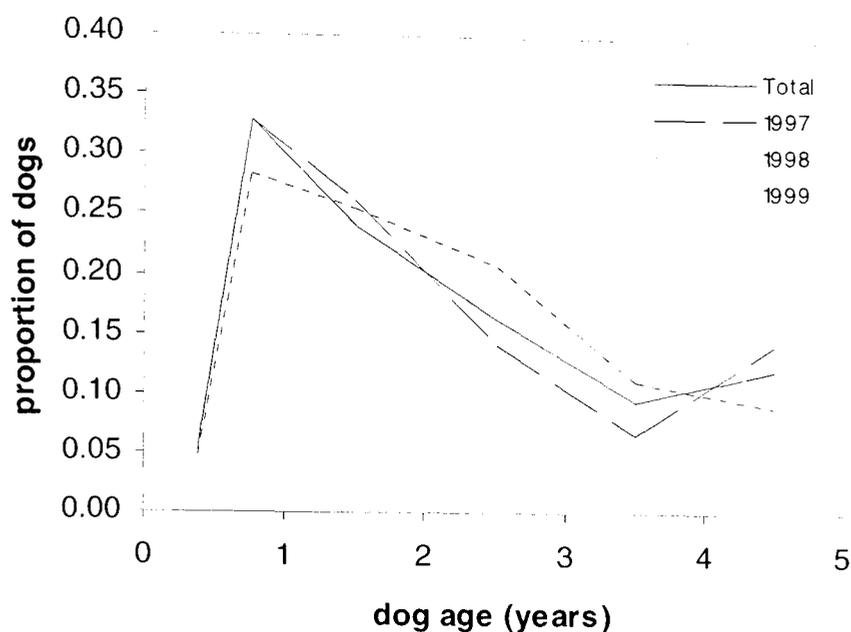
Prospective data. A total of 138 observations were used in the analyses: 49 dogs were surveyed in 1997 and 1998, 33 dogs in 1998 and 1999, 16 dogs in 1997 and 1999. Twenty dogs were sampled at all three time points. Among the 138 observations, 11/122 [9.0%] converted from a PCR negative to a PCR positive result during two surveys, and 8/16 [50%] recovered from a PCR positive to a negative result. 20/113 [17.7%] dogs sero-converted during any two surveys and 11/25 [44.0%] sero-recovered. Using diagnostic criteria set out in **Chapter 2**, 23/103 [22.3%] dogs were incident *Leishmania* infections during any two surveys, whilst 14/35 [40.0%] dogs had infections that resolved. Thus, solving equations E2 and E3 [using the average time between survey time points] the instantaneous rates of conversion and recovery for 'infection' were $\lambda_{infection}$ = 0.291/year [95% C.I. 0.195-0.387] and $\rho_{infection}$ = 0.520/year [95% C.I. 0.302-0.738], respectively [**Table 4.3.**].

Figure 4.2. Cumulative age-prevalence of canine ACL infection.

A
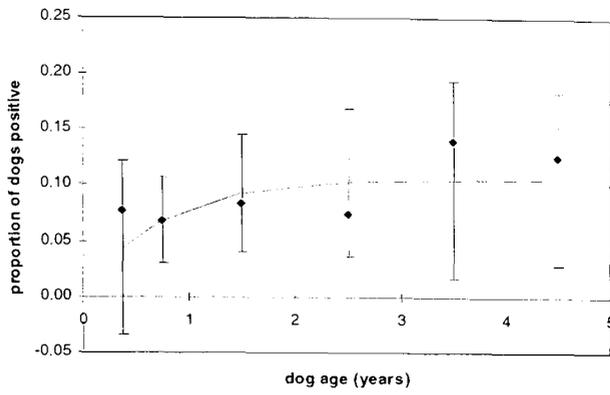
B
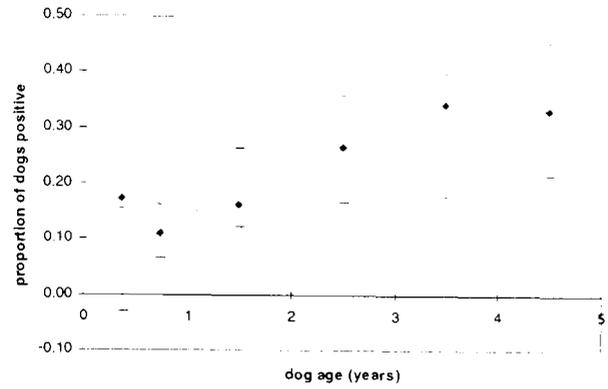
C
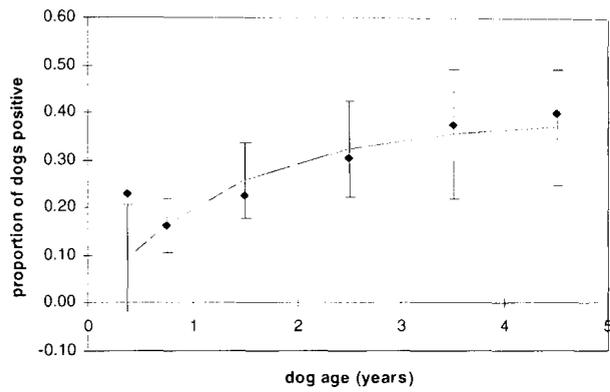
D
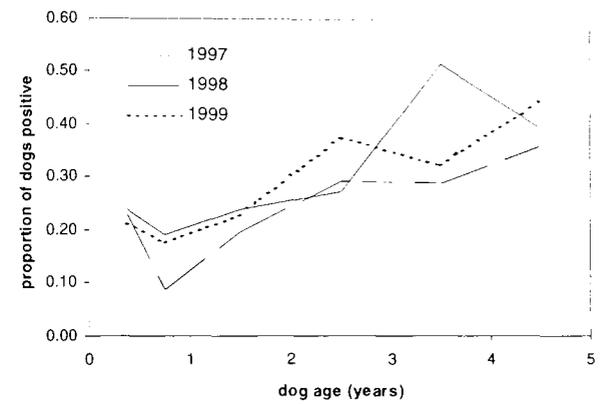
E
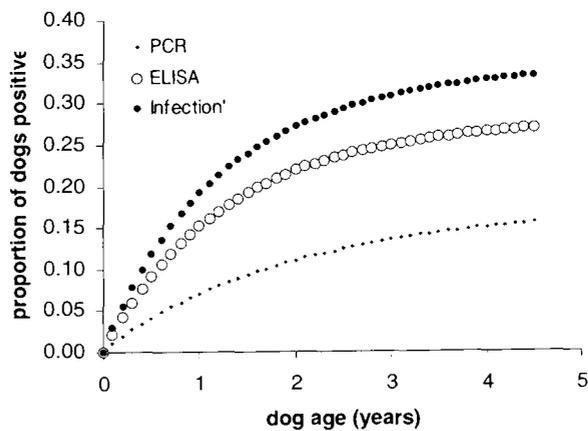
NOTE. Cumulative age-prevalence of ACL infection in dogs according to diagnostic technique: 'infection' [i.e. positive by ELISA or PCR, or both] [**A**], PCR [**B**], ELISA [**C**]. Lines are the best-fit to the data as estimated by maximum likelihood; for estimates of λ and ρ see **Table 3**. Age-prevalence curves ['infection'] in different survey years [**D**]. Using the incidence and recovery rates calculated from the prospective surveys, the cumulative age-prevalence was simulated [**E**] (591).

In order to better understand the dynamics of *L. [Viannia]* transmission in dogs, incidence and recovery rates were estimated in a four-compartmental model for different status of disease with twelve possible rates defining the movements between pairs of compartments. At any time point, each dog in the cohort can be in one of four states as they may have been either PCR negative or positive [PCR-/+] and either ELISA negative or positive [ELISA-/+] [**Figure 4.2.**]. As before, the parameters describing the yearly rates were derived using E4 and E5. Whereas no difference was observed between the sero-conversion rates of PCR- dogs [i.e. 0.192/year] and in PCR+ dogs [i.e. 0.228/year], the parasitological conversion rates of ELISA- dogs [i.e. 0.072/year] was half the rate in ELISA+ dogs [i.e. 0.156/year] [**Figure 4.2.**]. The serological recovery rate was higher in PCR- [i.e. 0.642/year] than in PCR+ [i.e. 0.384/year] dogs. Similarly, the parasitological recovery rate was much higher in ELISA– [i.e. 1.032/year] than ELISA+ [i.e. 0.168/year] dogs. The odds of susceptible [i.e. PCR-ELISA-] dogs becoming serologically positive was significantly higher than the odds of dogs becoming parasitologically positive [OR 3.34, 95% C.I. 1.08-11.02, Yates-corrected, $\chi^2 = 4.49$, p= 0.034], or becoming both serologically and parasitologically positive [OR 5.68, 95% C.I. 1.48-25.60, Yates-corrected, $\chi^2 = 7.37$, p= 0.007]. Using differential equations derived from the incidence and recovery rates in the compartmental model, the change in the proportion of dogs in each of the four states can be simulated over very short time intervals, as at time 0, all dogs will be susceptible to infection [PCR-ELISA-] [**Figure 4.3.E.**]. The simulations show that dog age prevalence and simulations from the prospective data are remarkably consistent with the rates that were calculated from the cross-sectional data using maximum likelihood [**Figure 4.3.A-4.3.C**].

Table 4.3. Epidemiology of *L. [Viannia]* in dogs: incidence and recovery rates estimated from cross-sectional and prospective surveys.

	'Infection[a]	PCR	ELISA
Incidence [$\lambda \pm$ s.e.]			
Cross-sectional data	0.285±0.064	0.147±0.008	0.180±0.041
Prospective data	0.291±0.049	0.113±0.028	0.227±0.041
Recovery [$\rho \pm$ s.e.]			
Cross-sectional data	0.456±0.207	1.237±1.018	0.288±0.176
Prospective data	0.520±0.111	0.630±-0.181	0.565±0.137

NOTE. Incidence and recovery rates using cross-sectional data were calculated as described in Materials & Methods. [a] dogs positive by either PCR or ELISA, or both.

Figure 4.3. Compartmental model of *L. [Viannia]* transmission in dogs.

PCR - ELISA - (103)

PCR - ELISA + (19)

PCR + ELISA - (10)

PCR + ELISA + (6)

0.642/year

0.192/year

0.024/year

0.072/year

1.032/year

0.168/year

0.156/year

0.228/year

0.384/year
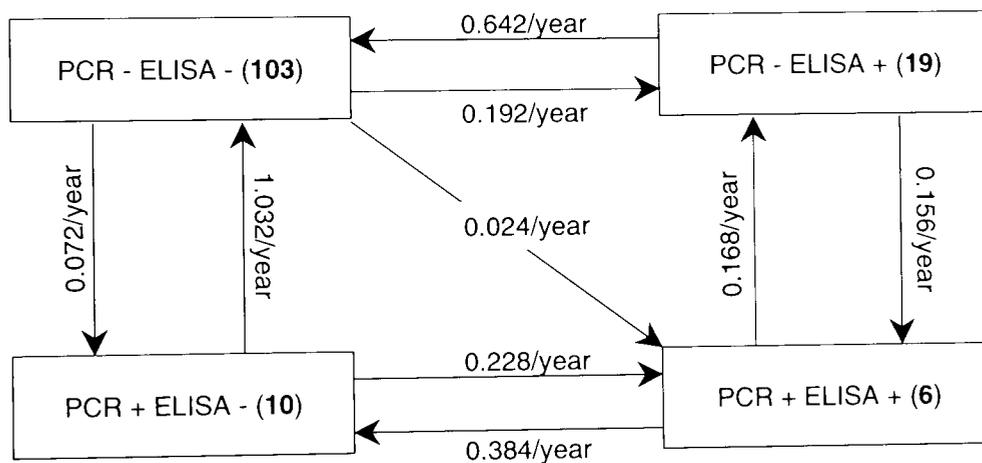
NOTE. Incidence rates [λ] and recovery rates [ρ] were calculated using prospective data and solving E4 and E5 [see Materials and Methods]. At any one time, there are four possible states cohort dog could have been in: PCR negative [PCR-] or positive [PCR+], and ELISA negative [ELISA-] or positive [ELISA+]; numbers in brackets represent the number of dogs in each state.

The basic reproduction number [R_0]. The basic reproduction number R_0 can be estimated, as:

[E6] $R_0 = 1 + \dfrac{L}{A}$

where L is the life expectancy of a dog [3.04 years, 95% C.I. 2.21-3.61] and A is the average time for a dog to acquire infection which can be estimated from the reciprocal of the force of infection [i.e. $1/\lambda_{infection}$] (440). Using cross-sectional and prospective data [**Table 4.3.**] A is estimated to be 3.51 [95% C.I. 2.44-6.25] and 3.44 years [95% C.I. 2.58-5.13], respectively. Hence, R_0 is estimated to be 1.87 and 1.88, respectively.

4.4. Discussion.

Epizoology of *L. [Viannia]* in dogs. This is the first large-scale study to describe the epidemiology of ACL in dogs. Considering the extensive literature suggesting the incrimination of domestic dogs as ACL reservoir hosts, it is surprising that data on the clinical, parasitological and immunological course of *L. [Viannia]* infection in dogs are scarce. Using both cross-sectional and prospective data the ACL incidence and recovery rates in the dog population surveyed in Huánuco was estimated. Although there was a slight difference in age-prevalence curves observed between years [**Figure 4.2.D.**], both cross-sectional and prospective analyses gave similar results with incidence and recovery rates estimated to be ≈0.3/year and ≈0.5/year, respectively [see also **Figures 4.2.A.** and **4.2.E.**]. The reason why estimates from cross-sectional and prospective data were slightly different is because ACL transmission is likely to be heterogeneous (582), as shown by the different prevalence of infection and incidence rates between study villages, valleys and years. This is not surprising because ACL transmission in Huánuco is seasonal, with a peak transmission season between May and August after the yearly rainy season [January-April]. This also may explain the somewhat lower prevalence of infection in 1998, a year of exceptionally

low rainfalls [Canales, *personal communication*]. Other factors that will affect *L. [Viannia]* transmission rates between villages or valleys are temperature and altitude, factors that will influence sandfly vector ecology and, hence, vectorial capacity [see also **Chapter 5**].

One major caveat of this study is that currently no diagnostic test exists that is able to differentiate between *L. peruviana* and *L. braziliensis* infections, both of which are endemic in Huánuco. Of note are the high recovery rates [as estimated by both cross-sectional and prospective data] observed in this study. A possible explanation for this is that dogs readily develop a cell-mediated Th1 immune response against *L. [Viannia]* parasites controlling infection [i.e. parasite numbers in the blood drop below the level detectable by PCR]. This hypothesis appears to be confirmed, as PCR+ELISA+ dogs sero-recover [i.e. they become ELISA-] at a higher rate than they cure parasitologically [i.e. becoming PCR-] [**Figure 4.2.**]. The high rates of sero- and parasitological recovery could also be explained if a proportion of infections is due to *L. peruviana*. *L. peruviana* is known to be very pathogenic, i.e. the parasites easily infect dogs, but -unlike *L. braziliensis-* infections self-cure and do not cause clinical disease (147, 322). Both cross-sectional and prospective surveys indicate that *L. [Viannia]* parasites infecting dogs in Huánuco are pathogenic, but not very virulent indicating that many infections may have been caused by *L. peruviana*. The cross-sectional surveys showed that 3.8% [see **Chapter 2**] of dogs had clinical disease and only one of the dogs with incident infections [i.e. 4.3%] was clinically positive. Unfortunately, parasite isolates were not made in this study, but a previous study had shown that most of the *Leishmania* dog isolates in the study villages are *L. braziliensis/L. peruviana* hybrids (400). It has to be noted that the course of *L. braziliensis/L. peruviana* hybrids in either natural hosts or experimental laboratory animals is unknown. Interestingly, the highest observed sero-recovery rate [1.032/year] was in those dogs that appeared to have low levels of parasites in their blood [i.e. PCR-ELISA+ dogs]. This again indicates that dogs are able to clear *Leishmania* infections readily [as otherwise a sustained antibody response would be observed], underlining the avirulence of *Leishmania* parasites infecting dogs in Huánuco.

The observed high recovery rates are in stark contrast to recovery rates [$\approx$0.100/year] observed in humans living in *L. peruviana*-endemic areas (147), where infections tend to result in life-long immunity to the parasite. This life-long immunity may be sustained by continuous antigen presentation due to persistent parasites (245, 446, 498, 499). The observed recovery rates are also in contrast with those observed in *L. infantum*-endemic areas, where most infections in dogs appear to be life-long with no detectable recovery (126, 440). Nevertheless, the recovery rates that were detected in *L. [Viannia]*-infected dogs mean that infection persists [i.e. $1/\rho = 2$ years] for almost the average life-time of an ACL-infected dog [2.5 years]. Thus, should a dog become infected at an early age, it will remain infected until death, and, hence, potentially infectious to the sandfly vector.

Implications for ACL control. The reason why canine ACL transmission dynamics at the dog population level are of interest is because it allows the quantification [in terms of R_0] of the putative reservoir role of domestic dogs. In epidemiological terms, a primary reservoir of a zoonosis can be defined as a host that is able to maintain the endemic transmission of the pathogen in the absence of any other host species, i.e. maintain R_0 above 1 (24). Secondary reservoirs are those host species whose presence significantly increases R_0, but who are unable to maintain R_0 above 1 in the absence of other host species. In contrast, accidental reservoir hosts play no role in the transmission cycle and their presence has no impact on R_0. Hence, were dogs primary ACL reservoir hosts, it would be feasible to eradicate domestic transmission by targeting dogs; but if dogs are secondary reservoir hosts, targeting dogs would only reduce [i.e. control] ACL. Of course, any dog control strategy would be ineffective were dogs accidental hosts of ACL.

If infected dogs are significantly infectious to sandflies and do play a role in the maintenance of *L. [Viannia]* transmission in Huánuco, they contribute an R_0 of approximately 1.9 [range between valleys: 1.3-2.5]. This estimate compares well with the R_0 for canine ZVL which have been estimated to be 1.1, 1.5, 5.9, 11 in Spain (23), Italy (587), Brazil (440) and Malta (177), respectively. Future analysis of human ACL data will have to determine to

what extent canine transmission rates contribute to overall ACL transmission in Huánuco. R_0 is probably the best indicator of the disease control problem. Because $R_0 \approx 1.9$ is calculated from the mean incidence, the mean yearly effort [i.e. coverage] of a dog control intervention [e.g. vaccination, culling or application of dog collars] to ensure the elimination of $L.$ $[Viannia]$ spp. transmission [i.e. to reduce $R_0 < 1$] can be estimated: $100[1 - 1/R_0] = 47\%$.

One has to note that R_0 does not measure the maximum seasonal potential for an outbreak, nor can that quantity be obtained from seasonal or yearly incidence rates. Thus, one cannot establish from these data how to prevent a dog population to have epidemic outbreaks of $L.$ $[Viannia]$ infection. Moreover, the R_0 that was calculated here did not account for heterogeneous $L.$ $[Viannia]$ transmission by sandfly vectors, although entomological and epidemiological studies indicate that this is the case in Huánuco; similarly different canine ACL prevalence was observed in study villages [**Table 4.2.**]. When including heterogeneity in the distribution of sandfly vectors and hosts across endemic areas and using mathematical modelling, it has been shown that the R_0 may be in fact 2-4 times higher than the R_0 calculated on the assumption of homogeneous transmission (582). Modelling theory predicts that 20% of all hosts will account for 80% of the total transmission [i.e. the so-called '20/80 rule'], a hypothesis that has recently been confirmed by a field study (125) showing that 20% of $L.$ $infantum$-infected dogs account for 80% of the transmission to sandfly vectors. Clearly, this has implications for a dog control strategy, as in the case of Huánuco R_0 could be as high as 7.6 and, thus, the control effort to eliminate transmission in Huánuco could be as high as 87%. The same principle could apply to ACL infections in dogs in Huánuco. The life expectancy of dogs in Huánuco was 3.04 years and the average time for a dog to acquire an infection was 3.4 years. As seen in **Table 4.2.** these figures vary between valleys, but generally appear to indicate that most dogs will not become infected during their lives. This in turn means that those dogs that will become infected will account for most $L.$ $[Viannia]$ transmission to dogs [and humans]. In order to account for heterogeneous transmission in the analyses, correlates of infectiousness will have to be known and a diagnostic test detecting highly infectious dogs will have to be available, neither of which is the case for either ZVL or ACL.

Conclusion. The results presented here show that if dogs are the main ACL reservoir host, a dog control strategy in the study villages in Huánuco would be very feasible, because the control effort to reduce $R_0 < 1$ would be comparatively small. Such an intervention should also provide the ultimate proof in the incrimination of dogs as ACL reservoir hosts, as –if such an intervention strategy were successful- it should have a significant epidemiologial impact on human ACL transmission.

CHAPTER 5:
Domestic dog ownership: a risk factor for human infection with Leishmania [Viannia] spp.

Abstract

An epidemiological study has shown that cumulative, village prevalence of L. [Viannia] infection in dogs ranges from 8% to 45% in Huánuco, Peru. Using data from a prospective survey of human ACL, it was shown that the risk of human ACL did not significantly increase with dog abundance, neither in absolute terms [p=0.659] nor in relation to dog:human ratios [p=0.213]. A significant positive association was observed for risk of human ACL and village dog ACL prevalence [p=0.022]. When controlled for village dog ACL prevalence, there also was an association between the average number of dogs per household and risk of ACL [p=0.033]. The results suggest that dogs play a role in the [peri-]domestic transmission of L. [Viannia] to humans in Huánuco and indicate that a control intervention targeting dogs to control human ACL is warranted.

5.1. Introduction.

As discussed in the **General Introduction** and **Chapters 2-4**, there is growing evidence that domestic dogs are not only reservoir hosts of zoonotic visceral leishmaniasis [ZVL] but of ACL as well, because [i] high ACL infection rates in dogs are found in ACL endemic areas across Latin America; [ii] *Leishmania [Viannia]* strains isolated sympatrically from dogs and humans are indistinguishable; and [iii] there is some reported coincidence between households with ACL patients and the presence of infected dogs. However, these observations alone are not criteria to incriminate dogs as ACL reservoir hosts, but are merely evidence that humans and dogs are likely to be exposed in the same way to the sandfly vector.

Xenodiagnosis studies are the 'golden standard' for reservoir host incrimination, but are notoriously cumbersome to carry out due to the requirements of a constant supply of colonised sandflies (125). Only two small xenodiagnosis studies have been carried out for *L. [Viannia]*-infected dogs. In the first study it was shown that *Lu. whitmani* became infected when made to feed on the lesions of 3/9 *L. braziliensis*-infected dogs, with a mean infection rate on the three infectious dogs of 2.7% [5/186; range: 1.8-8.3%] (557). In the second study, *Lu. migonei* was fed on the lesion of four *L. braziliensis*-infected dogs, with all dogs being infectious for up to two months after the appearance of the lesions. Infectiousness was also shown to change with time, with dogs infectious to 36%, 13%, 14%, 0% and 0% of total flies fed on dogs 11, 41, 56, 71 and 86 days after appearance of lesion (69). No generalisations can be extrapolated from those results due to the low number of replicates, and because sandflies were fed artificially on lesions. Indeed, the *L. braziliensis* infection rate reported for sandflies fed on dog lesions is not higher than that reported on humans: 10.4% [10/96] for *Lu. youngi* fed directly on the lesions of eight human patients, all of whom were infectious (466). *Lu. youngi* have also been infected when fed on 3/6 [asymptomatic] opossums, 40-60 days post-inoculation with *L. braziliensis*, with a mean infection rate on the three infectious hosts of 7.3% [4/55] (502).

These infection rates can be compared with those on ZVL-infected dogs: for example, 13/16 naturally *L. infantum*-infected, seropositive dogs were infectious to *Phlebotomus perniciosus* fed on the head, with a mean infection rate of 37% [149/404] (376). Studies on dogs either naturally or experimentally infected with *L. infantum* have shown that [i] asymptomatic dogs can be infectious to sandflies (22, 125, 238, 376, 558); [ii] dogs become infectious to sandflies after a median period of about 209 days (125); [iii] infectiousness may be associated with high antibody titres (22, 125) and the number of lymphoid T helper [$CD4^+$] cells (239); and [iv] the infectiousness of dogs that have serologically recovered and/or clinically healed after treatment tends to drop temporarily until the dogs relapse (22, 224). Whether any of these features are true for ACL is unknown.

One of the criteria for incriminating dogs as ACL reservoir hosts should come from studies demonstrating that either dog abundance or dog ownership are risk factors for ACL in humans. The epidemiological evidence to date is inconsistent. From four case-control studies in areas of domestic ACL transmission, a positive association between dog ownership and human ACL was identified in two studies [with odds ratios [OR] of 2.9 [95% C.I. 1.4-6.1] and 2.9 [1.0-8.1] in Argentina (186) and Costa Rica (467), respectively], but no association [and a negative trend] was detected in two other studies in Argentina [OR= 0.50 [0.2-1.6]] (585) and Peru [OR= 0.77 [0.5 - 1.2]] (321). Furthermore, using multivariate regression analysis, a cohort study of ACL incidence in Peru identified a slight negative association between village dog abundance and ACL incidence in humans after controlling for inter-village variation in sandfly vector abundance (146).

The objectives of the analyses presented here were to determine whether either village dog abundance, the dog:human ratio or dog ownership could explain the variation in the cumulative ACL prevalence amongst the human population within and between villages of the Department of Huánuco, Peru.

5.2. Materials and Methods.

Human data set and project description. Data on human demography and cumulative ACL prevalence were obtained from an epidemiological study carried out by LSHTM and UPCH in 14 of the 18 study villages of Huánuco included in the canine survey [Davies and Llanos-Cuentas, *unpublished,* see also **Map 1** in **Chapter 2**], and will be briefly outlined below. In 1994 [villages 901 to 909] and 1996 [villages 914 to 918] the houses of each study village were surveyed and mapped. The houses were given a unique code number and demographic as well as clinical information was collected on each household member by questioning (143). All villages were visited at 3-month intervals between 1994 [villages 901-909] or 1996 [villages 914-918] and 1998 to record changes in demographic and clinical data. MST were applied at yearly intervals to a subset of individuals between 1996 and 1998 to record immunological and parasitological changes in *Leishmania* infection status; these data were included in all risk analyses [see below]. The MST antigen was prepared according to standard procedure using *L. peruviana* [MHOM/PE85/LP052] promatigotes (143); the MST response was read 48-96hrs post-inoculation with an induration size >5mm being regarded as positive.

Dog data set. When collecting human data during the base-line household surveys in 1994 [villages 901-909] and 1996 [villages 914-919], data were also collected on the household number of dogs from which the total numbers of dogs in a village and the village dog:human ratio was calculated [for reasons of convenience these dog data are referred to as 1994* data]. Data on 1997 to 1999 [i.e. 1997*] ACL infection in dogs and total household number of dogs were obtained from surveys carried out as previously described, with a positive dog being defined as a dog that was either positive by PCR or ELISA, or both [see **Chapters 2** and **4**]. However, because the 1997* dog surveys were not carried out by house-to-house surveys, only households with dogs were included in the analyses using 1997* data; households from which no dogs were sampled had to be excluded from the analyses.

Data Analysis. Human ACL infection was defined as at least one positive MST response during any of the surveys between 1996 and 1998. The epidemiological impact of dogs was tested at two spatial levels, at the village and at the household level [i.e. after controlling for inter-village variation in ACL transmission]. Both analyses were carried out by logistic regression in STATA 7, i.e. assuming binomial errors in the outcome proportion [individuals MST positive]. In the village analyses the effect of the following variables was tested: [i] the number of dogs per house in 1994*; [ii] the ratio of dogs to humans in 1994*; [iii] the proportion of households with a dog in 1994*; and [iv] the total village population of dogs in 1994*. These variables were tested both in univariate analyses and in combination with an additional parameter, dog prevalence [based on 1997* surveys]. In all models over-dispersion was controlled with Pearson's χ^2. In the household analyses, the effect of [i] presence of a dog in the house in 1994*; and [ii] the number of dogs in the house in 1994* was tested. These variables were included in models which were clustered by households and controlled for gender and age [grouped into four age classes: 1, ≤4 years of age [yrs]; 2, ≤5 yrs; 3, ≤6 yrs; 4, ≥7 yrs; these groups were chosen as the proportion infected levels out at about 30% after 7yrs of age; Davies & Llanos-Cuentas, *unpublished*], and inter-village variability in transmission rates [i.e. clustering villages as categories]. In addition, we tested the effect of the number of infected dogs in a household – but only for those households where dogs were sampled in 1997*. For completeness, all analyses that were carried out are included in the **Appendix** of the chapter, with only the most significant findings being reported below.

5.3. Results.

Between 1996 and 1998, 1916 people were tested by MST for ACL infection, 437 of which tested positive at least once [**Table 5.1.**]. The mean village dog population size in 1994* was 57 dogs [range: 18–125], the mean village dog:human ratio was 0.34 [0.13–0.53], and 64% [326/510] surveyed houses owned a dog. Of surveyed dogs included in the analyses, 26% [251/953] were shown to have an ACL infection during 1997 to 1999 [**Table 5.1.**].

Effect of village dog population size, dog ownership patterns, and dog ACL prevalence on inter-village differences in risk of ACL. The risk of ACL was not associated with either [i] dog populations in 1994* [OR=1.00, 95% C.I. 0.99-1.02, z=0.44, p=0.659]; [ii] dog:human ratios [OR=1.03, 95% C.I. 0.98-1.07, z=1.24, p=0.213]; or [iii] proportion of households owning a dog [OR=1.00, 95% C.I. 0.97-1.04, z=0.28, p=0.783]. A significant positive association was observed for risk of human ACL and village dog ACL prevalence [OR=1.04, 95% C.I. 1.01-1.08, z=2.28, p=0.022], i.e. with every 10% increase in ACL canine village prevalence, there was a 4 % increase in human ACL prevalence. Controlling dog population size and dog ownership patterns for village dog prevalence parameters did not change the association between these parameters and risk of human ACL. Although there was no association between the average number of dogs per household and risk of ACL [OR=1.51, 95% C.I. 0.68-3.36, z=1.01, p=0.315], the association became significant when controlled for village dog ACL prevalence [OR=1.90, 95% C.I. 1.05-3.44, z=2.13, p=0.033], i.e. with every additional unitary increase in the average number of dogs per household in a village there was a 90% [5-344%] [1994*] increase in the odds of a village inhabitant having a positive MST.

Association between household variation in dog ownership patterns and infection rates in dogs and humans at the household level after controlling for variability in village ACL transmission rates. After controlling for inter-village variability in ACL transmission rates, the risk of

ACL was significantly greater in households owning at least one dog in 1994* as compared to households without a dog [OR 1.36, 95% C.I. 1.17-1.57, z=4.13, p<0.001]. When treated as a continuous explanatory variable, the number of dogs per household was also significantly associated with risk of human disease in 1994* [OR 1.07, 95% C.I. 1.02-1.12, z=2.75, p=0.006]. Thus in 1994*, within each village, with every additional dog per household there was a 7% [2-12%] increase in the odds of a household member having a positive MST response. There was no greater risk of ACL when households owned at least one [as compared to none] [OR 0.87, 95% 0.56-1.35, z=-0.64, p=0.525] or two infected dogs [as compared to one or less] [OR 1.23, 95% C.I.0.70-2.16, z=0.71, p=0.475]. Neither was the number of infected dogs per household in a village associated with higher risk of human disease when used as a continuous explanatory variable [OR 0.94, 95% C.I. 0.75-1.18, z=-0.52, p=0.606].

Table 5.1. Human and canine parameters of ACL in study villages used in the logistic regression analyses.

Village	ACL prevalence [proportion MST positive]	No. dogs 1994*	Dog: human Ratio[a]	ACL prevalence dogs 1997-1999
Coz [908]	0.56 [68/121]	37	0.26	0.43 [49/114]
Chinobamba [904]	0.46 [90/196]	122	0.53	0.33 [47/143]
Chullay [907]	0.34 [33/98]	45	0.25	0.22 [5/23]
Limapampa [906]	0.27 [28/103]	56	0.34	0.12 [8/66]
Virroy [914]	0.27 [31/113]	18	0.13	0.43 [6/14]
Conchumayo [902]	0.23 [39/170]	66	0.32	0.28 [33/120]
Quechualoma [903]	0.21 [63/305]	125	0.26	0.23 [33/146]
Parcoy [916]	0.18 [15/82]	19	0.20	0.37 [7/19]
Atahuayon [917]	0.14 [13/89]	37	0.36	0.45 [5/11]
Pomacucho [901]	0.12 [25/208]	69	0.24	0.24 [34/142]
Cochachinche [915]	0.12 [11/91]	32	0.31	0.30 [6/20]
Huancapallac [909]	0.11 [9/83]	54	0.24	0.22 [11/51]
Vilcabamba [905]	0.06 [11/170]	89	0.31	0.08 [6/75]
Mauca [918]	0.01 [1/86]	28	0.32	0.11 [1/9]
Total	0.23 [437/1916]	797	0.29	0.26 [251/953]

NOTE. Human ACL prevalence was defined as described in Materials & Methods, i.e. as a positive Montenegro skin test [MST] response in at least one of the three follow-up surveys during 1996 to 1998. ACL prevalence in dogs was established as described in **Chapters 2-4**. [a] based on 1994* dog data.

Table 5.2. Dog ownership patterns: number of dogs owned per household in 1994*and proportion MST positive.

Household number of dogs	Number of households	ACL prevalence [proportion MST positive ± S.E.]
0	184	0.19 ± 0.02 [117/601]
1	152	0.21 ± 0.02 [122/572]
2	106	0.29 ± 0.02 [120/412]
3	48	0.20 ± 0.03 [46/230]
4	16	0.32 ± 0.05 [24/76]
5	3	0.32 ± 0.11 [6/19]
6	-	-
7	1	0.33 ± 0.19 [2/6]
Total	510	0.23 ± 0.01 [437/1916]

Table 5.3. Dog ownership patterns: number of infected dogs owned per household [1997-1999] and proportion MST positive.

Household number of infected dogs	Number of households	ACL prevalence [proportion MST positive ± S.E.]
0	407	0.21 ± 0.01 [311/1471]
1	75	0.23 ± 0.02 [73/319]
2	19	0.44 ± 0.05 [39/81]
3	4	0.59 ± 0.12 [10/17]
4	3	0.23 ± 0.12 [3/13]
5	2	0.13 ± 0.12 [2/8]
Total	510	0.23 ± 0.01 [437/1916]

Figure 5.1. Changes in household number of dogs between 1994* and 1997 [n=174].

Change in number of dogs between 1994 and 1997
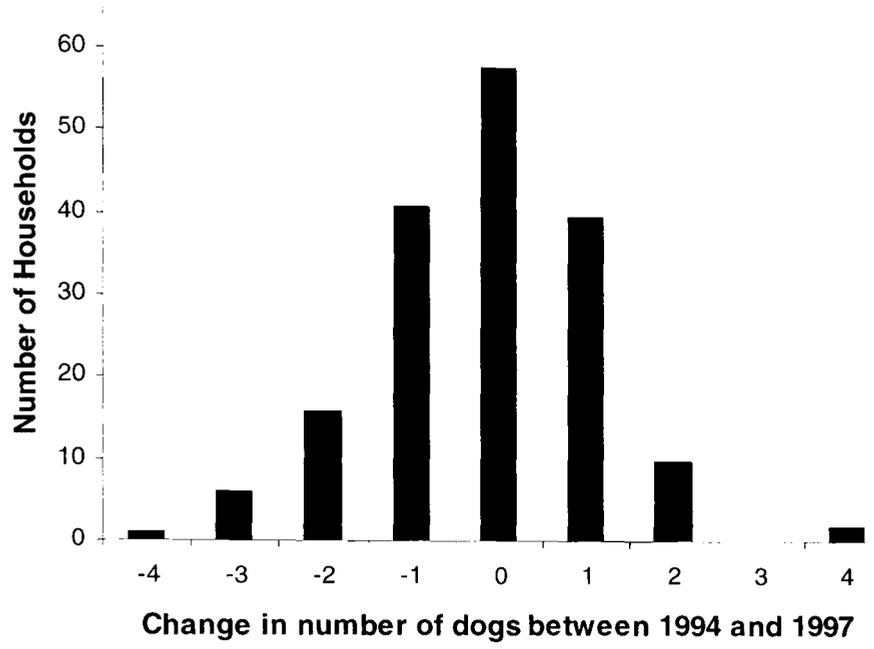
Figure 5.2. The risk of human ACL due to dog abundance, ownership patterns and canine ACL infection.

A
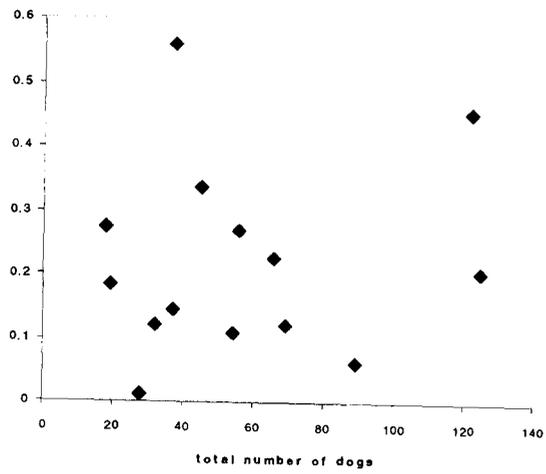
B
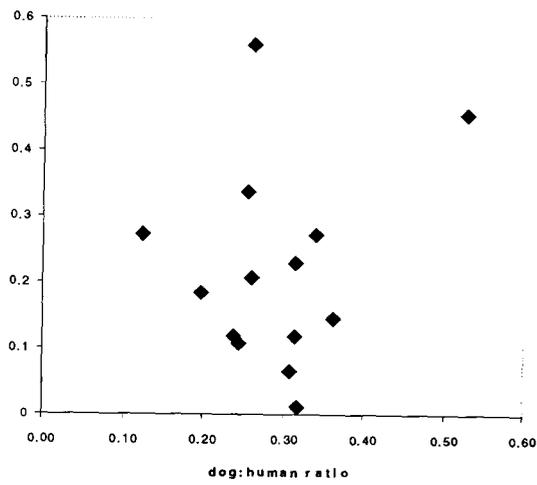
C
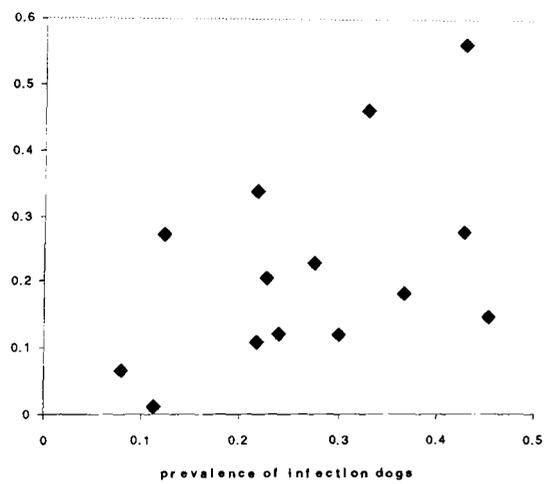
NOTE. The relationship between cumulative MST prevalence and [a] the total number of dogs in the same study villages; [b] the ratio of dogs to humans in the same study villages; and [c] the cumulative ACL prevalence in dogs in the same study villages.

5.4. Discussion.

Dog abundance, dog ownership patterns and dog prevalence and the risk of human ACL. To date case-control studies have been inconsistent in showing an association between dog ownership and risk of human disease. The reason why despite numerous studies on ACL infection rates in dogs such information is scarce is because reliable data sets on canine and human data are rarely available for analysis [**Part 1: Introduction**]. Also, comparative data sets on infection rates of other potential [sylvatic] ACL reservoir hosts do not exist. This is the first study to demonstrate the epidemiological significance of geographical variability in dog population size [in relation to dog ownership patterns] as a risk for human ACL – a key prediction of the hypothesis that domestic dogs are reservoir hosts of ACL. At both the village [when controlled for dog ACL prevalence] and household level [when controlled for inter-village ACL transmission] we show that the risk of human ACL increases with the number of dogs per household.

Because the human and one of the dog data sets were from different years, cumulative ACL prevalence between 1996 and 1998 [as opposed to 1994 to 1998] was chosen as an outcome variable in an attempt to link all three data sets. There are potentially a number of caveats in our analyses. First, dog ownership in itself could be a confounder, as dogs and humans could share a common ACL risk factor. However, to our knowledge, no behavioural difference between people owning or not owning dogs can be observed in Huánuco [e.g. dog owners might be more inclined to go hunting and thereby be at higher risk of ACL infection]. Second, we used cumulative MST positivity in all analyses. This was done in order to increase the sample size of the human data set and hence the power of the analyses; once infected, individuals remain MST positive for a long time due to the very low recovery rates (143). Third, ownership of infected dogs in 1997* was biased towards those households where owners were willing to have dogs sampled for ACL diagnosis. Although the human and dog data sets did not overlap completely [due to the timing of the different surveys], dog ownership did not vary dramatically over time as no change in dog number was recorded in 33% of

the households with dogs in 1994* and 1997. A change in one, two, three of four dogs between 1994 and 1997 was seen in 47%, 15%, 3% and 2% of households, respectively [**Figure 5.1.**]. Fourth, the number of regression analyses that were carried out will have increased the odds of finding a significant association between risk of human ACL and at least one of the dog variables. Nevertheless, bearing these caveats in mind, our analyses consistently show that village dog ownership patterns [in absolute terms] are associated with risk of human ACL both at the village [when controlled for ACL dog prevalence] and at the household level. The reason why no associations were found between dog abundance or dog:human ratio and increased risk of human ACL is probably due to other variables associated with ACL transmission, e.g. altitude, humidity and temperature, factors that are known to influence sandfly ecology and, hence, transmission heterogeneity (146). Above findings can be compared to observations made recently in a *L. infantum* endemic area in Iran, where it was shown that child seropositivity increased significantly with village dog abundance [OR 1.01, 95% C.I. 1.01-1.02] as well as with greater dog:human ratios [OR 10.9, 95% C.I. 1.3-92] (211).

Alternative domestic reservoir hosts of ACL. Considering that previous studies in Peru have shown relatively low *L. [Viannia]* infection rates in sylvatic animal hosts [e.g. *Didelphis albiventris* and *Phyllotis andinum*] as compared to dogs (322), the findings presented here suggest that the importance of sylvatic animals in the domestic transmission cycle of ACL in Huánuco is probably negligible. So far the only domestic animals other than dogs to have been reported to be associated with a higher risk of ACL are: pigs in Argentina [OR 6.91, 95% C.I. 1.47-36.67] (520) and Costa Rica [OR 2.1, 95% C.I. 1.1-4.3] (467). In contrast, chicken [OR 1.43, 95% C.I. 0.94-2.19], sheep [OR 1.51, 95% C.I. 0.94-2.42], goats [OR 1.20, 95% C.I. 0.73-1.96] and cows [OR 1.79, 95% C.I. 0.90-3.55] were not associated with a greater risk of ACL (321). These results, however, are not sufficient enough to incriminate pigs as ACL reservoir hosts, especially as no *L. [Viannia]* infections in pigs have been reported in the literature. The association with human ACL risk is likely to stem from the effect that pigs might have on sandfly abundance and distribution in the peridomestic environment.

Ultimately, the relative importance of dogs in the *L. [Viannia]* transmission cycle will depend on [i] the relative biting rates of sandfly vectors on different hosts including dogs; [ii] the susceptibility of dogs to *Leishmania* infection; and [iii] the infectiousness of infected dogs to sandfly vectors [**Part 1: Introduction**]. As outlined previously, data describing either the sandfly biting rate or the infectiousness to sandfly vectors is scarce and is hampered by a number of confounding variables [e.g. methods of sandfly collection to measure biting rates, exposure of sandlfies to either healthy skin or active lesion in xenodiagnosis experiments]. Data comparing the relative susceptibility of dogs, humans or other reservoir hosts to *L. [Viannia]* infection does no exist. However, assuming that there is no difference in [i] host susceptibility and [ii] sandfly host preference, the ratio of bites a sandfly takes on a dog and a human can be estimated by multiplying the ratio of dog and human incidence rates by the ratio of their respective population densities. As seen in **Chapter 4**, the incidence of ACL in dogs in Huánuco was estimated to be ˜ 0.290/year. The human ACL incidence in Huánuco is ˜ 0.029/year [Davies & Llanos-Cuentas, *unpublished*] and the dog:human ratio is 1:3.4 [**Table 5.1**]; hence, the estimated dog:human sandfly biting ratio is 2.8:1.

Conclusion. Direct incriminatory evidence that dogs are ACL reservoir host would come from a dog control trial, similar as the ones carried out for AVL (32, 171, 210). Were dogs reservoirs of ACL, dog control programmes should be effective in reducing not only canine incidence but human incidence as well. However, so far there is no evidence that targeting dogs with ACL is effective. Only one controlled intervention trial has been carried out to measure the epidemiological impact of dog control [treatment of infected dogs with 20mg/kg Glucantime *vs* no dogs treated] on human ACL. Although the reduction in human ACL prevalence was larger in the treatment area [from 24% to 4%] than in the control area [from 29% to 12%], the difference was not significant (504). The results presented here indicate that dog ownership and dog infection rates are associated with an increased risk of human disease in Huánuco, and appear to suggest that –at least in Huánuco- a controlled dog intervention trial could have a significant epidemiological impact on human disease [**Chapter 4**].

PART 2

CHAPTERS 6 - 8

TOPICAL INSECTICIDES TO CONTROL CANINE ZOONOTIC LEISHMANIASIS

As outlined in **Chapter 1**, *L. infantum* is the etiological agent of ZVL. *L. infantum* has been isolated from a range of mammalian hosts [e.g. *D. albiventris* and *D. marsupialis, R. rattus, Acomys capirinus*], and canids [e.g. *Vulpes vulpes, V. pallida, Cerdocyon thous*] and domestic dogs, in particular, have been incriminated as [peri-]domestic ZVL reservoir hosts. This incrimination is based on the following observations. First, dogs are highly susceptible to *L. infantum* infection with high infection rates observed in dogs throughout the *L. infantum* endemic range (220). Second, the distribution of canine and human ZVL overlap considerably, and identical *L. infantum* zymodemes have been isolated from dogs, man and sandfly vectors (168, 220, 285, 289). Third, both asymptomatic and symptomatic *L. infantum*-infected dogs have been shown to be infectious to sandfly vectors (22, 125, 224, 238, 239, 376, 540). Fourth, the risk of human ZVL infection has been shown to be significantly associated with dog ownership and abundance (211).

Similar to the disease in humans [**Chapter 1**], canine visceral leishmaniasis tends to be a systemic disease with a broad spectrum of clinical signs such as skin ulcers, lymphadenopathy, hepatoslpenomegaly, emaciation, conjunctivitis, onychogryphosis, epistaxis and alopecia (115, 201, 290, 513). In some cases, additional signs may include polyarthtritis, thrombosis and intestinal bleeding. ZVL in dogs is also associated with a number of biochemical changes in urine and serum, including proteinuria, hypergammaglobulemia, decreased packed cell volume and increased blood urea nitrogen (115, 201, 290, 353, 513).

As for ZVL in murine experimental models, resistance to infection is associated with a Th1-type CD4+ T cell response with high levels of INFγ, IL2 and TNFα. Susceptibility of infection and progression of disease is associated with a Th2 response. As the immune response to *L. infantum* in dogs is cell-mediated, no serological markers to distinguish susceptible and resistant dogs exist. Indeed, whereas some authors have shown that different IgG

subclasses appear to be associated with either acute or chronic infections (108, 397, 441), others failed to observe such association (311, 427, 428).

Dog control.

Thus, because domestic dogs are reservoir hosts of L. infantum, one of the approaches to reduce the Leishmania infection rates in sandfly vectors and to control human ZVL is by targeting dogs either through culling, treatment or vaccination.

Dog culling. The impact of dog culling programmes on canine and human ZVL incidence has been doubted on both theoretical (176, 532) and practical (85, 188, 416, 508) grounds. In particular, despite culling an average 20 000 out of 850 000 screened dogs per year since 1989, human ZVL incidence has increased steadily during the same period in Brazil (560). There are several problems associated with the culling strategy. First, a significant proportion of infected [and infectious] dogs are not culled, because the diagnostic tests used to screen dogs [e.g. IFAT or ELISA] are not 100% sensitive [e.g. the sensitivity of serology to detect infectious dogs after patency is 63-69%, (440)], and because of non-compliance by dog owners [e.g. up to 13% of seropositive dogs are not culled as owners hide their dogs from culling personnel, (508)]. Second, positive dogs are detected many weeks after they have been infected due to the infrequency of surveys and because the time-span between surveying, diagnosing and culling dogs is large [up to 120 days] (85, 188, 416). Third, culled dogs are rapidly replaced with susceptible animals through birth and immigration (32, 85, 171, 188, 416).

Results of two controlled intervention trials in Brazil are equivocal (32, 171). Thus, although temporary reductions in dog seroconversion rates [from 36% to 6% over the first two years] were observed when culling seropositive dogs at yearly intervals, no change in the cumulative incidence of canine ZVL could be shown after five years (32). There was also an observed 25% reduction in the number of human ZVL cases incidence; however, this

reduction was not significant showing that ZVL transmission was not fully interrupted. The authors argued that this was likely to be due to the high immigration rates of dogs into the intervention area, which meant that only 42-73% of the seropositive dog population was eliminated in any intervention year. Similarly, a study by Dietze and colleagues (171) demonstrated that a blanket cull of the seropositive dogs at two 6-monthly intervals did not significantly reduce the ZVL seroconversion rate in either dogs [36% to 14% seronconversion rate in the intervention rate compared to 52% to 11% in the control area] or humans [33% to 54% and 36% to 54% in intervention and control areas, respectively].

Where there is some anecdotal evidence that dog culling reduces canine and/or human ZVL incidence [e.g. China (234, 235, 312), Brazil (149, 336, 402, 415) and Italy (223)], it is difficult to clearly demonstrate or quantify the efficacy of this specific measure, as the culling of infected dogs was part of an integrated control strategy including insecticide spraying of houses (149, 235, 336) and active case detection and treatment of human cases (149, 235, 336) or infected dogs (223).

The failure of the epidemiological impact of mass-culling programmes of ZVL infected dogs on human ZVL incidence has led some authors to question the dogma that humans have no role in the ZVL transmission cycle. Most human ZVL infections tend to be asymptomatic with numerous parasites in skin and blood, and, hence, are potentially infectious to the sandfly vector (40, 269, 273). Thus, there have been calls to study the epidemiological role of humans played in the ZVL transmission cycle in more detail. However, so far only human ZVL cases have been shown to be infectious to sandfly vectors (121).

Treatment of infected dogs. In countries where ZVL is mainly a veterinary problem, such as Europe [where estimates suggest that up to seven million dogs are at risk of infection (220)] and United States [where there has been a recent, widely publicised outbreak in foxhounds in Pennsylvania (209)] culling of infected dogs is considered unacceptable and dogs are treated with antileishmanial drugs [e.g. pentavalent antimonials (165, 223, 339, 385,

406, 436, 460, 473, 514, 524, 543, 544), amphotericin B (302, 303, 405, 545), aminosidine (406, 436, 559) or allopurinol (108, 165, 199, 318, 385, 554, 555)]. Treatment of infected dogs is not a very practical control policy, not only because of the prohibitive cost involved [up to US$800/dog with liposomal amphotericin B (302, 391)], but also because of the high relapse rates [up to 74% (514)] amongst treated and clinically cured dogs. Moreover, a high proportion of clinically cured dogs remain parasitologically positive (302, 303, 339, 385, 460, 473, 514, 524) and infectious to the sandfly vector (22, 224, 376). There is also great concern that large-scale use of drugs for ZVL-infected dogs may enhance the emergence of drug-resistant parasite strains.

Dog vaccination. Several vaccine candidates have been promising in experimental animal models, phase 1 and phase 2 vaccine trials, only two were tested in phase 3 trials (221). Surprisingly, only two vaccine candidates have been evaluated in phase 3 trials. In the first trial no protection in vaccinated dogs could be demonstrated; in fact, vaccinated were shown to be more susceptible to *L. infantum* infection than non-vaccinated dogs (175). In a recent trial in Brazil, 92% protection was reported for dogs vaccinated with a fucose mannose ligand [FML]-based vaccine (136). However, the sample size [n=117, 58 cases, 59 placebo controls] and trial protocol [e.g. no data were presented on the sensitivity and specificity of the diagnostic test used to differentiate incident clinical and asymptomatic cases in the vaccine and placebo group] are not rigorous enough to support the data and conclusions.

The next three chapters will discuss present issues regarding the control of canine ZVL control policies. First, a rapid immunochromatographic dipstick to detect ZVL infection was evaluated as a possible solution to the epidemiological consequences of the delay [i.e. the time between surveying, sampling, diagnosing and culling dogs] in canine control implementation [**Chapter 6**]. Second, the potential usefulness of insecticide-impregnated dog collars was tested as an alternative canine leishmaniasis control

strategy, by protecting dogs from sandfly bites [**Chapter 7**] and from disease [**Chapter 8**].

CHAPTER 6:

Rapid detection of Leishmania infantum infection in dogs: a comparative study using an immunochromatographic dipstick test, ELISA and PCR.

Abstract

Current ZVL control programmes in Brazil include the culling of L. infantum-infected reservoir dogs, a strategy which has failed to prevent a rise of canine and human ZVL cases over the past decade. One of the main reasons why this strategy has failed is because of a long delay between sample collection, sample analysis and control implementation. A rapid, sensitive and specific diagnostic tool would be highly desirable, as it would allow control interventions to be implemented in situ. We compared an immunochromatographic dipstick test to ELISA and PCR for detecting L. infantum infections in dogs from a ZVL-endemic area in Brazil. The dipstick test was shown to have 61-75% specificity and 72-77% sensitivity, compared to 100% specificity for both ELISA and PCR and 71-88% and 51-64% sensitivity for ELISA and PCR, respectively. Of the tested field samples, 92/175 [53%], 65/175 [37%] and 47/175 [27%] were positive by dipstick, ELISA and PCR, respectively. The positive and negative predictive values for tested dipstick were 58-77% and 75%, respectively. Efforts should be made to develop a more specific dipstick test for leishmaniasis diagnosis, as they may ultimately prove more cost-effective than currently used diagnostic tests, when used in mass-screening surveys.

6.1. Introduction.

As outlined in **Part 2: Introduction**, one of the reasons why dog culling programmes have failed is because of the long delay between sample collection, sample analysis and control implementation [i.e. culling of infected dogs]. This delay typically is 30 days long, but can be as long as 120 days, with infected dogs remaining infectious to sandfly vectors during this period, thereby transmitting ZVL to susceptible dogs and humans. In a study in Brazil, it was shown that whereas a standard culling strategy implemented 80 days post sample collection resulted in only a 9% decrease in dog seroprevalence, culling implemented 7 days post sample collection resulted in a 27% decrease in seroprevalence (85). Current diagnostic methods used for *Leishmania* mass-screening surveys [mainly ELISA, IFAT or DAT] lack sensitivity or specificity [**Chapter 2**], require technological expertise and specialised laboratory equipment, and can be labour-intensive and time-consuming. Hence, a rapid, sensitive and specific diagnostic test would be extremely valuable in mass-screening surveys and intervention campaigns, as results could be read immediately and control measures could be implemented *in situ*. Implementation coverage rates would be improved [e.g. dog owners would have less opportunity to hide their dogs from culling personnel] and the control intervention would be more effective.

Immunochromatographic dipstick tests for *Leishmania* diagnosis have recently been developed and are all based on recombinant K39 [rK39], a protein predominant in *L. infantum* and *L. donovani* tissue amastigotes (95). rK39 dipstick tests have shown to be quite sensitive [reported sensitivities: 67-100%] and very specific [reported specificities: 97-100%] when tested on kala-azar patients (66, 274, 276, 481, 526, 594), with similar results to rK39-ELISA assays (38, 95, 293, 414, 438, 480, 494, 512, 592, 593). Although rK39-ELISA assays have been used to detect ZVL infection in dogs (38, 414, 457, 494, 592), there are no published reports on the use of the rK39 dipstick to detect ZVL in dogs.

Here the sensitivity and specificity of a commercially available immunochromatographic rK39 dipstick test were compared to serological and molecular diagnostic tests [ELISA and PCR] used in canine leishmaniasis diagnosis. Epidemiological and control intervention implications are discussed.

6.2. Materials & Methods.

Sampling. Blood samples [2-10ml] were taken from 148 dogs in the municipality of Capitão Eneas [16°30'S, 44°00'W], a *L. infantum*-endemic area in Minas Gerais, Brazil. 27 dogs were sampled again after 5 months. Samples were taken by venepuncture and put into sterile, EDTA-coated 10ml polypropylene tubes, and processed 4-10hrs after collection. The blood was centrifuged at 800g for 20min, the buffy coat layer and sera were separated and stored at -20°C. Dog age was estimated by tooth wear and when interviewing dog owners. Mean dog age was 34 months [range: 2 to 180 months], 57 of sampled dogs were female, 91 male. No *L. [Viannia]* spp. or *T. cruzi* transmission was reported in the area [**Plate 5E-G**].

Polymerase chain reaction [PCR]. DNA from buffy coat samples was extracted using the DNeasy® DNA extraction kit [Qiagen, UK] according to the manufacturer's protocol. All samples were amplified with *L. donovani* complex-specific AJS31 [5'-GGGGTTGGTGTAAAATAGGGCC-3'] and DBY [5'-CCAGTTTCCCGCCCCGGAG-3'] primers according to previously published conditions (439). Amplification products were analysed by electrophoresis on 1.5% agarose gels in 1xTAE buffer. To evaluate sample degradation or PCR inhibition, sample DNA was also amplified for a canine housekeeping gene, acidic ribosomal phosphoprotein fragment, using PO3 [5'-GGAGAAGGGGGAGATGTT-3'] and PO5 [5'-TCATTGTGGGAGCAGACA-3'] primers [**Chapter 2**]. When samples did not yield amplification with PO3/PO5 primers, they were extracted again, until positive amplification was obtained. Each amplification cycle included negative [no DNA, DNA from uninfected dog] and positive [water-lysates of reference strain cultures] controls. PCR-

grade H_2O was used throughout. To avoid cross-contamination, separate areas were used for DNA extraction, PCR sample preparation and amplification.

Hybridisation. Agarose gels were processed according to standard procedures, i.e. in denaturation and neutralisation buffer for 20min each, Southern blotted onto a nylon membrane [Boehringer Mannheim, Switzerland], and DNA was fixed by UV-crosslinking. Membranes were prehybridised at 42°C and hybridised with a [$\gamma^{32}P$]-ATP-labelled B4RsaB [5'-GACCTGAAACCCTGGGTCCTGGGCGT-3'] probe for 8-12hrs (439), then washed at 65°C for 2x15min in 2xSSC/0.1% SDS and in 0.1xSSC/0.1% SDS, before being exposed for autoradiography for 36hrs and 72hrs at -70°C.

Enzyme-linked immunosorbent antibody tests [ELISA]. Log-phased _L. donovani_ promastigotes [MHOM/ET/67/L82] were harvested at a concentration of ca. 2.5×10^9 cells/ml, centrifuged at 5000rpm, washed in PBS and frozen at -20°C. Cells were freeze/thawed and briefly sonicated. Antigen [10^5 promastigotes/well] was added to polysterene microtitre plates [Immunolon® 2, Thermo LabSystems, UK] in 100µl of carbonate coating buffer [pH9.6] and incubated overnight at 4°C. Plates were washed [x3] with PBS and blocked with 100µl/well 2% milk powder in coating buffer for two hours at 37°C. They then were washed [x3] with PBS, and serum samples were added at 1/100, 1/400 and 1/800 dilutions in 100µl incubation buffer [PBS/0.05% Tween20+2% milk powder], and again incubated for two hours at 37°C. After washing [x6] with PBS/0.05% Tween20, peroxidase-conjugated, affinity-purified rabbit anti-dog IgG [Sigma, Poole, UK] was added at 1/1500 in 100µl incubation buffer, and plates were incubated for two hours at 37°C. Plates were washed [x6] with PBS/0.05% Tween20 before 100µl of substrate solution [O-phenylenediamine dihydrochloride in phosphate-citrate buffer, pH5.5] was added. The reaction was stopped with 50µl 2M H_2SO_4 and plates were read at 490nm in an ELISA plate reader.

ELISA standardisation. The method used is similar as to the one used in **Chapter 2**. Briefly, on each plate a positive control serum was titrated two-fold from 1/20 to 1/327680. The positive control serum was assigned an

arbitrary number of units/ml, 81920/ml, which was defined as the reciprocal of the highest dilution at which absorbance was greater than the mean + 3 s.d. of background [i.e. no antibody] wells. Absorbance was calculated as observed absorbance minus mean background absorbance. A standard line was fitted over the range 1/80 to 1/81920 to the positive control absorbance values using a log-logit transformation. Absorbances of the three test sera dilutions were expressed as antibody units/ml using the standard line, from which the test sample's geometric mean number of antibody units/ml was calculated. Where the dilution curve for any test serum was noticeably non-parallel to the standard, test sera were repeated at dilutions of 1/100, 1/720 and 1/4320. Samples were considered positive when their antibody level was greater than the arithmetic mean of antibody body units/ml + 3 s.d. of negative controls.

Dipstick test. The dipstick test [*Leishmania* RAPYDTEST®, Intersep, UK] was carried out according to the manufacturer's instructions. The dipsticks were briefly placed into 50μl of serum. After 5-8min, a red control line and, if positive, a second line appeared on the test field. The test is based on a combination of protein A colloidal gold conjugate and rK39 *Leishmania* antigen to detect anti-*Leishmania* antibody in serum or plasma [**Plate 5A**].

Negative and positive controls. Three groups of uninfected dog sera were used as negative controls for all diagnostic tests. The sera came from [i] dogs of various ages and breeds which had attended a veterinary clinic in Lima [Peru; n=17]; [ii] mongrel dogs from Belém [Brazil; n=12]; and [iii] dogs of various ages and breeds which had attended a veterinary clinic in Cambridge [UK; n=11]. The positive standard control serum as well as nine other high-titre positive control sera came from dogs with confirmed *L. infantum* infection [by either culture, microscopy or xenodiagnosis] from Marajó, Brazil [see **Chapter 2**].

6.3. Results.

Table 6.1. summarises the dipstick, ELISA and PCR results of field samples, negative and positive controls, and the frequency distribution of log units of anti-*Leishmania* antibody units/ml depending on dipstick or PCR positivity is represented in **Figure 6.1.-6.3.** The mean antibody levels for the three groups of negative controls were 2562 [s.d.=558] [Lima], 1099 [s.d.=689] [Marajó] and 1854 [s.d.=783] [Cambridge] units/ml. The mean antibody units of all negative controls was 1831 [s.d.=978] units/ml, hence the cut-off for positivity was 4765 units/ml [i.e. 3.68 log [antibody units/ml]]. The mean for positive control sera was 170237 [s.d.=97281] units/ml.

None of the 40 negative control samples was positive by ELISA or PCR [**Table 6.1.**], but 10 out of 40 negative control sera were positive using the dipstick test. Thus, specificity of PCR, ELISA and dipstick was 100%, 100% and 75%, respectively. The 10 negative control dogs that were positive in the dipstick test came from Cambridge [n=4], Peru [n=2] and Brazil [n=4] [mean antibody units/ml=1851, s.d.=1230]. All 10 positive controls were positive by both ELISA and dipstick [i.e. 100% sensitivity], while 8 of 10 were positive by PCR. The proportion positive of the field samples with each diagnostic technique was 53% [92/175], 37% [65/175] and 27% [47/175] for dipstick, ELISA and PCR, respectively.

To estimate the sensitivity and specificity of each test in the field samples, the true number of infected dogs must be estimated. Two approaches were used to estimate the number of infected and uninfected field dogs. In approach 1, we use the estimates of 100% specificity for ELISA and PCR from the control samples [i.e. all ELISA and/or PCR positives are true positives], and assume that all ELISA-/PCR- samples were true negatives [i.e. all 39 RAPYD+ but ELISA-/PCR- samples were false positives]. Approach 1 gives an estimated 74 positives and 101 negatives. In approach 2, we again use the estimated 100% specificity for ELISA and PCR, but also the estimated 75% dipstick specificity. Thus, only 21 [62/3] of the 39 RAPYD+/ELISA-/PCR- samples are false positives, and 18 are true positives.

Approach 2 gives a total of 92 positives and 83 negatives. **Table 6.2.** summarises the sensitivity, specificity and positive and negative predictive values [PPV and NPV, respectively] of each test using each approach. With approach 1, the sensitivity and specificity of the dipstick test were 72% and 61%, respectively; approach 2 increases the estimated sensitivity to 77%. The sensitivities of ELISA and PCR were 88% and 64% using approach 1, and 71% and 51% using approach 2 [**Table 6.2.**].

Table 6.1. Comparative diagnosis of L. infantum in dog blood.

	Negative Controls[a] [n=40]		Positive Controls[a] [n=10]		Field Samples [n=175]	
	RAPYD+	RAPYD-	RAPYD+	RAPYD-	RAPYD+	RAPYD-
ELISA+/PCR+	-	-	8	-	30	8
ELISA-/PCR+	-	-	-	-	6	3
ELISA+/PCR-	-	-	2	-	17	10
ELISA-/PCR-	10	30	-	-	39	62

NOTE. [a] PCR on positive and negative Marajó controls were carried out on sera, not buffy coat.

Table 6.2. Sensitivity, specificity, negative and positive predictive values for used diagnostic tests.

	PCR		ELISA		RAPYDTEST	
Approach	1	2	1	2	1	2
PPV	100%	100%	100%	100%	58%	77%
	[47/47]	[47/47]	[65/65]	[65/65]	[53/92]	[71/92]
NPV	79%	65%	92%	75%	75%	75%
	[101/128]	[83/128]	[101/110]	[83/110]	[62/83]	[62/83]
Sensitivity	64%	51%	88%	71%	72%	77%
	[47/74]	[47/92]	[65/74]	[65/92]	[53/74]	[71/92]
Specificity	100%	100%	100%	100%	61%	75%
	[101/101]	[83/83]	[101/101]	[83/83]	[62/101]	[62/83]

NOTE. See Text for different approaches used to estimate PPV, NPV, sensitivity and specificity of each diagnostic test; the different assumptions made for each approach are in italic. PPV, positive predictive value; NPV, negative predictive value.

Figure 6.1. Frequency distribution of log units/ml of anti-*Leishmania* antibody in the tested field samples.
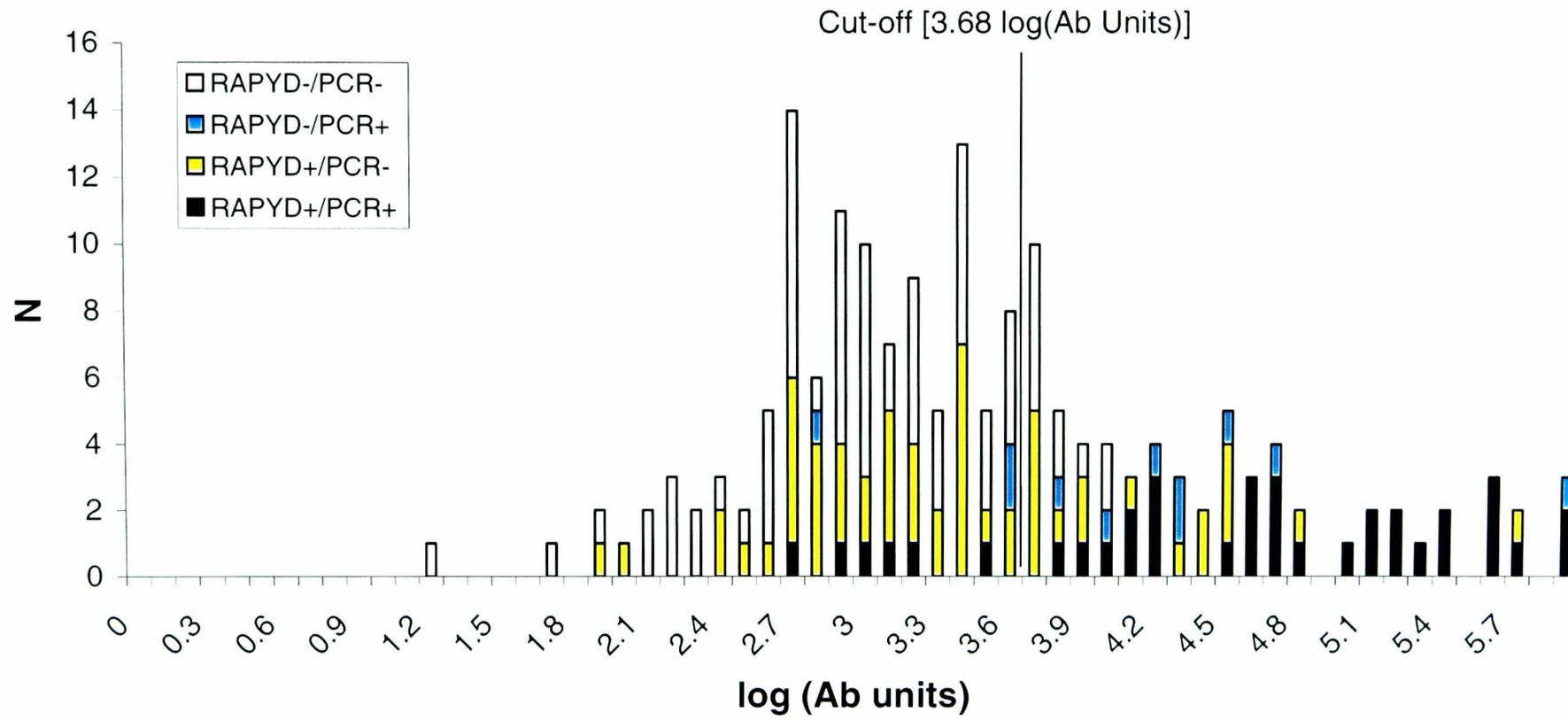
6.4. Discussion.

Mass-screening in epidemiological surveys: a choice of tests. This is the first study to use a dipstick test to detect *Leishmania* infection in dogs. Our study indicates that the RAPYDTEST has a comparable sensitivity to ELISA, but that its specificity is very low [61-75%]. Thus, use of the dipstick tests would lead to a high proportion of dogs being misdiagnosed as false positives [up to 39 out of 92 positive field samples, **Table 6.1.**]. The reason for this is unknown, but it could include test cross-reactivity to some factor present in dog blood, as tested rK39 dipsticks were highly specific when tested on kala-azar patient blood. Previous studies using the rK39-ELISA assay reported responsiveness to rK39 in 2/33 Chinese toxoplasmosis patients [though the authors reported that the two responsive patients may have had sub-clinical ZVL (512)], 2/61 Sudanese healthy endemic controls (593), 1/10 Turkish malaria patients (414), and 6/83 Turkish cutaneous leishmaniasis patients (414). The rK39 antigen is not known to cross-react to *L. braziliensis* or *T. cruzi* (38, 95, 163, 438). Also, rK39 responsiveness appears to be restricted to active kala-azar infections as opposed to asymptomatic, self-healing, cured or treatment-resistant patients (38, 293, 512) or dogs (457, 494), though other studies failed to show such association (593, 594).

Five reported studies used a rK39-ELISA assay to detect *Leishmania* infection in dogs (38, 414, 457, 494, 592). The rK39-ELISA was 100% sensitive in 90, parasitologically confirmed, high-antibody titre dogs in Brazil (38) and in 37 parasitologically confirmed dogs in Venezuela [negative controls were not included in either study] (592). In a Turkish study 18/494 dogs were positive by rK39-ELISA, sensitivity and specificity were reported to be 93% and 100%, respectively (414); in a large epidemiological survey in Italy rK39-ELISA sensitivity and specificity were 97% and 99%, respectively (494). Finally, in a Moroccan study the rK39-ELISA was 100% sensitive in detecting 11 parasitologically confirmed, clinically symptomatic dogs, but failed to detect ZVL infection in 9 parasitologically confirmed, clinically asymptomatic

dogs (457). Variability in dipstick performance will depend on factors such as the type of diagnostic antigen and conjugate used. Previous experience on malaria dipstick tests show that these tests can be highly variable in terms of sensitivity and specificity (580). False positive rates for malaria dipsticks can be as high as 28%, which may for example be due to cross-reactivity to rheumatoid factor (297).

A number of PCR protocols to detect *L. infantum* have been developed and PCR has been shown to be a sensitive and highly specific technique for the detection of symptomatic or parasitologically proven infections [see also **Chapter 2**] (31, 67, 202, 439, 472, 473, 518). Evidence suggests that PCR is less sensitive in detecting asymptomatic dogs (31, 439). The results presented here confirm this observation, as PCR only detected 79% of ELISA positive and 39% of dipstick positive field samples, with PCR positivity being associated with ELISA antibody units [linear regression after arc-sine transformation of data, d.f.= 39, r= 0.84, p< 0.001]. The sensitivity and specificity of the PCR assay depend on several factors including PCR primers, DNA extraction protocol and source of biopsy material [**Chapter 2**] (294, 296). The advantage of using blood [buffy coat] is that the sampling is less invasive than bone marrow, spleen or lymph node aspirates, and samples can be processed readily. On the other hand, parasite load in blood tends to be lower than in bone marrow, spleen or lymph node aspirates, and blood may contain a number of PCR inhibitors [e.g. heme] that may affect PCR assay sensitivity.

Using a conservative cut-off [i.e. mean + 3 s.d.] (440), ELISA was 100% sensitive in detecting culture positive dogs and 84% sensitive to detect parasitologically confirmed [PCR] field dogs. Interestingly, 4 out 9 PCR positive but ELISA negative samples were from dogs that had recovered serologically by the time the second samples were taken. This demonstrates that the sensitivity of the diagnostic technique used can change with course of infection (439), and that these dogs appear to have developed an immune response controlling infection. The sensitivity and specificity of ELISA depend on type of antigen used [e.g. parasite species, promastigotes or amastigotes]

and changes to the standard experimental protocol [e.g. incubation time or type of microtitre plates used].

Conclusion. Research into developing a more specific *Leishmania* dipstick test should be pursued, as the advantage of such a tool with respect to other diagnostic methods such as microscopy, ELISA or PCR are many-fold. Using dipsticks, a vast number of samples can be processed quickly and with minimum effort. Compared to microscopy, ELISA or PCR, minimal technological expertise [i.e. training of personnel] and specialised laboratory equipment are required. Another advantage of dipstick tests is that patients [in this case, dog owners] can see the results for themselves, which should contribute to a better working relationship between local communities and people carrying out the surveys, and increase compliance rates. Epidemiologically, the most important characteristic of a dipstick test is that it allows interventions to be implemented *in situ*. The outcome should be to significantly reduce the mean duration of infectiousness of dogs that have become infected, thereby significantly enhancing the impact of the intervention on the basic reproductive number, R_0. Immunochromatographic dipstick tests are comparatively expensive, but considering the above, a sensitive and specific dipstick test could prove a very cost-effective alternative to currently available diagnostic tests, especially when used in mass-screening surveys.

CHAPTER 7:
A comparative trial of topical insecticide treatments to protect dogs from bites of sandfly vectors of leishmaniasis.

Abstract

We compared the susceptibility of sandfly vectors to four topical insecticide treatments applied to domestic dogs, a reservoir of human leishmaniasis. Dogs were exposed to sandflies pretreatment and at 1 week, 1 month, and 2 months posttreatment. Sandfly bloodfeeding and survival rate of both fed and unfed flies were significantly reduced by the permethrin, deltamethrin, and fenthion treatments, but diazinon had no effect. The survival of bloodfed sandflies was reduced by up to 86% with deltamethrin collars. The antifeeding effect suggests that deltamethrin collars may be recommended to dog owners to protect their pets from sandfly-borne diseases. The combined effects on sandfly feeding and survival indicate that epidemiologic, community-based trials are warranted to test whether deltamethrin collars could reduce the incidence of canine and, hence, human leishmaniasis.

7.1. Introduction.

The first evidence that topical insecticides could be used to control ZVL came from experimental studies in China. After showing that deltamethrin bath treatment reduced the bloodfeeding rate and survival rate of *Phlebotomus chinensis* sandflies exposed to an unnatural host [hamsters: (110)], it was found that the bloodfeeding rates of *P. chinensis* exposed to dogs for 8 hrs were significantly reduced from 62% to 4% by dipping dogs in 25ppm deltamethrin, and none of the sandflies [unfed or fed] exposed to treated dogs survived (236). The effect persisted for up to 104 days. Field evidence for the impact of topical insecticides comes from a community-based trial in China, where ZVL transmission was apparently interrupted following 2 years of treatment [two rounds per year] of all village dogs in 50ppm deltamethrin baths (237). However, the trial results must be interpreted with caution, as no control villages were included in the study.

In contrast to insecticide lotions [Molina *et al.,* unpublished], experimental trials in France have shown that the effects of deltamethrin-impregnated collars on sandfly bloodfeeding and survival can persist for up to 8 months (287). Once collars had been applied for 2 weeks, blood-feeding consistently dropped by 90% and mortality rates increased by 51% during the course of the experiment. This level of persistence was not fully achieved when similar trials were carried out in Spain (329), where the anti-feeding effect dropped significantly from >90% during the first 4 months to 84% after 6 months, and the lethal effect dropped steadily from 76% after 2 weeks to 42% after 6 months. Nevertheless, both trials demonstrate that, when using collars, the effects persist for much longer than when dogs are dipped in deltamethrin. The potentially wide applicability of DMC collars for protecting dogs against sandflies has since been demonstrated by experimental trials with *P. papatasi* in Iran (247) and *Lu. Longipalpis* and *Lu. migonei* in Brazil (142).

The work reported in this chapter directly compared the anti-feeding and lethal effects of deltamethrin-impregnated dog collars versus the effects of alternative topical insecticide applications. The study also increases the

number of sandfly species against which insecticide-impregnated dog collars have now been tested, and was the first such study to test their impact on a vector of ACL [*Lu. intermedia*], which is known to feed on both humans and dogs (102).

7.2. Materials & Methods

Study Site and Protocol. All 17 dogs used in the experimental trial came from the Fazenda Palmital, a large farm 40km from Maringá, Estado do Paraná, Brazil [23°40'S, 52°25'W]. Mongrel dogs were stationed individually inside cages [50cm width x 60cm height x 60cm depth] and were exposed overnight [±22.30-5.30hrs, i.e. for 7 hours] to an average 96 [range: 69-121] wild-caught sandflies introduced through sleeves of closed net curtain tents [100cm x 180cm x 130cm] [**Plate 5B**]. Sandflies had been caught the same night by mouth aspirator inside open chicken pens at the Fazenda Marista [21.00-22.00hrs], a neighbouring farm. Previous studies have shown that >85% of all collections made at the Fazenda Marista are *Lu. intermedia* [Teodoro et al., unpublished data], which was confirmed by microscopically identifying to species a subset of caught sandflies: of 200 unfed and 40 bloodfed sandflies, 172 [86%] and 35 [88%] were identified as *Lu. intermedia,* respectively; the remainder being *Lu. whitmani.* Sandflies were collected from the tents the following morning using a mouth aspirator, placed into suspended gauze cubic cages, and maintained on sucrose-solution for a further 17hrs at 24-26°C and 90-95%RH. After a total of 24hrs, flies were sexed and scored as either dead or alive, and bloodfed or unfed.

All dogs were exposed to sandflies prior to treatment [day 0] and again at 5-12 days post treatment [dpt], 32-36dpt, and 58-65dpt. For brevity, these time points are referred to in this paper as 1 week [1w], 1 month [1m] and 2 months [2m]. Four treatments were compared: [i] 40mg/g deltamethrin-impregnated collars [DMC, Scalibor®, Intervet International GmbH, Wiesbaden, Germany] [n=5]; [ii] 15% diazinon-impregnated collars [DZ, Canovel®, Pfizer, UK] [n=3]; [iii] application of 1ml 0.65g/ml permethrin

topical lotion [PM, Pulvex®, Coopers Brasil Ltda, São Paulo, Brazil] [n=3]; and [iv] application of 1ml 15% fenthion topical lotion [F, Pulfim®, Bayer S.A. Brasil, Barueri, Brazil] [n=3] [**Plate 5C**]. The collars were attached around the neck of the dogs. The topical lotions were applied directly onto the skin after the dog's hair was separated at the nape of the neck. Three untreated dogs [negative controls, C] were exposed to sandflies at the same time points in order to adjust for any background changes in sandfly feeding and survival rates over time. Changes in climatic conditions [temperature and relative humidity] were measured at the start and end of each bioassay.

Data Analysis. The effects of the different treatments [in relation to the negative control] at each time point were tested using General Linear Models (129) in the computer package STATA, i.e. by analyses of deviance, specifying binomial errors, of the effect of treatment on the log odds of sandfly bloodfeeding and mortality. Any significant overdispersion was corrected by re-scaling the model using the ratio of the residual deviance to residual degrees of freedom. Maximal models incorporated the effects of dog age, dog size and climatic conditions along with treatment. The significance of each variable was tested by back-step analysis of deviance, i.e. by observing whether these variables explained a significant [$P<0.05$] proportion of the deviance remaining after their removal from the model. Variables were excluded from the models in order of least significance until only significant variables were retained in the minimum adequate model.

7.3. Results.

An average of 49 [range: 19-86] female and 22 [range: 2-47] male sandflies were recovered from the tents the following morning [i.e. a mean recovery rate of 74%]. The sex ratio was remarkably constant throughout the experiment, with no significant differences detected with time or treatment [median proportion of females recovered: 0.68; 25%-75% quartiles: 0.61 - 0.75]. Sandfly bloodfeeding and mortality rates were unaffected by dog age, dog size or climatic conditions throughout the trial. No dogs had visible side-effects against the different treatments.

Table 7.1. Experimental dog trial comparing effects of topical insecticide applications on the feeding and survival rates of female sand flies.

Dog	TM	0dpt		5-12dpt		32-36dpt		58-65dpt	
		B	U	B	U	B	U	B	U
1	C	40 (1)	37 (3)	37 (0)	38 (6)	25 (0)	34 (4)	23 (1)	30 (4)
2	C	43 (1)	34 (8)	28 (0)	27 (2)	26 (1)	39 (6)	25 (1)	33 (3)
3	C	31 (1)	44 (5)	24 (2)	40 (7)	30 (0)	29 (2)	14 (3)	37 (8)
4	DM	20 (1)	28 (2)	21 (2)	31 (10)	14 (3)	34 (14)	8 (1)	36 (11)
5	DM	24 (0)	54 (4)	16 (1)	27 (5)	7 (3)	37 (8)	ND §	ND §
6	DM	47 (1)	39 (2)	9 (1)	53 (7)	8 (2)	37 (14)	5 (1)	43 (9)
7	DM	26 (0)	28 (3)	3 (0)	20 (10)	4 (2)	31 (20)	4 (2)	34 (15)
8	DM	25 (1)	34 (1)	14 (0)	26 (5)	6 (2)	26 (23)	5 (1)	23 (19)
9	DZ	38 (0)	40 (5)	32 (0)	20 (3)	23 (1)	18 (6)	ND ¥	ND ¥
10	DZ	26 (0)	38 (4)	36 (2)	8 (4)	29 (1)	27 (7)	ND ¥	ND ¥
11	DZ	16 (0)	34 (5)	15 (1)	21 (2)	13 (0)	39 (6)	17 (0)	29 (4)
12	PM	17 (0)	27 (6)	6 (2)	35 (7)	9 (1)	21 (4)	8 (1)	23 (8)
13	PM	16 (1)	39 (3)	10 (1)	24 (11)	21 (1)	38 (19)	11 (0)	38 (11)
14	PM	22 (0)	14 (3)	15 (1)	31 (9)	13 (2)	36 (10)	10 (1)	39 (9)
15	F	22 (1)	47 (7)	15 (2)	15 (6)	6 (0)	17 (9)	9 (1)	28 (13)
16	F	29 (1)	22 (8)	27 (1)	15 (13)	11 (4)	28 (19)	16 (0)	37 (7)
17	F	10 (1)	21 (2)	16 (0)	44 (5)	4 (3)	15 (10)	8 (3)	33 (8)

NOTE. Dead sand flies (after 24hrs) are in brackets. Abbreviations: B, bloodfed sand flies; C, control; DM, deltamethrin-impregnated dog collar; dpt, days post treatment; DZ, diazinon-impregnated dog collar; F, fenthion topical lotion; PM, permethrin topical lotion; ND, not done; TM, treatment; U, unfed sand flies. §, dog 5 was killed by his owner; ¥, dog 9 and dog 10 moved.

Sandfly bloodfeeding rate. In the absence of treatment, the average sandfly bloodfeeding rate was 42% [**Table 7.1.** and **Figure 7.1.**]. There was no significant difference between the bloodfeeding rate on negative control dogs and the rates on any of the four 'treatment groups' prior to treatment [$P > 0.2$ for all four comparisons]. Neither were there any significant differences in bloodfeeding rates on treated and untreated dogs at the first time point [i.e. 1w], although there was some suggestion of a reduction on the dogs treated with PM [$P = 0.088$] and DMC [$P = 0.083$]. Bloodfeeding rates

Table 7.2. Percentage reduction in sand fly bloodfeeding after application of topical insecticides.

TM	Reduction in sand fly bloodfeeding (%) [95% C.I.]					
	5-12dpt		32-36dpt		58-65dpt	
DM	37.6	[5.3, 68.0]	56.7	[38.7, 70.5]	68.5	[42.8, 77.6]
DZ	-37.0	[-77.1, 13.3]	1.4	[-23.1, 24.7]	3.4	[-40.1, 40.0]
PM	44.1	[-7.2, 76.2]	31.6	[7.8, 51.4]	49.2	[14.3, 61.6]
F	4.2	[44.8, 48.3]	41.4	[13.3, 62.9]	43.1	[53.0, 56.0]

NOTE. Abbreviations are as in **Table 7.1.**

were significantly less on dogs treated with DMC, PM and F treatment [as compared with untreated dogs] at both 1m [$P<0.001$; $P=0.010$; and $P=0.005$, respectively] and 2m [$P<0.001$; $P=0.004$; and $P=0.018$, respectively]. At both time points the reduction in bloodfeeding rate was greatest on DMC treated dogs [**Table 7.2.**], though the difference with PM and F treated dogs was not statistically significant. The greatest anti-feeding effect in all trials was detected on DMC-treated dogs after 2m, when bloodfeeding rates were reduced by 69% [95% CI: 43–78%]. A similar pattern was demonstrated when bloodfeeding rates post-treatment were compared to bloodfeeding rates pre-treatment on the same dogs [rather than to bloodfeeding rates on control dogs at the same time points]. In these analyses, a significant reduction in bloodfeeding was detected on DMC treated dogs at all three time points: at 1w [$P=0.012$], 1m [$P<0.001$] and 2m [$P<0.001$]. In contrast, bloodfeeding rates only dropped significantly by 1m on FL treated dogs, and by 2m on PM treated dogs. No significant reduction in bloodfeeding was detected on DZ treated dogs at any time point, when compared to either negative control dogs or pre-treatment controls.

Sandfly mortality. In the absence of treatment, the average sandfly mortality (i.e. the proportion of sandflies dying during the first 24hrs post-treatment) of unfed and bloodfed sandflies was 12% and 2%, respectively [**Table 7.1, Figure 7.1.**]. There was no significant difference between the

mortality of bloodfed sandflies on negative control dogs and the equivalent rates on any of the four 'treatment groups' prior to treatment [P>0.4 for all four comparisons]. In comparison with untreated dogs, mortality of bloodfed flies was significantly increased at 1w by 11%, i.e. 5.7-fold [95% CI: 1.1–20.5], as a result of PM treatment [P=0.037], and at 1m by 33%, i.e. 27-fold [4.4–66], by F treatment [P=0.001] and by 30%, i.e. 25-fold [4.2–63], by DMC treatment [P=0.001]. No significant treatment effects on the mortality of bloodfed sandflies were detected at 2m.

Prior to treatment, there was no significant difference between the mortality of unfed sandflies on negative control dogs and the equivalent rates on three of the 'treatment groups' [P> 0.4 for all three comparisons], but the mortality of unfed sandflies on the group allocated with DMC treatment was significantly less [P= 0.020] than on the control group. The effect of this minor pre-treatment bias was to make it harder to detect any significant increase in mortality as a result of DMC treatment. With this caveat, we were unable to detect any significant differences in the mortality of unfed sandflies at 1w. However, in comparison with untreated dogs, mortality of unfed sandflies was significantly increased by 41%, i.e. 4.1-fold [1.7–6.6], at 1m by DMC treatment [P=0.004] and by 58%, i.e. 5.4-fold [2.3–7.6], by F treatment [P=0.001]. Mortality of unfed sandflies at 2m was significantly increased by 29%, i.e. 2.6-fold [1.02–4.7], by DMC treatment [P=0.046].

Finally, we analysed the combined effects of treatment on bloodfeeding and the mortality of bloodfed flies, i.e. how treatment affected the proportion of females that both took a bloodmeal and survived 24hrs. As expected, the DZ collar had no effect at any time point. None of the treatments had a significant effect at 1w [although the effects of DMC and PM were of borderline significance: P= 0.064 and P= 0.053, respectively]. At 1m DMC [P< 0.001], PM [P= 0.003] and F [P< 0.001] all caused a significant reduction; but the effect of DMC was significantly greater than that of PM [P= 0.001]. A significant reduction was again detected at 2m for DMC [P< 0.001], PM [P= 0.008] and F [P= 0.015]; and the effect of DMC was significantly greater than that of both PM [P=0.019] and F [P=0.01].

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Figure 7.1. Comparison of various topical insecticide applications to protect dogs from sand fly bites.

1A. Deltamethrin.
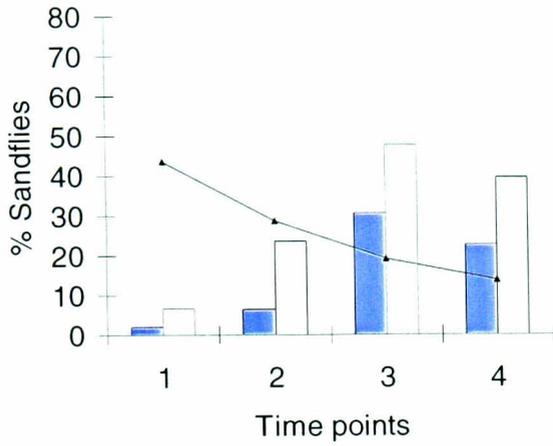
1B. Diazinon.
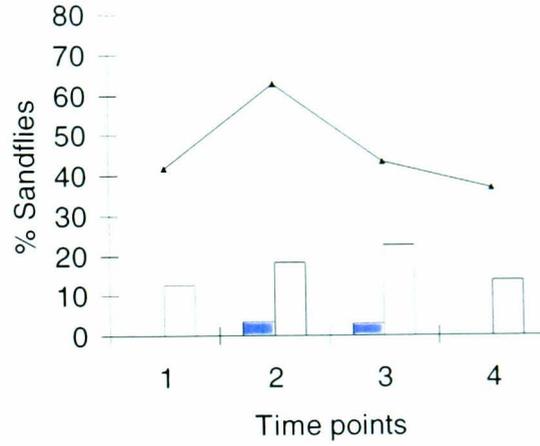
1C. Permethrin.
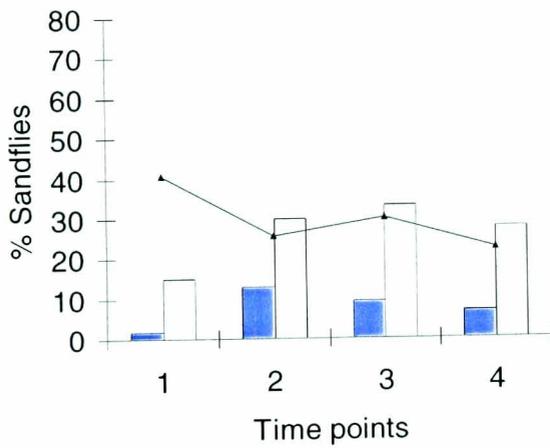
1D. Fenthion.
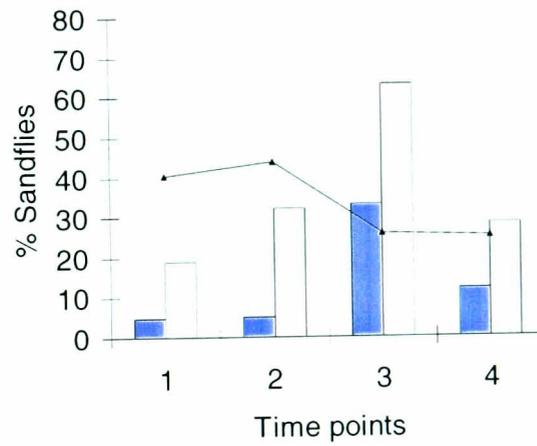
1E. Control.
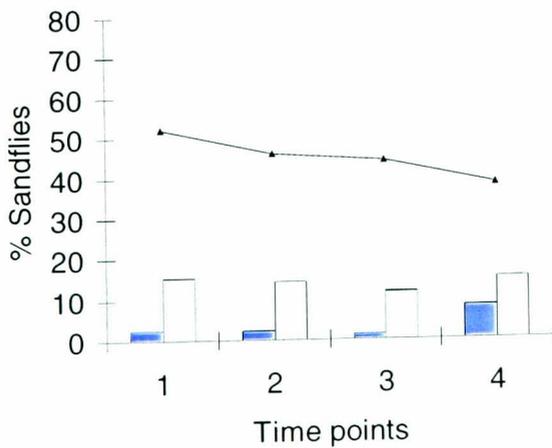
NOTE. Represented are percentage bloodfed (line), dead bloodfed (dark columns) and dead unfed (white columns) sand flies. Time point (TP) 1: 0dpt; TP2: 5-12dpt; TP3: 32-35dpt and TP4: 58-65dpt.

7.4. Discussion.

The effects of topical insecticides on sandfly blood-feeding. The observed reduction in *Lu. intermedia* bloodfeeding and increase in sandfly mortality supports the hypothesis that topical insecticides, including collars, could protect dogs against leishmaniasis. Bloodfeeding rates of sandflies were reduced from 1m post-treatment not only on dogs with deltamethrin collars [DMC], but also on dogs treated with topical lotions of permethrin [PM] and fenthion [F]. No effect was detected for dogs with diazinon collars [DZ]. Although no significant difference between the anti-feeding effects of DMC, PM and F were detected, the reduction due to DMC was greater than that due to either PM or F at both 1m and 2m post-treatment [**Table 7.2.**]. The failure to detect any significant effect on bloodfeeding 1w post treatment is probably due to the time required for the lipophilic insecticides to spread in the dermal secretions over the dog's body (370); and the manufacturers currently recommend that the DMC collar is put on approximately 2 weeks prior to an anticipated sandfly challenge. However, a significant 5-fold increase in mortality rates was detected for bloodfed sandflies on PM-treated dogs after 1w, suggesting that topical application of PM lotion can have a comparatively immediate effect. But this effect is short-lived as no lethal effect on bloodfeds was detected at 1m or 2m post treatment, and no effect on unfeds was detected at any time points. In contrast, topical F application showed greater persistence, significantly increasing mortality rates of both unfeds and bloodfeds at 1m post treatment. The lethal effect of the DMC collar was the most persistent of all the treatments, with significant effects on bloodfeds after 1m and on unfeds at both 1m and 2m post-treatment. The failure to detect a significant impact on bloodfeds after 2 months may be due to the relatively few bloodfeds at that time point [due to the high anti-feeding effect]. The DZ collar failed to cause any impact on mortality rate of either bloodfed or unfeds at any time-point.

Comparisons of the results of our trial with those of similar studies with DMC collars (142, 247, 287, 329) are difficult, due to variation in experimental protocol. For example, we used wild-caught *Lu. intermedia* whereas Killick-

Kendrick *et al.* (287) and Lucientes (329) used 7-15 day-old, colonised *P. perniciosus*, and David *et al.* used 7-15 day-old, colonised *Lu. longipalpis* and *Lu. migonei* (142). This may account for the relatively low lethal effect detected in our trial, as old and colonized flies tend to be more sensitive to low doses of insecticide. Secondly, whereas dogs were exposed to high sandfly densities [mean 155 female flies/dog recovered after 2hrs] in the study by Killick-Kendrick et al. (287), our dogs were exposed to low fly densities [mean 49 female flies/dog recovered after 7hrs]. The longer exposure time in our experiments, which was chosen in order to increase sandfly bloodfeeding rates and contact time with the various treatments, may have contributed to the decreased sandfly recovery rates observed in our study [see below]. Thirdly, in contrast to all previously reported studies, we chose not to sedate the dogs, in order to make the trial conditions as 'natural' as possible. This provides a further reason for the slightly lower recovery rates of sandflies after exposure, as compared to previous studies.

The potential protection against sandfly transmitted diseases afforded by collars to individual dogs depends solely on their anti-feeding effect. Although trials measuring impact on dog infection rates are still required, the entomological results reported here strongly indicate that DMC collars may be recommended to dog owners wishing to protect their dogs from leishmaniasis. This could include dog owners travelling to leishmaniasis-endemic countries, such as Southern Europe, as imported leishmaniasis cases of dogs with a travel history to Mediterranean leishmaniasis-endemic areas are increasingly common (219, 513). With the recent change in the UK quarantine laws, this is likely to be of increasing concern to British dog owners.

The effects of topical insecticides on sandfly mortality. The putative epidemiological impact of DMC collars on leishmaniasis transmission will depend not only on reducing the number of sandflies feeding on dogs, but also on reducing the survival of those flies that do feed so that they are less likely to transmit *Leishmania* when taking a subsequent bloodmeal on a susceptible dog or human. Combining the effects on the bloodfeeding and mortality rates, we calculate that DMC collars reduced the number of

bloodfeds that survive 24hrs by an average of 91% at 1m post-treatment and by 81% at 2m post-treatment. This was greater than the reductions of 61% [ns] and 37% [$P=0.010$] for F treatment, and of 37% [$P=0.001$] and 41% [$P=0.019$] for PM treatment, at 1m and 2m respectively. Thus, there appears to be a clear advantage in terms of effectiveness in using DMC collars versus the two topical lotions. Another advantage of collars is that their presence on dogs, when following-up treated dogs during a control campaign, is proof that the insecticide is applied. Balancing these advantages, pour-on lotions are probably easier to use, and wear and tear is not a concern. Further studies are clearly needed to decide the optimal mechanisms of insecticide application and delivery [e. g. topical lotions, sprays, powders, dips, collars, or ear tags], before widespread implementation can be recommended.

Conclusion. The entomological results reported here are sufficiently encouraging to warrant the undertaking of trials measuring the epidemiological impact of community-wide DMC collar implementation [see **Chapter 8**].

CHAPTER 8:

The use of insecticide-impregnated collars to control canine leishmaniasis: a matched cluster intervention trial in Brazil.

Abstract

We tested the effectiveness of deltamethrin-impregnated dog collars [DMC] to reduce the incidence of canine zoonotic visceral leishmaniasis [ZVL] in a community-based intervention trial in Brazil. After blood was taken from 441 dogs in two different neighbourhoods [D1 and D2], DMC were attached to surveyed dogs in D1. Using PCR and/or ELISA ZVL infection was detected in 18.7% and 12.6% of dogs in D1 and D2, respectively. After five months dogs were surveyed and sampled again in both areas. Although ZVL incidence was 32% lower in collared as compared to uncollared dogs, the difference was not significant. However, DMC did reduce the odds of dogs increasing their anti-Leishmania antibody titre by 50% [95% C.I. 29-87, p=0.01]. The effect of the community-wide application of DMC was compared to the currently practised dog culling control strategy using mathematical modelling; epidemiological implications are discussed.

8.1. Introduction.

Domestic dogs are established reservoir hosts of ZVL caused by *L. infantum* [**Part 2: Introduction**]. Hence, one of the approaches to reduce the incidence of human ZVL is to cull infected dogs. Critics have cast doubt on the impact of such dog culling programmes on human and canine ZVL incidence on both theoretical (176) and practical grounds (85, 416), and because results of controlled intervention trials are equivocal (32, 171). Others remain adamant that culling is effective in reducing canine and human ZVL incidence (415). The costly treatment of ZVL infected dogs is not a feasible control strategy because treated and clinically cured dogs often relapse as they remain parasitologically positive and, hence, are infectious to the sandfly vector (452). Whilst waiting for an effective canine (221) or human (249) vaccine, alternative control strategies are paramount.

As seen in **Chapter 7**, several experimental trials have demonstrated that topical insecticides, and deltamethrin-impregnated dog collars [DMC] in particular, can protect dogs from >85% of sandfly bites for periods of up to 6 months. The objective of this study was to test the effectiveness of DMC to control canine ZVL in a matched cluster intervention trial and to highlight possible constraints associated with a community-wide implementation of a dog collar ZVL control strategy.

8.2. Materials & Methods.

Study site. The intervention trial was carried out in Capitão Eneas [16°30'S, 44°00'W], a *L. infantum*-endemic area in Minas Gerais State, Brazil [see **Chapter 6**]. Capitão Eneas is a small town of 9800 inabitants, located 72km from Montes Claros in an arid plain. Main economic activities are commerce and a local computer chip plant. Most people own chickens, pigs, and dogs. Houses tend to be surrounded by abundant vegetation [either garden or crops] [**Map 2, Plate 5**]. Transmission of ZVL by the vector *Lu. longipalpis*

occurs throughout the year; there is no documented *L. braziliensis* or *T. cruzi* transmission in the area.

Dog survey and diagnoses. In September 1999, a cohort of 441 dogs was surveyed clinically, parasitologically and immunologically in two different neighbourhoods [D1 and D2, respectively] of Capitão Eneas [**Box 2**]; D1 and D2 were 500m apart and selected at random. After blood samples [2-10ml] had been taken from all dogs, 40mg/g DMC [Scalibor, Intervet International, The Netherlands] were attached to all dogs above three months of age in D1 [**Plate 5D**], whereas dogs remained uncollared in D2. All dog owners were informed about the objective of the study and possible side-effects due to collar use; participation was voluntary. After five months, i.e. February 2000, dogs in both areas were re-surveyed and a second blood sample was taken in order to estimate effectiveness of DMC in reducing canine ZVL incidence. Collar loss and side effects were also recorded. All blood samples were processed as described **Chapter 6**. Briefly, for parasitological diagnosis, samples were screened by a PCR-hybridisation protocol using *L. donovani* complex-specific AJS31 [5'-GGGGTTGGTGTAAAATAGGGCC-3'] and DBY [5'-CCAGTTTCCCGCCCCGGAG-3'] primers and a [γ^{32}P]-ATP-labelled B4RsaB [5'-GACCTGAAACCCTGGGTCCTGGGCGT-3'] probe. Samples were screened by ELISA for immunological diagnosis, with log-phased *L. donovani* promastigotes [MHOM/ET/67/L82] as antigen [at a concentration of 10^5 promastigotes/microtiter plate well] and peroxidase-conjugated, affinity-purified rabbit anti-dog IgG as antibody [at a concentration of 1/1500]. As shown in **Chapter 6**, using these protocols PCR and ELISA had both 100% specificity and 53-64% and 74-88% sensitivity, respectively.

Statistical analysis. To measure the epidemiological impact of DMC, chi-squared tests were used to compare the odds of dogs from D1 and D2 getting infected. In a second analysis, logistic regression was used to compare these odds after controlling for pre-intervention prevalence in each locality [i.e. the proportion of dogs that are positive by either ELISA or PCR, or both] and dog gender. Neither dog age nor loss of collar during the trial were shown to significantly affect the odds of infection in dogs after analyses and so these factors were excluded from the final regression model. The

outcome variables in all analyses were [i] positive by ELISA or PCR, or both, and [ii] increase in anti-*Leishmania* log antibody units [LAU]/ml [as a binomial variable]. All analyses were done in STATA 7.

Mathematical modelling. The potential effectiveness of DMC as compared to current dog culling programmes was simulated by using the vector-borne disease model of Ross-Macdonald (24). Total dogs and sandflies are divided into proportions that are uninfected and susceptible [D_S and F_S], latent [i.e. infected but not infectious] [D_L and F_L] and infectious [D_I and F_I]. Changes in the proportions of dogs and flies in each group through time are given by the following equations:

[E1] $\dfrac{dD_L}{dt} = D_S abmF_I - D_L\sigma - D_L\delta$

[E2] $\dfrac{dD_I}{dt} = D_L\sigma - D_I\,\rho - D_I\,\delta$

[E3] $D_S = 1 - D_L - D_I$

[E4] $\dfrac{dF_L}{dt} = acF_S D_I - F_L\dfrac{1}{\tau} - F_I\,\mu$

[E5] $\dfrac{dF_I}{dt} = F_L\dfrac{1}{\tau} - F_I\,\mu$

[E6] $F_S = 1 - F_L - F_I$

Box 2. Flow diagram of model parameters
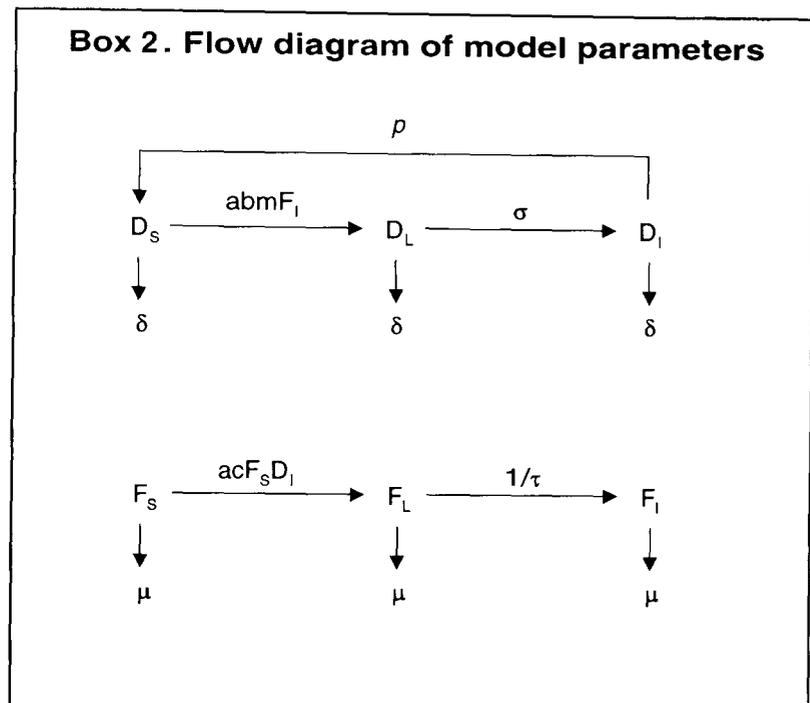
where δ and μ are the dog and sandfly mortality rates, respectively [note that the model assumes that both populations are constant, with instantaneous replacement of dead dogs and sandflies, and that mortality rates are independent of age and infection status], σ the rate at which latent dogs become infectious, ρ the rate at which infectious dogs recover from infection and, hence, infectiousness, a the sandfly biting rate on dogs, b the probability that an infectious sandfly transmits Leishmania when feeding on a susceptible host, c the probability that a sandfly becomes infected when feeding on a infectious dog, m the ratio of vectors to dogs, and τ the extrinsic incubation period [i.e. time required for the development of infective metacyclics in infected sandflies]. Using a series of difference equations

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derived from E1-6, simulations were run at quarter day intervals until an equilibrium prevalence of infectious dogs was obtained. The effect of collaring or culling dogs at 180-day pulses was then simulated. In additional simulations, the rates of collar coverage and collar loss were varied in order to test their effect on prevalence of infectious dogs. All simulations were carried out in Microsoft Excel.

8.3. Results.

Pre-intervention data. Of the 441 surveyed dogs, 267 were male [mean age ± standard error: 30.6 ± 1.7 months, range: 3-180 months] and 174 female [28.2 ± 2.1 months, range: 2-168 months]. Results for both pre –and post-intervention surveys are represented in **Table 8.1.** Although the local Ministry of Health [MOH] had apparently killed all IFAT-seropositive dogs one month prior to our survey as part of their leishmaniasis control programme, baseline prevalences in D1 and D2 were very high, 18.7% [47/251] and 12.6% [24/190], respectively [Yates-corrected, χ^2=2.54, p=0.11].

Post-intervention data. After five months, 136/251 [54%] and 97/190 [51%] dogs were re-surveyed in D1 and D2, respectively; 44/251 [18%] and 40/190 [21%] dogs had died and 40/251 [16%] and 21/190 [11%] dogs had emigrated from D1 and D2, respectively. 56/136 [41%] dogs in D1 lost their collar during the study, of which 10/136 [7%] had reportedly experienced side-effects [skin irritation [n=5], loss of appetite and epitaxis [n=4] and trembling [n=1]], so that collars had to be removed.

Using the diagnostic criteria set out in **Chapter 6,** an incident ZVL infection was defined as a dog that either converted by PCR or ELISA, or by both PCR and ELISA. The ZVL incidence in D1 was 13.5% [13/96] [95% C.I. 6.7; 20.4] and 21.4% [15/70] [95% C.I. 11.8; 31.0] in D2 [**Table 8.1.**], i.e. the odds ratio [OR] for a ZVL ELISA/PCR conversion in D1 as compared to D2 was 0.63 [95% C.I. 0.26; 1.51, Yates-corrected, χ^2=0.84, p=0.36]. A logistic regression analysis incorporating dog sex and pre-intervention ZVL

prevalence confirmed the trend [OR 0.62, 95% C.I. 0.28; 1.39, p=0.24]. The mean change in LAU/ml in D1 dogs was −0.04 LAU/ml [95% C.I. −0.16 to 0.08] compared to +0.10 LAU/ml [95% C.I. −0.04 to +0.24] in D2 dogs, the frequency distribution of which is represented in **Figure 8.1**. The proportion of dogs whose titre increased in D1 [55/136] was significantly less than in D2 [55/97] [OR 0.52, 95% C.I. 0.30; 0.91, Yates-corrected, χ^2=5.37, p=0.02]. Using multiple regression analysis adjusting for dog sex and pre-intervention ZVL prevalence it was estimated that collars reduced the odds of dogs increasing their antibody titre by 50% [95% C.I. 0.29-0.87, p=0.01].

## Box 3. Study design of the intervention trial.

August 1999 All infected dogs were culled as part of the Brazilian MOH leishmaniasis control campaign, and control and intervention areas for current trial were defined.

September 1999 441 dogs included in the trial

251 collared in D1

190 uncollared in D2

44 died
40 emigrated
31 lost to follow-up

40 died
21 emigrated
32 lost to follow-up

March 2000
136 dogs at the end of the trial

March 2000
97 dogs at the end of the trial

To measure the epidemiological impact of collars on canine leishmaniasis incidence, ELISA and PCR carried out on blood samples taken from D1 and D2 dogs in September 1999 and in March 2000.

Table 8.1. Pre-intervention and post-intervention infection prevalence.

Treatment area [D1].

1999	2000					
	ELISA+/PCR+	ELISA+/PCR-	ELISA-/PCR+	ELISA-/PCR-	-1	Total
ELISA+/PCR+	10	-	2	4	12	28
ELISA+/PCR-	5	1	-	2	7	15
ELISA-/PCR+	-	-	1	2	1	4
ELISA-/PCR-	5	3	5	96	95	204
-1	-	1	-	2	2	5
Total	20	5	8	106	117	256

Control area [D2].

1999	2000					
	ELISA+/PCR+	ELISA+/PCR-	ELISA-/PCR+	ELISA-/PCR-	-1	Total
ELISA+/PCR+	2	-	-	3	9	14
ELISA+/PCR-	5	-	-	1	3	9
ELISA-/PCR+	-	-	-	1	-	1
ELISA-/PCR-	6	4	5	70	81	166
-1	-	-	1	14	3	18
Total	13	4	6	89	96	208

Figure 8.1. Frequency distribution of changes in anti-*L. infantum* log antibody units/ml.
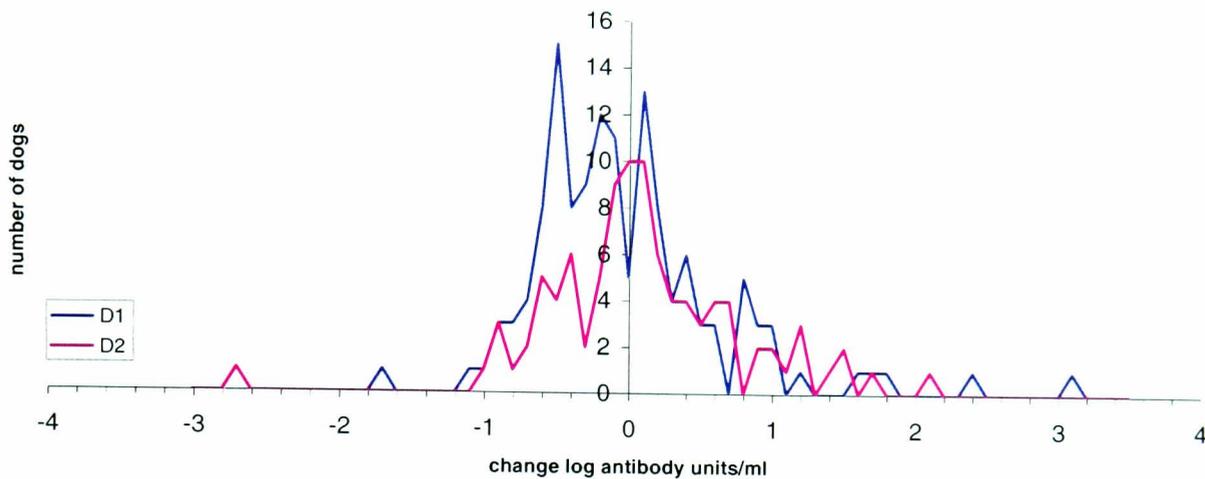
NOTE. D1, intervention area with collared dogs; D2, control area with uncollared dogs.

Mathematical modelling. The total dog population was kept constant, i.e. $\delta = \beta = 0.0025/day$ [data from this study], and the other parameters used were $\sigma = 0.0048/day$ (125) and $\rho = 0.0036/day$ [data from this study]. Parasites take approximately seven days to mature in the sandfly gut [i.e. $\tau = 7$] (176), flies have a life expectancy of 8 days [i.e. $\mu = 0.125$] (442) and bite dogs every four days (176). The other parameters describing sandfly population dynamics are unknown [i.e. b and m] and were chosen so that equilibrium prevalence of infectious dogs was 31%, which corresponds to a basic reproduction number $R_0 \approx 10$, similar to the R_0 observed for canine ZVL in the field (177, 440). The effect of culling dogs on the prevalence of infectious dogs was simulated by assuming that at 180-day pulses, 64% of infected and infectious dogs are culled with a 60-day delay between sampling, diagnosis and culling (85). Similarly, the effect of collaring dogs was simulated assuming that collars are applied to 80% of all dogs at each 180-day pulse, that collars protect dogs from 80% of the sandfly bites, and that they remain on dogs for a mean 167 days [i.e. the collar loss rate is 0.006/day, data from this study]. The comparison of collars compared to culling is represented in **Figure 8.2.A.** using parameters as outlined above. The impact of coverage rates on prevalence of infectious dogs is represented in **Figure 8.2.B.**,

whereas the impact of collar loss rates [collar longevity tested: 100, 200 and 300 days] on prevalence of infectious dogs is represented in **Figure 8.2.C.**

Using given parameters in our model simulations, the equilibrium prevalence of infectious dogs was 31% [**Figure 8.2.A.**]. When dogs are collared at each 180-day pulse, the prevalence of infectious dogs should drop by 64% because 80% of the dogs are protected from 80% of the sandfly bites due to the collars. Although the proportion of infective flies initially drops in the simulations due to the action of collars, prevalence of infectious dogs then increases between pulse intervals as the number of unprotected dogs increases due both to new-born dogs, and to latent and infectious dogs losing their collars [**Figure 8.2.A.**]. In contrast, assuming that 64% of all latent and infectious dogs are culled, prevalence of infectious dogs drops to 12% upon the first culling pulse. The prevalence then rapidly increases until the next culling pulse due to susceptible dogs becoming latent and infectious. **Figure 8.2.A.** demonstrates that collars have a bigger impact on the prevalence of infectious dogs than culling. Sustained implementation of each method during 6.5 years should lower the prevalence of infectious dogs to 4.6-12.9% [prevalence after and before last pulse] for collaring and to 8.4-17.4% [prevalence after and before last pulse] for culling. However, our simulations also show that the effectiveness of a community-wide application of collars would be significantly affected by collar coverage and loss rates [**Figure 8.2.B.** and **8.2.C.**]. A 25% lower coverage rate at each pulse reduces by about half the epidemiological impact on the prevalence of infectious dogs after 6.5 years of collaring. Increasing collar longevity by 100 days reduces by approximately 40% the prevalence of infectious dogs prior the last collaring pulse after 6.5 years of collaring.

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Figure 8.2. Epidemiological impact of insecticide-impregnated dog collars on zoonotic visceral leishmaniasis.
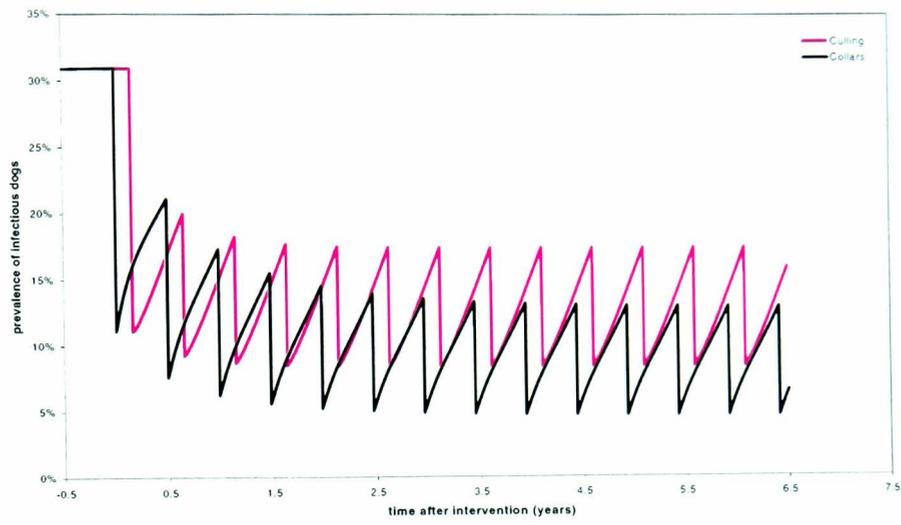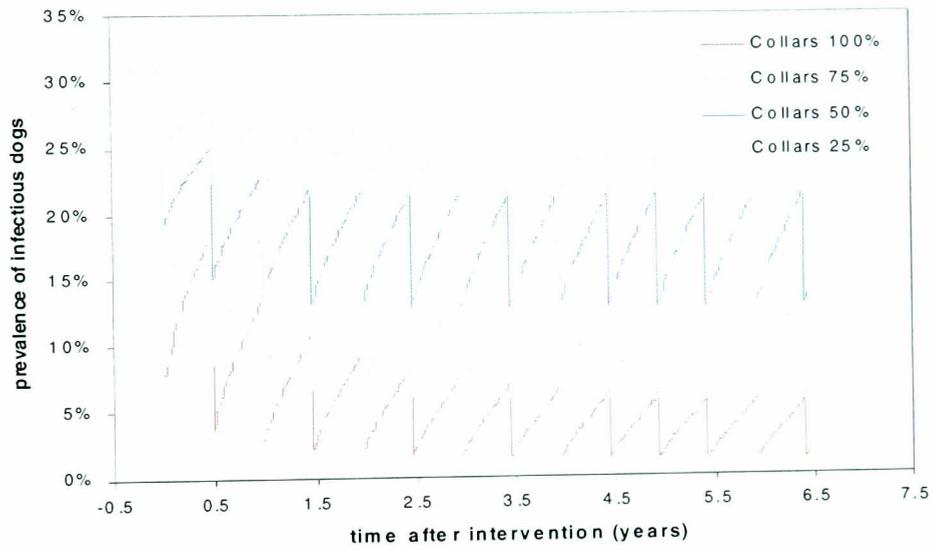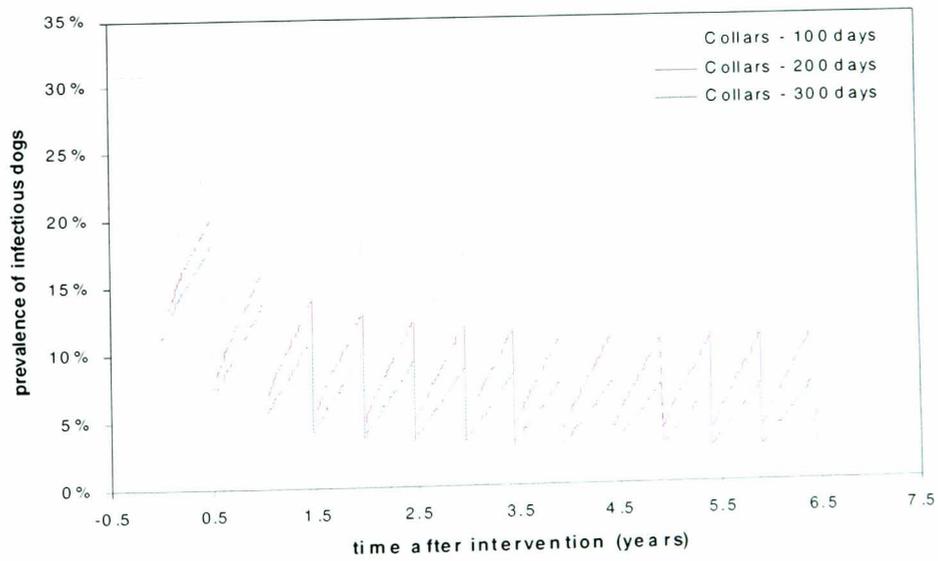
8.4. Discussion.

Epidemiological impact of DMC on canine ZVL in Capitão Eneas.
Previous experimental trials have shown that DMC reduce the biting rate of *Lu. longipalpis* [the ZVL vector in Capitão Eneas and most of Latin America] from 81% to 100% for up to 35 weeks [see **Chapter 7**]. Based on these and similar observations by others, an intervention trial was carried out to measure the epidemiological impact of DMC on canine ZVL incidence.

Although we observed a lower ZVL incidence in collared dogs than in uncollared dogs, the effect was not significant; possibly due to the low number of tested animals, the high rate of collar loss as compared to other studies [8% in Italy (342) and 10% in Iran (210)], the high number of animals with pre-patent infections during the baseline survey [see below], and the possibility that a number of dogs infected during the trial had yet to seroconvert by the end of the trial. However, we show that five months after collar application, the likelihood of dogs having higher LAU/ml was significantly decreased. The change in antibody titre can be seen as an approximation for infection, as a positive change in LAU/ml indicates that the dog has been exposed to *Leishmania* parasites. The discrepancy between the analyses of the two outcome measures may be because by taking into account all positive changes in antibody titre we are able to include new cases in the process of seroconverting [i.e. samples which otherwise are excluded from the relatively arbitrary ELISA+ category in **Table 8.1.**] [see also **Chapter 2**]. This approach probably represents a more accurate estimate of the ZVL transmission rate, because the duration of the trial was short [five months] and the number of incident infections may have been significantly underestimated due to the long [ca. 2 months] ZVL pre-patent period (177, 178, 439, 440).

Mathematical modelling and implications for dog control. We compared the epidemiological impact of collars and culling using a simple vector-borne disease model. Although we used conservative estimates for the effect of collars [i.e. 80% coverage rate and 80% protective effect] and optimistic

estimates for the effect of culling [i.e. 64% of infected and infectious dogs are culled], the model exposes –despite recent contrary claims (415)- the problems associated with the culling strategy. In endemic transmission areas, the remaining non-culled, infected and infectious dogs are sufficient to drive transmission over time, with newly recruited susceptible dogs rapidly acquiring ZVL infection (32, 85, 171, 176, 416, 440). The effect of culling programmes will additionally be undermined by the immigration of infected dogs into culling areas (85, 416). It is probable that far more infected and infectious dogs than the 36% in our model are not culled because they remain undetected [in part due to the significant ZVL pre-patent period] or because owners refuse to have their dogs culled. Also, the diagnostic test used [IFAT on filterpaper eluate] to mass-screen dogs is not sensitive enough to detect all ZVL infected animals: one study suggests that up to 65% of ZVL dogs are not detected by IFAT-based ZVL diagnosis (85). Moreover, culling strategies are hampered by the long delay between dog sampling, ZVL diagnosis and culling. This delay has been shown to be as high as 90-days [we used 60 days in our simulations] (85), with infected dogs remaining infectious to sandfly vectors and driving ZVL transmission. These observations appear to be confirmed in our study site, as despite the culling of infected dogs by the local MOH one month prior to the intervention trial, pre-intervention prevalence in D1 and D2 was surprisingly high [mean prevalence: 16%].

Our results can be compared with results from another commmunity-based intervention trial in Italy (342) where the epidemiological impact of DMC on canine ZVL was tested during two consecutive transmission seasons. Although seroconversion rates in intervention and control villages were not different [2.7% and 5.4%, respectively] after the first transmission season, they were shown to be significantly different after the second transmission season [3.5% to 25.8%, respectively], with collars providing an estimated 86% [95% C.I. 55; 96] protection to dogs.

Whether topical insecticides such as DMC will be effective as leishmaniasis control tools will depend on several factors. Firstly, the strategy will be most effective in those endemic areas where domestic dogs are the main ZVL

reservoir and the epidemiological significance of wild reservoirs or stray dogs contributing to ZVL transmission is negligible. Secondly, our simulations show that in order to achieve a significant epidemiological impact on ZVL transmission, high dog collar coverage rates are essential [**Figure 8.2.C.**]. This will not only require the rapid replacement of lost collars [**Figure 8.2.B.**], but also the collaring of new dogs recruited into the population; where population turnover rates are high, maintaining high coverage rates will be a greater logistic challenge, which invariably applies to dog populations in tropical, ZVL-endemic countries.

Conclusion. There is now increasing evidence that DMC not only protect dogs from sandfly bites, but also from ZVL. These findings are not only of interest to dog owners travelling with their dogs to ZVL-endemic countries, but are of potential public health importance: because dogs are ZVL reservoirs, the findings imply that DMC could be effective in controlling human disease as well. Indeed, preliminary results from a trial in Iran indicate that collars significantly reduce the ZVL incidence in both dogs and children after one year of collar application (210). Ultimately, the decision to replace the dog culling strategy with community-wide application of DMC will depend on [i] the relative cost of the interventions (8); and [ii] the practical applicability of DMC in the field [e.g. the willingness of the community to apply DMC and the efficiency with which they replace collars which have detached]. Clearly, the implementation of DMC on dogs is more likely to have the consent of the population at risk than the highly unpopular dog culling policy that continues to be practised in some ZVL-endemic countries.

PART 3

CHAPTER 9

DISCUSSION & CONCLUSION

Dogs and their role as zoonotic reservoirs of human disease. It is now known that dogs have been part of human activities and households ever since the emergence of human settlements 12 000 to 15 000 years ago (333). Today, dogs continue to occupy a major role in human societies. While dogs can significantly contribute to the well-being of people [e.g. either as a pet, play companion, guard or hunting dog], people can also suffer from detrimental effects due to the contact with dogs, because of injury from dog bites and because dogs are reservoir host for a range of zoonosis. Over 50 zoonotic diseases are associated with domestic dogs [see for review Refs. (117, 333)], including infections caused by arthropods [e.g. skin ulcerations caused by the sand flea *Tunga penetrans* and the cat flea *Ctenocephalides felis felis*], cestodes [e.g. cystic echinococcosis caused by *Echinococcus granulosus* and alveolar echinococcosis caused by *E. multilocularis*, or diphyllobothriasis caused by the tapeworm *Diphyllobothrium latum*], nematodes [e.g. visceral larva migrans due to *Toxocara canis*, hookworm infections due to *Ancylostoma* spp. and *Ucinaria* spp.], trematodes [e.g. paragonomiasis caused by *Paragonimus* spp. Flukes], bacteria [e.g. pasteurellosis caused by *Pasteurella canis* and salmonellosis caused by *Salmonella* spp.], and viruses [e.g. rabies]. Finally, dogs are also reservoir hosts of a range of intestinal [e.g. *Entamoeba histolytica* and *Giardia* spp.] and blood-borne protozoan parasites [e.g. *Trypanosoma* spp.], including *Leishmania infantum*, the aetiological agent of ZVL.

Are domestic dogs reservoirs of human American cutaneous leishmaniasis? As discussed in **Part 1: Introduction** there is a growing belief that domestic dogs dogs may also act as reservoir hosts of human ACL, because [i] transmission of ACL has become increasingly evident in the [peri-]domestic environment; [ii] several studies have reported high ACL infection in dogs throughout the Latin American continent; [iii] parasites isolated sympatrically from ACL patients and dogs are indistinguishable; and [iv] some studies have shown an association between ACL infection in dogs and risk of human ACL. However, this evidence to incriminate dogs as ACL reservoirs is largely circumstantial, because the isolation and identification of parasites from both patients and dogs as well as the observed high ACL prevalence in dogs only suggests that humans and dogs are exposed to the

sandfly vector in the same way. Also, natural ACL infections have been detected in a range of sylvatic animals frequenting the domestic environment [e.g. opposums and rodents], and their potential role in the transmission cycle of ACL has rarely been addressed. In **Part 1** of this thesis data are presented on a three-year, prospective study of ACL in dogs which was carried out to establish whether dogs could act as *L. [Viannia]* reservoir hosts in an area where domestic transmission of ACL to humans is apparently widesdpread. Though planned xenodiagnosis experiments could not be carried out due to shortage of sandflies, several findings presented in **Chapters 2-5** are strong enough to incriminate dogs as reservoir hosts of [peri-]domestic *L. [Viannia]* transmission in Huánuco, Peru. First, whereas the prevalence and incidence of clinical disease is low, the prevalence and incidence of *L. [Viannia]* infection in dogs is comparatively high. Second, the average duration of infection can be as long as the mean life expectancy of a dog. Third, *L. [Viannia]* parasites in dogs do not remain localised at the site of inoculation but are able to disseminate to both viscera and mucosa. Fourth, the detection of *L. [Viannia]* parasites by PCR in the blood of a high proportion of both symptomatic and asymptomatic dogs suggests that infected dogs are potentially infectious to sandfly vectors. Fifth, after controlling for inter-village differences in transmission rates, household dog ownership was shown to be a significant risk factor for human ACL. This incriminatory evidence is further strengthened because of the apparent absence or low abundance [e.g. opposums] of alternative [sylvatic] reservoir hosts that would be able to maintain domestic *L. [Viannia]* transmission in Huánuco. Rodents are at a density that would be able to maintain domestic *L. [Viannia]* transmission over such a wide area and their role as ACL reservoir host should be investigated in future epidemiological studies. The results presented here show that were dogs the main ACL reservoir host, a dog control strategy in the study villages in Huánuco would be very feasible and effective, because the control effort to reduce $R_0 < 1$ would be comparatively small. Such an intervention should also provide the ultimate proof in the incrimination of dogs as ACL reservoir hosts, as −if such an intervention strategy were successful- it should have a significant epidemiologial impact on human ACL transmission.

Are PCR-based diagnostic methods or immunochromatographic dipstick test useful for mass-screening of dogs in epidemiological studies or control programmes? Several studies have suggested that PCR-based methods for *Leishmania* diagnosis may provide the best gold standard for determining the presence and identity of leishmanial infections not only when diagnosing active cases, but also for monitoring parasitological cure of patients after chemotherapy and as a mass-screening tool to detect *Leishmania* infections in vertebrate hosts or sandfly vectors. Various PCR protocols have been reported for the detection of ACL-causing *Leishmania* in humans using either purified DNA [from cultured parasites or clinical specimens, including lesion and scar biopsies or blood] and have consistently proven to be more sensitive than other diagnostic methods, including *in vitro* culture of biopsies, biopsy smears and hamster inoculation. In the work presented in **Chapter 2** the utility of a PCR as a diagnostic mass-screening tool in epidemiological studies was evaluated. Specifically, the sensitivity and specificity of established PCR protocols on canine blood and bone marrow were compared to an ELISA for detecting *L. [Viannia]* spp. infection in dogs. A PCR-based assay on blood is advantageous as samples can be obtained less invasively from the patient [human or dog] and are easy to process. Although a positive PCR-hybridisation was associated with a higher anti-*Leishmania* antibody titre, the ELISA test was much more sensitive than PCR in detecting *Leishmania* infection in symptomatic and asymptomatic field dogs. We also show that the choice of biopsy material does not appear to be as critical as for studies using PCR to diagnose human ACL patients [skin snips, lesion aspirates], as the sensitivity of the PCR on GBL, BCS or BMS was not significantly different. Thus, PCR on blood alone does not appear to be the elusive 'gold standard' for diagnosing ACL infections in dogs [or humans] and to be used as a mass-screening tool in epidemiological and intervention studies. Unless a more sensitive PCR protocol [e.g. PCR-ELISA] is developed to detect asymptomatic ACL infections, the use of PCR will be restricted to the diagnosis of active cases. The use of PCR on blood could, however, have an important epidemiological application in studies to monitor the clinical and chemotherapeutic follow-up of patients with ACL. Detection of disseminating *Leishmania* parasites in patient blood would indicate that they are at risk of developing mucocutaneous lesions, the treatment of which is more

complicated than the treatment of the single lesions characteristic of ACL. Also, PCR combined with specific DNA probing and sequencing should help to identify and characterise those strains that are drug resistant and that cause the different clinical pathologies associated with ACL.

As outlined in **Part 2: Introduction**, one of the reasons why dog culling programmes have failed is because of the long delay between sample collection, sample analysis and control implementation [i.e. culling of infected dogs]. This delay typically is 30 days long, but can be as long as 120 days, with infected dogs remaining infectious to sandfly vectors during this period, thereby transmitting ZVL to susceptible dogs and humans. Current diagnostic methods used for *Leishmania* mass-screening surveys [mainly ELISA, IFAT or DAT] lack sensitivity or specificity, require technological expertise and specialised laboratory equipment, and can be labour-intensive and time-consuming. Hence, a rapid, sensitive and specific diagnostic test would be extremely valuable in mass-screening surveys and intervention campaigns, as results could be read immediately and control measures could be implemented *in situ*. Implementation coverage rates would be improved [e.g. dog owners would have less opportunity to hide their dogs from culling personnel] and the control intervention would be more effective. In **Chapter 6** the sensitivity and specificity of a commercially available immunochromatographic rK39 dipstick test were compared to the sensitivity and specificity of standard serological and molecular diagnostic tests [ELISA and PCR] used in canine ZVL diagnosis. Although the dipstick test was comparable to ELISA and PCR in terms of sensitivity, specificity was surprisingly low. This finding contradicts previous studies reporting high sensitivities and specificities of the rk39 dipstick when tested to detect ZVL infections in humans. The research into developing a more specific *Leishmania* dipstick test should be pursued, as the advantage of such a tool with respect to other diagnostic methods such as microscopy, ELISA or PCR are many-fold. Using dipsticks, a vast number of samples can be processed quickly and with minimum effort. Compared to microscopy, ELISA or PCR, minimal technological expertise and specialised laboratory equipment are required. Another advantage of dipstick tests is that patients [in this case, dog owners] can see the results for themselves, which should contribute to a

better working relationship between local communities and people carrying out the surveys, and increase compliance rates. Epidemiologically, the most important characteristic of a dipstick test is that it allows interventions to be implemented *in situ*. The outcome should be to significantly reduce the mean duration of infectiousness of dogs that have become infected, thereby significantly enhancing the impact of the intervention on the basic reproductive number, R_0. Immunochromatographic dipstick tests are comparatively expensive, but considering the above, a sensitive and specific dipstick test could prove a very cost-effective alternative to currently available diagnostic tests, especially when used in mass-screening surveys.

Can topical insecticides be used to control canine and human leishmaniasis? Domestic dogs are established reservoir hosts of ZVL caused by *L. infantum* [**Part 2: Introduction**]. Hence, one of the approaches to reduce the incidence of human ZVL is to cull infected dogs. Critics have cast doubt on the impact of such dog culling programmes on human and canine ZVL incidence on both theoretical and practical grounds, and because results of controlled intervention trials are equivocal. Others remain adamant that culling is effective in reducing canine and human ZVL incidence. The costly treatment of ZVL infected dogs is not a feasible control strategy because treated and clinically cured dogs often relapse as they remain parasitologically positive and, hence, are infectious to the sandfly vector. Whilst waiting for an effective canine or human vaccine, alternative control strategies are paramount. The findings of a comparative study testing topical insecticides and applications to protect dogs from sandfly bites were presented in **Chapter 7**. It was shown that whilst permethrin and fenthion pour-on lotions had a more immediate effect on sandfly biting rates and mortality, deltamehtrin-impregnated collars [DMC] had a more prolonged protective effect, with the survival rate of bloodfed sandflies reduced by up to 86% after two months of deltamethrin collar application.

In the work described in **Chapter 8** the effectiveness of DMC to control canine ZVL was tested in a matched cluster intervention trial. Possible constraints associated with a community-wide implementation of a dog collar ZVL control strategy were also investigated using mathematical modelling.

Although ZVL incidence was 32% lower in collared as compared to uncollared dogs after five months collar application, the difference was not significant. However, DMC did significantly reduce the odds of dogs increasing their anti-*Leishmania* antibody titre. Whether topical insecticides such as DMC will be effective as leishmaniasis control tools will depend on several factors. Firstly, the strategy will be most effective in those endemic areas where domestic dogs are the main ZVL reservoir and the epidemiological significance of wild reservoirs or stray dogs contributing to ZVL transmission is negligible. Secondly, in order to achieve a significant epidemiological impact on ZVL transmission, high dog collar coverage rates are essential. This will not only require the rapid replacement of lost collars, but also the collaring of new dogs recruited into the population; where population turnover rates are high, maintaining high coverage rates will be a greater logistic challenge, which invariably applies to dog populations in tropical, ZVL-endemic countries. Ultimately, the decision to replace the dog culling strategy with community-wide application of DMC will depend on [i] the relative cost of the interventions; and [ii] the practical applicability of DMC in the field [e.g. the willingness of the community to apply DMC and the efficiency with which they replace collars which have detached]. Clearly, the implementation of DMC on dogs is more likely to have the consent of the population at risk than the highly unpopular dog culling policy that continues to be practised in some ZVL-endemic countries.

Future recommendations. The objectives of this thesis were to [i] investigate whether domestic dogs are important reservoir hosts of ACL; and [ii] to identify the shortcomings of currently practiced dog control programmes and to evaluate whether topical insecticides could be used to control canine leishmaniasis. Based on the findings presented in this thesis, the following recommendation can be made:

[1] Research into the development of a sensitive and specific, rapid diagnostic test for leishmaniasis [e.g. dipstick test, or field-based PCR-ELISA] should be pursued, as not only would this facilitate the implementation of epidemiological studies, but also considerably improve the effectiveness of implemented control strategies.

[2] Further epidemiological studies should be carried out in Huánuco in order to exclude that [peri-]domestic animals [e.g. rodents] other than dogs can maintain the domestic transmission of *L. [Viannia]* to humans.

[3] A dog control trial [e.g. using deltamethrin-impregnated dog collars] should be carried out in Huánuco, Peru, as data presented in this thesis suggests that such an intervention strategy could have a significant epidemiological impact on ACL transmission to humans. This also would provide the irrefutable proof to incriminate dogs as ACL reservoir hosts.

[4] The dog culling programmes [such as the one carried out in Brazil] should be stopped as they have only a limited epidemiological impact on the ZVL transmission to humans due to several logistical and methodological problems associated with its implementation.

[5] A dog control programme using deltamethrin-impregnated dog collars should be implemented in [highly] endemic ZVL areas in order to reduce the incidence of human disease, because data presented here showed that collars can protect dogs from both sandfly bites and exposure to *L. infantum*. Data from other collar intervention trials currently being carried out in Brazil and Iran should also show the effect of collar loss, collar coverage and collar field efficacy on the effectiveness of the collar intervention strategy.

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Plate 1. American cutaneous leishmanasis in Huánuco, Peru: clinical characteristics of *L. braziliensis* and *L. peruviana* infection.
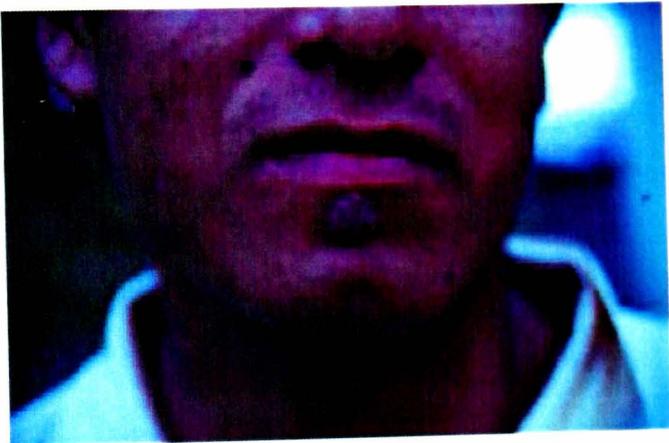
Plate 1 Legend:

A	B
C	D
E	F
G	H

Plate 1A. Localised cutaneous leishmaniasis: facial ulcer in a child, a common disease manifestation in Huánuco. It highlights the impact of ACL on the local community: children often know about ACL, why and how it is transmitted because most of them have suffered from the disease and consequent course of treatment during their childhood.

Plate 1B. Localised cutaneous leishmaniasis: involvement of extremities. Lesions tend to appear on body sites which are exposed to the bites of sandfly vectors, i.e. the face, arms and lower legs. Of note is the typical appearance of the lesion, a large ulcer with raised borders, characteristics that are typical of *L. braziliensis* lesions.

Plate 1C. Localised cutaneous leishmaniasis: reactivation. Of note is the depigmented area around the active lesion, characteristic of *L. braziliensis* scars.

Plate 1D. Localised cutaneous leishmaniasis: verrucous lesion. Verrucous lesions are extremely rare and can often be mistaken to be of other aetiology than *Leishmania*.

Plate 1E. Localised cutaneous leishmaniasis: scar. As in **Plate 1C**, this lesion is characteristic of *L. braziliensis* infection, because of the depigmented area in the centre of the scar.

Plate 1F. Mucocutaneous leishmaniasis: palate and nose involvement. This is a non-ulcerative mucosal lesion of the hard and soft palate. This particular patient was aphonic, as the infection had severely compromised vocal cords.

Plate 1G. Mucocutaneous leishmaniasis: palate involvement. This is a non-ulcerative mucosal lesion of the hard and soft palate. It is an exophytic plaque rich in granulation tissue; eventually erosion of the palate may result in bleeding.

Plate 1H. Differential diagnosis: maleolar ulcers. The crusted ulcers of venous aetiology on or around the ankles are commonly mistaken for LCL.

Plate 2. American cutaneous leishmanasis in Huánuco, Peru: clinical characteristics of *L. braziliensis* and *L. peruviana* infection.

Plate 2 Legend:

A	B	C
D	E	F
G	H	I

Plate 2A. Localised cutaneous leishmaniasis. This child has LCL due *L. peruviana*. Though there are no published reports of MCL caused by *L. peruviana*, it may cause MCL by continuity when lesions are close to the mucosae.

Plate 2B. Localised cutaneous leishmaniasis. Localised cutaneous leishmaniasis: facial ulcer in a child, a common disease manifestation in Huánuco. It highlights the impact of ACL on the local community: children often know about ACL, why and how it is transmitted because most of them have suffered from the disease and consequent course of treatment during their childhood.

Plate 2C. Mucocutaneous leishmaniasis: facial destruction. This adult has active MCL with destructive polypoid lesions on the left ala nasae, scarring of the nose and upper lip infiltration.

Plate 2D. Mucocutaneous leishmaniasis: palate involvement. This is a non-ulcerative mucosal lesion of the hard and soft palate. It is an exophytic plaque rich in granulation tissue; eventually erosion of the palate may result in bleeding.

Plate 2E. Localised cutaneous leishmaniasis: cutaneous lesion due to *Leishmania (Viannia)* spp.

Plate 2F. Cutaneous lesion due to *Leishmania (Viannia)* spp. Note, that in this case the patient used battery acid to cure the lesion, which makes differential diagnosis of ACL difficult.

Plate 2G. Mucocutaneous leishmaniasis. Metastatic lesions usually begins within the deep nasal mucosa. There is heavy cellular infiltration around the small blood vessels, proliferation of the vascular endothelial cells, progressive necrosis and fibrosis, erosion of the nasal septum, liquefaction of the cartilage. As a result, the inflammation spreads to the surface of the skin causing superficial necrosis, desquamation of the surface epithelium and ulceration. In some cases the inflammation can also spread to the eye.

Plate 2H. Mucocutaneous leishmaniasis: facial destruction due to cure after progressive disease. This particular patient [908/2/9] suffered from MCL for >10 years and underwent three courses of antimonial therapy. The picture displays one of the facets of ACL: because the lesions tend not to be painful, patients do not seek treatment in local health posts, with progressive MCL causing severe facial destruction which ultimately may be fatal due to bacterial contamination. Patients suffering from progressive MCL also can suffer from severe stigmatisation and may face the exclusion of the community.

Plate 2I. Differential diagnosis: malignant neoplasm of the skin. This adult has a squamous cell carcinoma, with aggressive exophytic growth on cheek and upper neck. Skin cancers are often misdiagnosed as LCL.

Plate 3. American cutaneous leishmaniasis in Huánuco, Peru: field sites and community participation.
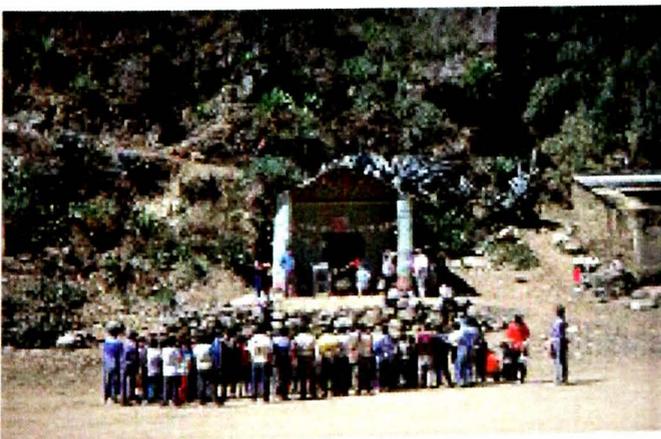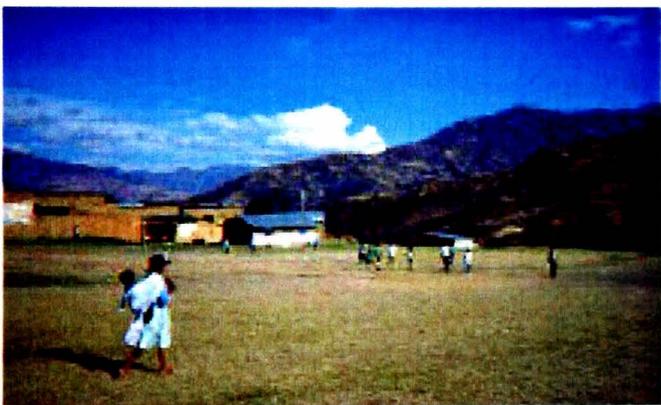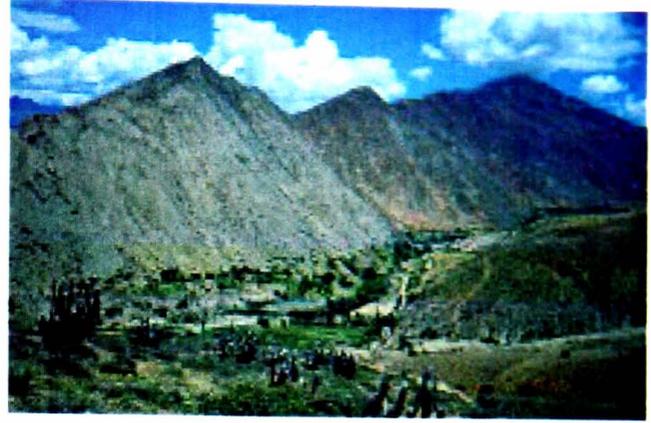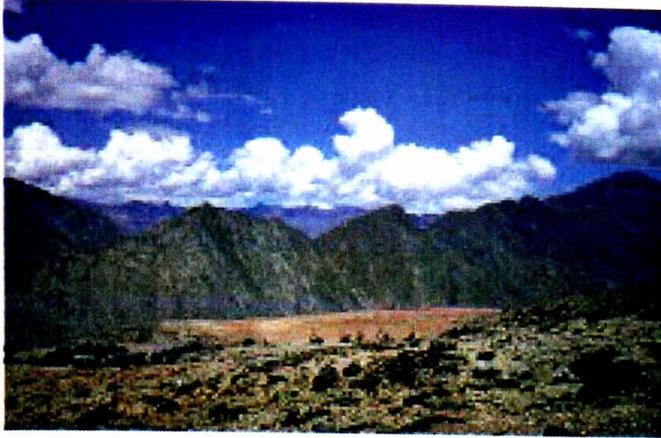
Plate 3 Legend:

A	B
C	D
E	F
G	H

Plate 3A. Topography of the study site. A typical view of one of the field sites showing the dry slopes of the Huanuqueñan valleys where both *L. braziliensis* and *L. peruviana* are endemic. Pictured: Conchumayo [902].

Plate 3B. Topography of the study site. A typical view of one of the field sites showing the dry slopes of the Huanuqueñan valleys where both *L. braziliensis* and *L. peruviana* are endemic. Of note are the dry mountain slopes and the fertile valley around the creek, where potatos, maize, manioc and wheat are grown. Pictured: Conchumayo [902].

Plate 3C. Topography of the study site. A typical view of one of the study villages. Of note are the dry mountain slopes and the fertile valley around the creek, where potatos, maize, manioc and wheat are grown. Pictured: Quechualoma [903].

Plate 3D. Seasonality of the study site: after the rainy season, the normally dry Andean valleys become green and lush.

Plate 3E. Study site. Study villages are at high altitude (2000-3500m) in the Andes and tended to have between 150-650 inhabitants. Each village tends to have a main square with adjacent church and school.

Plate 3F. Sandfly vector habitat. Many houses in the study sites have animal shelters close to the house, which provide optimal conditions for sandflies regarding temperature, humidity, and an abundance of bloodmeal sources. This animal shelter was next to the house of 'Tarzan' [908/97/27] (see **Plate 4E**).

Plate 3G. Project logistics: community participation and involvement. Prior to the start of the surveys, reunions were organised in each village to outline the study's purpose, objectives and implementation. Pictured: meeting the adults on the village square in Coz [908].

Plate 3H. Project logistics: community participation and involvement in epidemiological surveys. During 1994 and 1998 an epidemiological survey on human ACL was carried out by the Universidad Peruana Cayetano Heredia and the London School of Hygiene & Tropical Medicine (**Chapter 5**). People in study villages were followed-up clinically at three-month intervals during this period, with yearly Montenegro skin tests being applied (pictured: Juan Canales Espinoza applying MST in Coz).

Plate 4. American cutaneous leishmaniasis in *Canis familiaris*.

Plate 4 Legend:

A	B
C	D
E	F
G	H

Plate 4A. Sampling of dogs during the house-to-house surveys in study sites. Dogs were examined clinically for scars and ulcers indicative of ACL. After blood sampling, dogs were vaccinated against rabies (**Chapter 2**).

Plate 4B. Sampling of the canine population in study sites. Of a subset of sampled dogs bone marrow aspirates were taken for diagnostic purposes (**Chapter 2**).

Plate 4C. American cutaneous leishmaniasis in *Canis familiaris*. Clinical characteristics of ACL in dogs include small cutaneous ulcers or scars, which tend to be found at sites exposed to sandfly vectors, i.e. nose and ears. Dog: 902/97/30 aka 'Oso'; had an active lesion between 1997 and 1998, was PCR-/ELISA+[6.1 LAU/ml] in 1997, PCR+/ELISA+[7.2 LAU/ml] in 1998 and ELISA+, and PCR+/ELISA+ [6.3 LAU/ml] in 1999.

Plate 4D. American cutaneous leishmaniasis in *Canis familiaris*. As the dog in **Plate 4C**, the dog pictured has an ulceration on the snout. Additionally is suffers from severe conjunctivitis ('lunettes') around the eyes, a common clinical sign for zoonotic visceral leishmaniasis. Dog: 904/98/17 aka 'Alicia', had an active lesion in 1998, PCR-/ELISA+[5.5] in 1998.

Plate 4E. American cutaneous leishmaniasis in *Canis familiaris*. As the dogs in **Plate 4C-D**, the dog pictured has an ulceration on the snout. Additionally is suffers from severe conjunctivitis ('lunettes') around the eyes, a common clinical sign for zoonotic visceral leishmaniasis. Dog: 908/97/27 aka 'Tarzan', active lesions on the snout, around the eyes, and in the ear (not shown); PCR+/ELISA+ [5.1], culture positive [CAN/PE/94/LC56].

Plate 4F. Clinical characteristics of ACL in *Canis familiaris*. Same dog as in **Plate 4F**, close-up of the lesion on the snout.

Plate 4G. American cutaneous leishmaniasis in *Canis familiaris*. Advanced mucocutaneous leishmaniasis in a dog, where –similar to ACL in man- disease has led to the necrosis of the snout and partial collapse of the septum. Dog: 903/97/71 aka 'Marquesa', had an active lesion between 1997 and 1998, PCR-/ELISA+ [5.7LAU/ml] in 1998).

Plate 4H. American cutaneous leishmaniasis in *Canis familiaris*. Advanced mucocutaneous leishmaniasis in a dog, where –similar to ACL in man- disease has led to the necrosis of the snout and partial collapse of the septum. Other clinical signs included emaciation, onychogryphosis, dry and brittle hair. Dog: 921/97/1 aka 'Princesa'; ELISA+[5.5] PCR and histology detected parasites in blood, conjunctiva, lymph node, lung and ovary biopsies (**Chapter 3**).

# Plate 5. PCR as mass-screening tool to detect *Leishmania (Viannia)* infection in dog blood
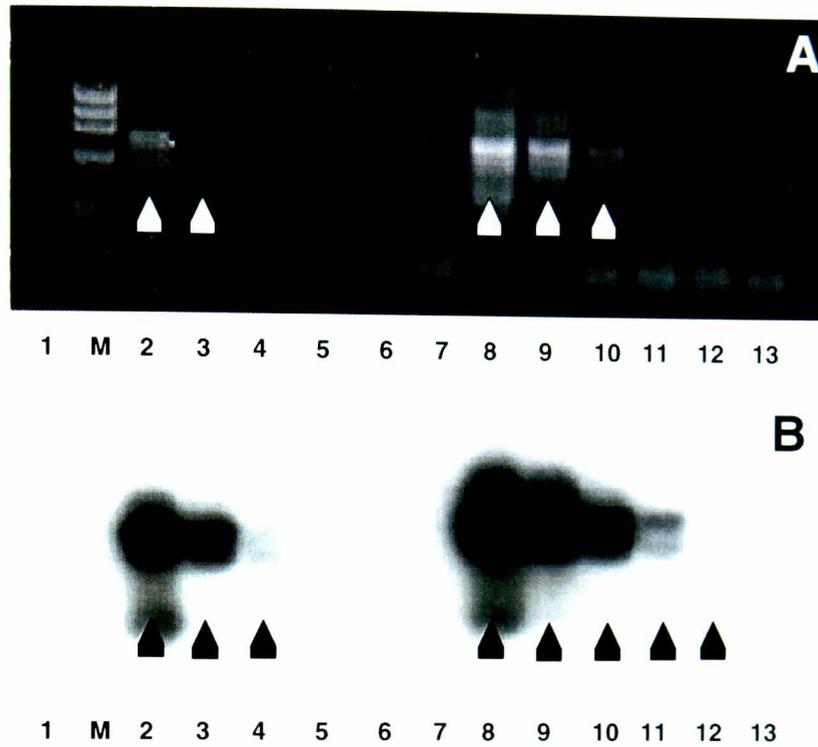
Plate 5A. Sensitivity titration assay (STA). As outlined in **Chapter 2**, an STA was set up to determine which DNA extraction protocol and DNA primers to use for PCR-hybridisation analysis of field samples. DNA was extracted from samples using four different extraction protocols, amplified using four different PCR primer pairs, Southern blotted and hybridised to a specific radioactive probe. Samples were amplified with B1/2 PCR primers (**A**) and hybridised to a B3 probe (**B**) (**Box 1**). Lane 1, no-DNA negative control; M, X174 base pair marker; Lanes 2-6, phenol/chloroform-extracted samples; Lane 7, no-*L. braziliensis* phenol/chloroform-extracted negative control; Lanes 8-12; DNeasy-extracted samples; Lane 13, no-*L. braziliensis* DNeasy-extracted negative control.
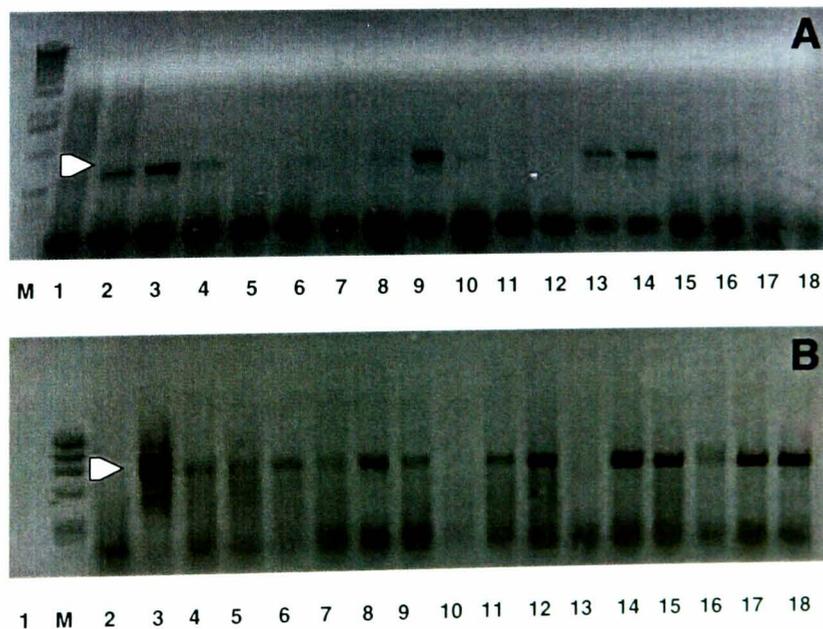
Plate 5B. PO3/PO5 PCR amplification of guanidine blood lysate field samples to evaluate sample degradation or PCR inhibition. Arrow indicates the 469bp amplification product (**Box 1**). M, X174 base pair marker; Lanes 1-18, field samples.

Plate 5. PCR as mass-screening tool to detect *Leishmania (Viannia)* infection in dog blood (guanidine blood lysate, GBL)
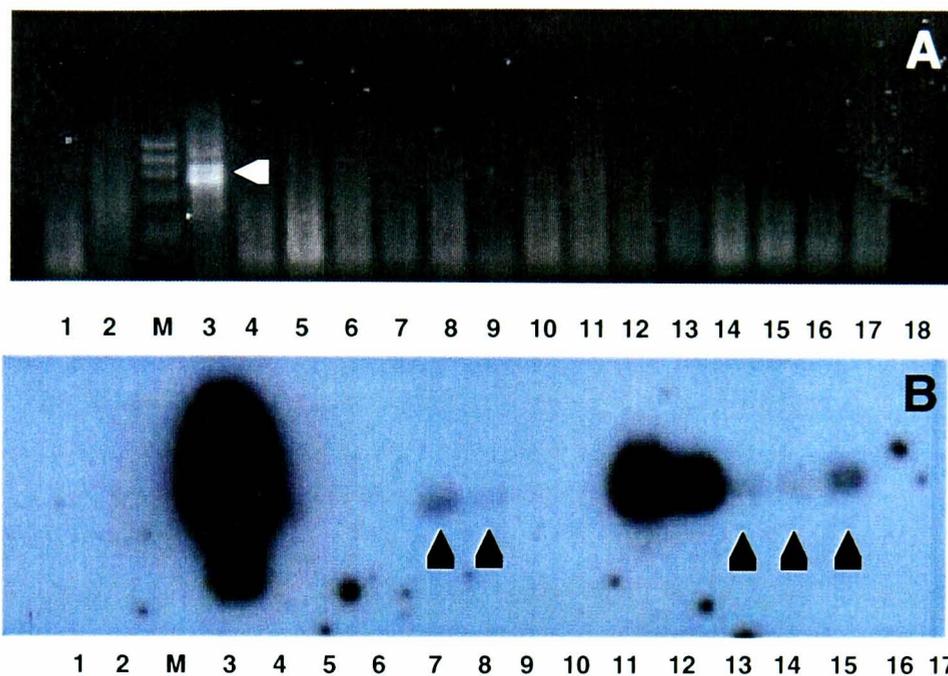

Plate 5C. B1/2 amplification of guanidine blood lysates (**A**) and hybridisation to B3 probe (**B**) (**Box1**). Lane 1, no DNA guanidine blood lysate negative control; Lane 2, dog DNA guanidine lysate negative control; M, X174 base pair marker; Lane 3, *L. braziliensis* positive control (MHOM/BR/75/M2903), Lanes 4-17, field samples. Though not visible by gel electrophoresis, 750bp B1/2 amplification products are detected by hybridisation for samples 7, 8 and 11-15.
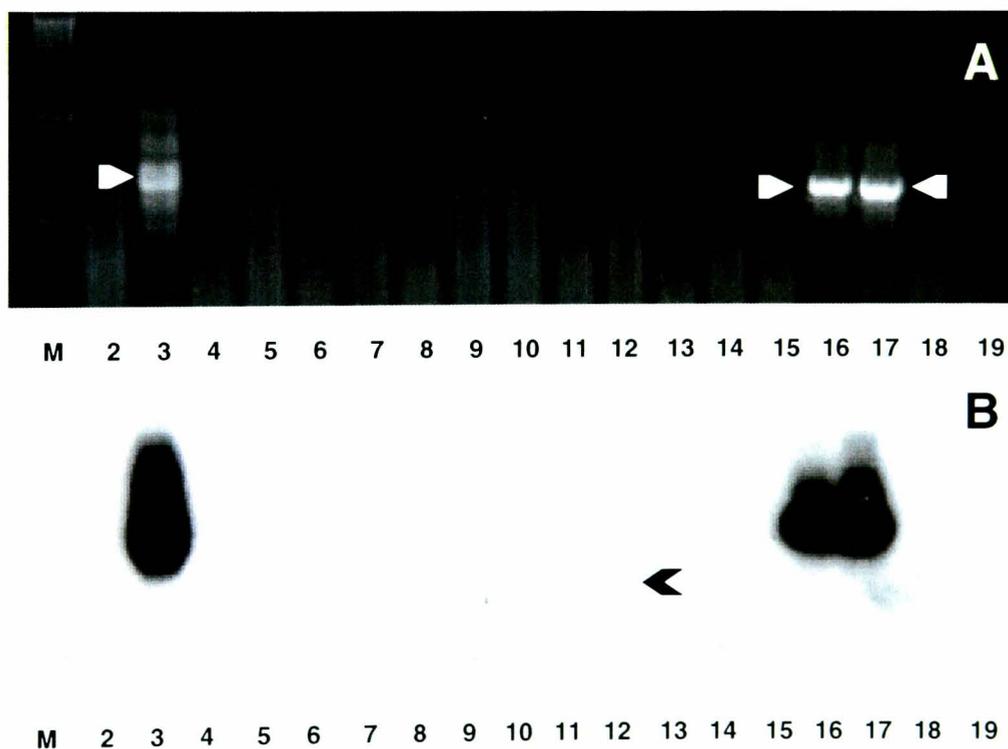
Plate 5D. B1/2 amplification of guanidine blood lysates (**A**) and hybridisation to B3 probe (**B**) (**Box1**). M, 1 kilo base pair ladder; Lane 2, dog DNA guanidine lysate negative control; Lane 3, *L. braziliensis* positive control (MHOM/BR/75/M2903), Lanes 4-19, field samples. 750bp B1/2 amplification products from samples 16 and 17 were visible by gel electrophoresis and hybridised to the B3 probe.

## Plate 5. PCR as mass-screening tool to detect *Leishmania (Viannia)* infection in dog blood (buffy coat, BCS)
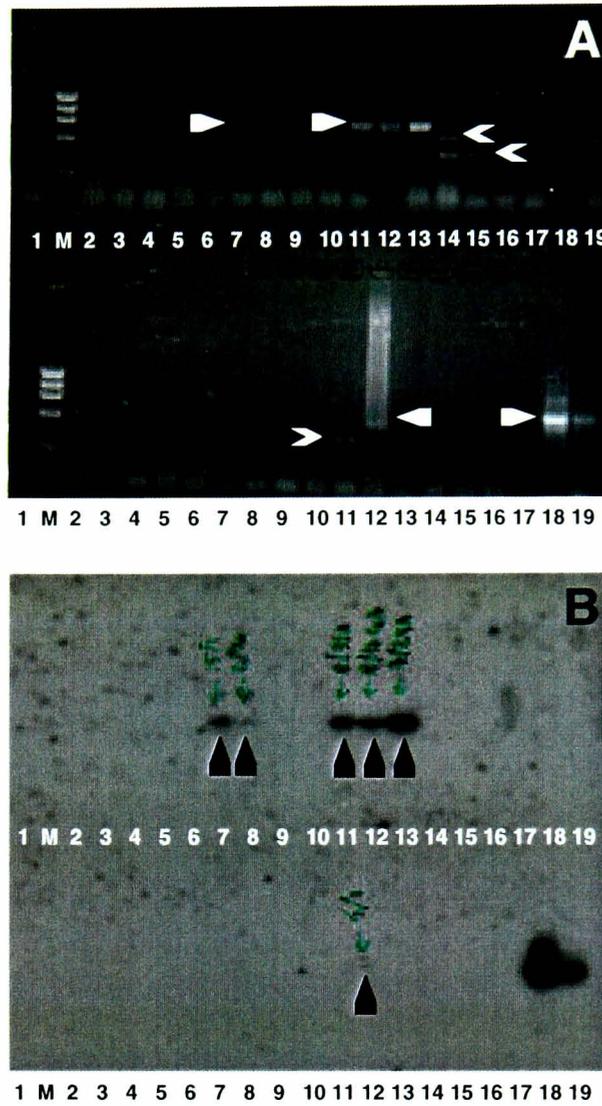
Plate 5E. B1/2 amplification of buffy coat samples (**A**) and hybridisation to B3 probe (**B**) (**Box1**). Lane 1, no-DNA buffy coat negative control; M, X174 base pair marker; Lanes 2-19, field samples; Lane 20, dog DNA negative control; Lanes 21-36, field samples; Lanes 37-38, *L. braziliensis* positive control (MHOM/BR/75/M2903). 750bp B1/2 amplification products from samples 7,8, 11-13 and 29 were hybridisation positive. Broken arrows indicate non-specific bands seen on the gel, which prevented classification of samples into gel electrophoresis or hybridisation positive or negative (see **Chapter 2** for details).

Plate 5. PCR as mass-screening tool to detect *Leishmania (Viannia)* infection in dog bone marrow (BMS).
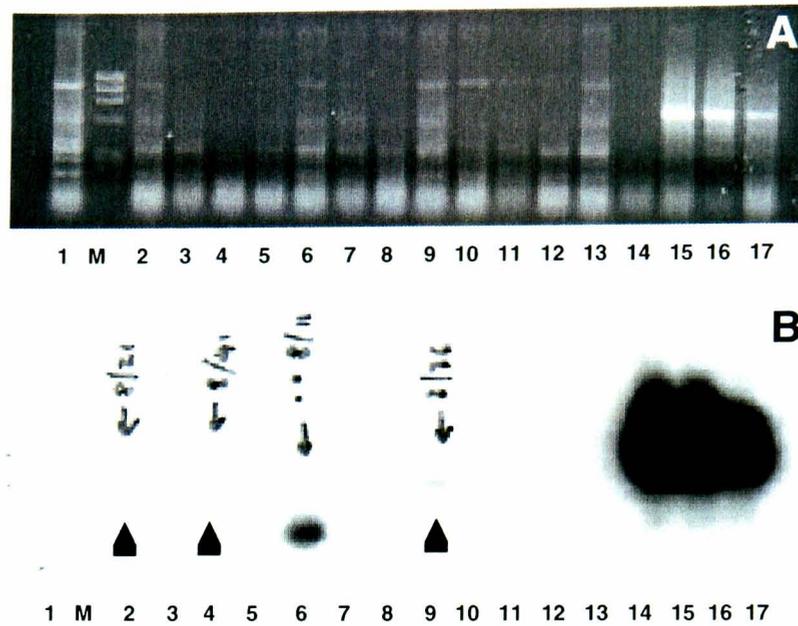

Plate 5F. B1/2 amplification of bone marrow samples (**A**) and hybridisation to B3 probe (**B**) (**Box1**). Lane 1, dog DNA bone marrow negative control; M, X174 base pair marker; Lanes 2-14, field samples; Lanes 15-17, *L. braziliensis* positive control (MHOM/BR/75/M2903). 750bp B1/2 amplification products from samples 2,4 and 9 were hybridisation positive. Broken arrows indicate non-specific bands seen on the gel, which prevented classification of samples into gel electrophoresis or hybridisation positive or negative (see **Chapter 2** for details).

Plate 6. Topical insecticides to control canine zoonotic visceral leishmaniasis: experimental and matched cluster intervention trial.
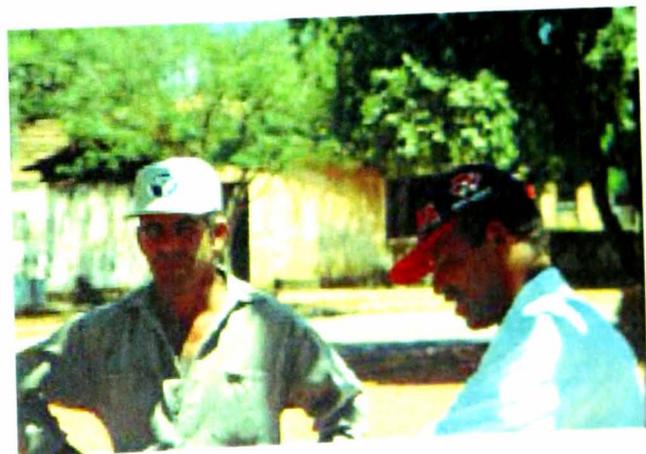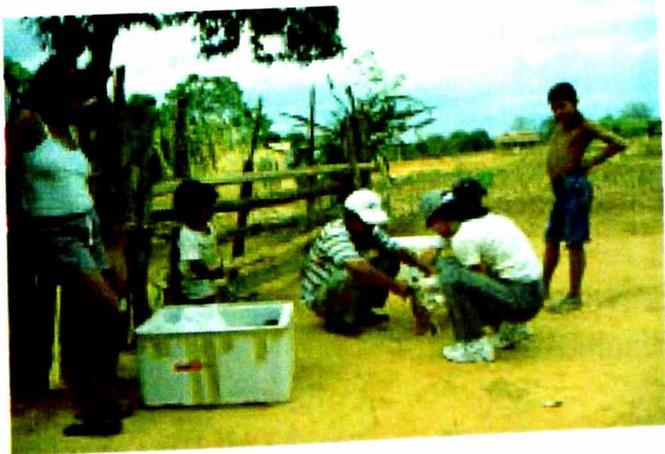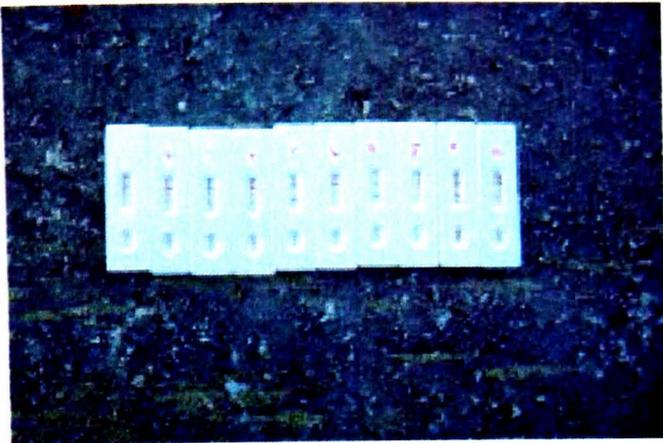
Plate 6 Legend:

A	B
C	D
E	F
G	H

Plate 6A. Diagnosis of canine zoonotic visceral leishmaniasis. Immunochromatographic dipsticks were compared to PCR and ELISA in detecting ZVL infection in dogs (**Chapter 6**).

Plate 6B. Experimental trial to test the efficacy of topical insecticides in protecting dogs from sandfly bites. Caged dogs were exposed to wild-caught sandflies overnight. After 7-8 hrs, sandflies were retrieved from the tents and scored as either dead or live, bloodfed or unfed (**Chapter 7**).

Plate 6C. Experimental trial: Fazenda Palmital. Topical insecticides used in the trial included deltamethrin and diazinon-impregnated dog collars as well as permethrin and fenthion pour-on lotions (**Chapter 7**).

Plate 6D. Matched cluster intervention trial: Capitão Eneas. Dog with 40mg/g deltamethrin-impregnated dog collar (**Chapter 8**).

Plate 6E. Matched cluster intervention trial: Capitão Eneas. Capitão Eneas has been a focus for urban ZVL for a number of years and canine culling campaigns are carried out every 1-2 years; area shown: D1 (**Chapter 8**).

Plate 6F. Matched cluster intervention trial: Capitão Eneas. Capitão Eneas has been a focus for ZVL for a number of years and canine culling campaigns are carried out every 1-2 years; area shown: D2. Note the proximity of houses to crops and vegetation, ideal resting and breeding places for *Lutzomyia longipalpis* vectors (**Chapter 8**).

Plate 6G. Matched cluster intervention trial: Capitão Eneas. House-to-house visits and sampling of dog population (**Chapter 8**).

Plate 6H. Matched cluster intervention trial: Capitão Eneas. Fundação Nacional de Saúde staff helping with the intervention trial (**Chapter 8**).