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Development of new diagnostic tools to identify canine reservoir super-spreaders of *Leishmania infantum*

by

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Declaration

I hereby declare that the work presented in this thesis is the result of original research carried out by the author, Aurore Lison, under the supervision of Dr. Orin Courtenay and Dr. Steven Reed. No part of this thesis has been submitted for a degree at another University.

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Summary

Zoonotic visceral leishmaniasis (ZVL) is a vector-borne infection induced by protozoan parasite *Leishmania infantum* and transmitted from animal reservoir to human. Domestic dogs are the main proven reservoir, and the detection of their transmission potential is a research priority. Longitudinal xenodiagnosis studies of natural infection in dogs showed that a large fraction of transmission events to the sand fly vector is due to a small fraction of the infected canine reservoir population, known as super-spreaders. The management of visceral leishmaniasis requires a different approach to current blanket control operations that otherwise require extremely high intervention coverage to successfully include the super-spreaders.

The aims of the study were to discriminate super-spreaders in a mixed reservoir population by developing novel diagnostic tools, and to complete mathematical models based on collected data including transmission potential and tool-implementation in the field. Existing and novel anti-*Leishmania* antigens were tested in enzyme-linked immunosorbent assays (ELISA) on archived sera collected from a naturally infected cohort population of Brazilian dogs. Their transmission potential was measured by xenodiagnoses during a two years longitudinal study.

Results from serological assays showed that carefully selected threshold-based antigens allowed a more specific test towards reducing transmission; and some of the novel proteins (rK28, K26, rK34) out-performed the currently available test antigens for infection. These antigens were tested for the serodetection of infectiousness and were able to discriminate super-spreaders of *Leishmania* within the mixed canine population. Based on these results, a prototype of rapid diagnostic test (RDT) was developed based on a brand-new antigen, KL914, and specifically designed for detecting super-spreaders. The aim was to setup a field-friendly screening method. The impact of the novel diagnostic tool to detect and remove super-spreaders from the population before the onset of infectiousness was modelled and quantified under different population dynamic scenarios. The mathematical model offered a notice on the diagnostic tool to be applied in the field, and pointed out the limitations and the possible improvement.

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List of abbreviations

AUC	area under the ROC curve
CL	cutaneous leishmaniasis
CLA	crude *Leishmania* antigen
CVL	canine visceral leishmaniasis
DAT	direct agglutination test
DHT	delayed type hypersensitivity
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
IDRI	Infectious Disease Research Institute
IFAT	indirect fluorescence antibody test
IFI	indirect immunofluorescence test
IgG	immunoglobulin G
kDNA	kinetoplast DNA
MOH	Ministry of Health (Brazil)
NPV	negative predictive value
PCR	polymerase chain reaction
PPV	positive predictive value
RDT	rapid diagnosis test
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROC	receiver-operator characteristic
UK	United Kingdom
USA	United States of America
VL	visceral leishmaniasis
VLCSP	Visceral Leishmaniasis Control and Surveillance Programme
WHO	World Health Organization
ZVL	zoonotic visceral leishmaniasis

CHAPTER 1.   Introduction

1.1   General background

Visceral leishmaniasis is considered to be a major tropical and subtropical disease by the World Health Organization (WHO), and affects impoverished people in Africa, Asia and Latin America. Leishmaniasis is endemic in 98 countries, and over 90% of cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, Sudan and South Sudan (Alvar et al., 2012). Over 12 million people are affected, and 0.2 to 0.4 million new cases are thought to occur each year (Alvar et al., 2012; Van der Auwera et al., 2015). Visceral leishmaniasis (VL) can be life threatening in the absence of treatment. The mortality rate also remains significant ranging from 5 to 15%, even with treatment. The overall case-fatality rate is 10%, meaning that VL causes between 20,000 and 40,000 deaths each year worldwide (Alvar et al., 2012). The disease is characterized by irregular fevers, weight loss, hepatosplenomegaly (enlargement of liver and spleen) and anaemia. Death usually occurs following an opportunistic secondary infection.

Visceral leishmaniasis is caused by an obligate intracellular protozoan of the *Leishmania* species (Kinetoplastida, Trypanosomatida). In Latin America, the main *Leishmania* species responsible for infection are *L. donovani* and *L. infantum* (Alvar et al., 2012). *Leishmania* has two successive morphological forms: the amastigote form is replicating intracellularly and spreading within the host tissue, while the promastigote form is the infective stage (Sacks et al., 2001). As a vector-borne parasite, *Leishmania* is transmitted by the bite of female phlebotomine sandflies from the genera *Phlebotomus* and *Lutzomyia*, respectively restricted to the Old World and the New World. In Latin America, *Lutzomyia longipalpis* is responsible for the transmission of the protozoan to various mammalian hosts. During blood meal, the sandflies inject promastigotes into the host circulation. These parasites are phagocytosed by macrophages, and transform into amastigotes for intracellular replication. Infected cells implode and release their contents in amastigotes in the blood and lymph. They can then infect tissues. During a new blood meal, sandflies retake cells infected with amastigotes. In the intestines of the sandflies, parasites are released and differentiate into promastigotes, which migrate to the salivary glands, ready to infect other

individuals (Sacks et al., 2001). In Latin America, especially in Brazil, there is a seasonal variation in transmission of *Leishmania* based on the natural vector cycle; therefore, the transmission is the lowest in the wettest months from January to March, and increases during the dry season up to December (Quinnell et al., 1997; Grimaldi et al., 2012b).

Animal reservoir hosts, along with sand flies, maintain the transmission cycle of the parasite. In *L. infantum*-endemic areas, domestic dogs are considered to be the main proven reservoir host to the sandfly vector (Courtenay et al., 2014; Quinnell et al., 2009). Studies have shown that infection in dogs precedes infection in humans (Oliveira et al., 2001). Subsequent transmission between dogs and humans then leads to the zoonotic infection (Connolly, 2005). In zoonotic VL, humans are incidental hosts and do not contribute to the transmission of *Leishmania infantum* (Quinnell et al., 2009). The first case of infection of *L. infantum* from domestic dogs by the bite of sandflies was demonstrated in the 1930s (Parrot et al., 1930; Adler et al., 1932). Since then, many studies have confirmed the role of the domestic dog as the primary reservoir of ZVL. Dogs often have a high prevalence of both infection and infectiousness, have long-lasting infections, and are common in the peridomestic environment in which most ZVL transmission occurs (Quinnell et al., 2009). Nowadays, little is known about canine transmission, but it is essential to understand this, as human disease is dependent upon canine infection. This project focuses on the transmission of the *Leishmania* infection from the canine reservoir to the sandfly vectors, with the aim of limiting incidence in humans.

1.2   Canine Visceral Leishmaniasis

1.2.1   *Course of infection in reservoir dogs*

The course of *Leishmania* infection varies greatly among the canid population, and these heterogeneities are essential to the understanding of the transmission. Leishmaniasis may, for instance, become patent after initial infection in some dogs, approximately 2 or 3 months (82 to 111 days, average of 94 days) (Quinnell et al., 1997). Alternatively, infections in other dogs remain sub-patent for more than a year. This prepatent period appears to correlate to the period of time between infection and seroconversion. Seroconversion allows the infection to be detected through a serology

diagnosis test (Coura-Vital et al., 2013a). A further complication is that the definition of infection varies within the literature. Infection does not invariably lead to illness, nor does it necessarily lead to infectiousness. The clinical symptoms manifested by dogs are weight loss, fever, lethargy, lymphadenopathy (swollen lymph nodes), onychogryphosis (abnormal nails), conjunctivitis (ocular disease), splenomegaly (enlarged spleen), alopecia and several dermal affectations including dermatitis, hyperkeratosis (excessive epidermal scaling) and depigmentation on the face, ears and feet (Ozbel et al., 2000). Besides these clinical signs, dogs are considered to be positive for infection when they test positive for parasite culture, PCR and serology (Quinnell et al., 2001) which involves the seroconversion of the dog. Moreover, about 20 to 30% of infected dogs never develop clinical signs; they are referred to as asymptomatic (Sundar et al., 2006b). The canine population in endemic areas has been described as being composed of four mutually exclusive groups of hosts: **(1)** those susceptible to the infection, **(2)** those resistant to the infection, **(3)** those susceptible that become latent after a sandfly bite (called asymptomatic), **(4)** and those infectious to sandflies that emerge from latent canine infection (Grimaldi et al., 2012b). Dogs that are born resistant are able to maintain an effective cellular immune response against the parasite, and thus never become infectious. The reasons why some dogs are resistant while others become latent are unknown (Courtenay et al., 2002b; Grimaldi et al., 2012b).

1.2.2 Infectiousness and super-spreaders

In infectious diseases, heterogeneity in the transmission potential of reservoirs and vectors is well known; where infectiousness is defined as the ability of reservoirs to transmit infection to the vector. Moreover, a small percentage of infected individuals within any population is observed to control most transmission events. This is known as the 20/80 rule, in which an average of 20% of the host population is expected to responsible for over 80% of all transmission events. This highly infectious individuals are called super-spreaders and infect disproportionately more secondary contacts. Over time, super-spreading events (in humans and animals) have been documented, in many diseases such as for tuberculosis, Ebola haemorrhagic fever, West Nile viral infection, Typhoid fever and so many other. The first symbolic case, named Mary Typhoid, was detected during an outbreak of Typhoid fever in the 1900's when a single asymptomatic person infected more than 200 individuals over several years. However, the real first evidence of the existence of super-spreading events was demonstrated during the severe

acute respiratory syndrome (SARS) outbreak of 2003. During this pandemic, majority of the infected individuals had low infectivity and the super-spreaders were maintaining up to 75% of the infections on their own (Li et al., 2004). A stochastic model showed that the average transmission was 2.7 secondary cases for one normal person (Riley et al., 2003), whereas super-spreaders would infected over 10 secondary contacts (Lipsitch et al., 2003). This outbreak revealed the epidemiologic importance of this heterogeneous transmission. Many other evidences confirmed it throughout the following infections, for which the most recent examples are the outbreaks of Ebola virus in 2014-2015 or MERS coronavirus (middle-east respiratory syndrome) in 2015. Even if it is still unclear what makes super-spreaders; according to the review of Stein (2011), the super-spreading events are influenced by host, pathogen and environment factors. Examples are multiple: the virulence of *Salmonella* is associated with super-spreading; co-infection with HIV induces higher shedding; the host behaviour (length and frequency of contacts) is correlated with the intensity of transmission for the West Nile viral infection; the immune response of the host is linked super-spreading in SARS; crowding increases the number of secondary contacts in SARS; and finally, misdiagnoses or mismanagement of cases would be the environment related factors (Stein, 2011).

In the case of ZVL, the presence of heterogeneity in dogs' infection and infectiousness was demonstrated first by Courtenay et al. (2002b) during a longitudinal study on Marajo Island, Brazil. Indeed, not all dogs were equally susceptible to infection: a small number of infected dogs became infectious, while others never became infectious to sandfly vectors (Courtenay et al., 2002b). Moreover, the presence of variable *L. infantum* loads between dogs suggests that the bulk of the transmission is due to a small proportion of infectious dogs (Courtenay et al., 2014). Combined results for the Marajo study showed that less than half (43%) of infected dogs became infectious and a much smaller proportion of dogs (17%) became highly infectious. They are the super-spreaders. Results coincide with the "20/80 rule" detailed above. Later studies have confirmed the presence of heterogenicity of infectiousness in ZVL. In Brazil, studies from Michalsky et al. (2008) and da Costa-Val et al. (2007) aimed to measure the infectivity of dogs based on their clinical status and the immune response; whereas in Europe, a study form Guarga et al. (2000) on dogs' infectiousness was positively associated with the immune response of the hosts. It is still unclear why certain dogs

become super-spreaders and infect disproportionately large numbers of contacts. One potential explanation is that highly infectious dogs have greater than average parasite loads compared to mildly and non-infectious dogs. Another question is how we might identify super-spreaders a mixed canid population. The present project seeks to address this question. Identifying super-spreaders would enable to reduce approximately 80% of pathogen transmission events in the population. As super-spreaders infect disproportionally more secondary contacts (Stein, 2011), they hinder the successful implementation of infection control strategies. Little is known about super-spreaders or their public health implications. Although the concepts of super-spreaders are not new, current models do not sufficiently take super-spreaders into consideration. A meta-analysis of the available canine infectiousness data for the New and Old Worlds, indicated that the proportion of infectious dogs in the infected dog population may be higher in European than in Brazilian studies, 0.86 and 0.45 respectively (Quinnell and Courtenay, 2009). A further reason for heterogeneity is that infectiousness varies over time (Courtenay et al., 2002b). Indeed, infectiousness develops on average 6 months after infection in endemic areas. According to Courtenay et al. (2002b), the mean proportion of flies infected reached a peak of 20% at 135 days after patent infection and declined significantly thereafter. Infected dogs became infectious a median of 105 days after seroconversion, 135 days after patent infection, and 333 days after the dogs were placed in the study site. As previously mentioned, Quinnell et al. (1997) estimated that seroconversion occurs on average 94 days after infection. Combined with Courtenay's results, this indicates a latent period of 199 days between infection and infectiousness (Courtenay et al., 2002b; Quinnell et al., 1997).

Heterogenicity in infectiousness is also present in other hosts of VL; for example, humans can be transmitters in *L. donovani* anthroponotic infection in the Indian subcontinent (discussed in Chapter 9), as well as in the murine model of visceral leishmaniasis (Doehl et al., 2017). However, it has been demonstrated that foxes, while they are the wildlife host of *Leishmania infantum*, are not considered to maintain the transmission cycle (Courtenay et al., 2002a). Indeed, foxes have the same infection level as dogs, but none of them were infectious to the sandflies in a longitudinal study (Courtenay et al., 2002a). One explanation for the lack of infectiousness of foxes may be their low parasite loads. A conservative estimate of the possible contribution of foxes to transmission was 9%, compared to 91% by domestic dogs.

1.2.4 Role of asymptomatic carries

In the Indian subcontinent, where the transmission of *L. donovani* is anthroponotic, it is well known the asymptomatic infectious people are an important source to start and/or maintain epidemics. The identification of these cryptic carriers is a priority for the elimination of VL. Controverly, in ZVL, neither the role of these asymptomatic dogs in transmission, nor the prognosis of asymptomatic infection at the individual level has been fully elucidated. Moreover, results differ concerning the transmission potential of asymptomatic dogs compared to symptomatic dogs. Some studies have shown that asymptomatic dogs are unable to transmit the parasite (Travi et al., 2001; Verçosa et al., 2008). Other studies have demonstrated that transmission occurs in similar proportion for symptomatic dogs (Courtenay et al., 2002b; Costa-Val et al., 2007; Michalsky et al., 2007). Finally, a single other study demonstrated that asymptomatic dogs are an important source of amastigotes for the infection of phlebotomine, which contribute to the transmission of *L. infantum* (Molina et al., 1994; Michalsky et al., 2007; Courtenay et al., 2002b). In 2013, Laurenti et al. observed that asymptomatic dogs are highly able to transmit *L. infantum* to sandflies. In their study, all asymptomatic dogs except one (93%) were able to transmit the parasite. Additionally, it seems that sandflies fed more on asymptomatic dogs than on symptomatic dogs. However, according to a longitudinal study by Courtenay et al. (2002b) on the role of asymptomatic dogs in transmission, the majority of those infectious asymptomatic dogs (75%) were in fact pre-symptomatic, developing symptoms after becoming infectious. Only 2 of 9 dogs that clearly remained asymptomatic were infectious, thus, pre-symptomatic or symptomatic dogs were responsible for the vast majority of sandfly infections (99.6%). Overall, the transmission potential of asymptomatic still remains highly controversial.

1.3   Interventions, control and prevention programs

Different prevention and control programs to limit transmission have been created in areas where infection is endemic. There is currently no efficient vaccine, and treatment of infected dogs is not usually curative (Baneth et al., 2002). Thus, most control programs are based on early diagnosis and treatment of human cases, health education, vector control by spraying residual insecticides (Desjeux, 1966), and canine removal

(Quinnell et al., 2009). These efforts to control canine leishmaniasis are essential to prevent the disease and reduce transmission to humans.

1.3.1 Vaccines in dogs

As studies have shown, dog infection is usually followed by human infection, so vaccination of dogs is a key research focus. In 2006, several vaccines designed for dogs became available on the Brazilian market, including Leishmune® and Leish-Tec® (Fort Dodge Wyeth, Brazil), a preventive second-generation vaccine. In 2010, the Brazilian Ministry of Agriculture and Health discontinued the prescription of Leishmune® as it did not prove its efficacy in Phase III studies (Lima et al., 2010). Since 2014, Leish-Tec® is the only commercial vaccine approved in Brazil and has 71% proven efficacy for prophylaxis of canine infection (Regina-Sliva et al., 2016). A comparative trial, conducted in 2014 with vaccinated dogs, has shown no significant difference between Leishmune® and Leish-Tec®, based on parasitology, serology, clinical signs, or infectiousness (Testasicca et al., 2014). The advantage of Leish-Tec® compared to Leishmune® is that vaccinated dogs can be differentiated from naturally infected dogs, since the vaccine does not interfere with the diagnostic tests. Indeed, the diagnostic tests are based on antigens from promastigotes, while the Leish-Tec® vaccine is based on antigens from amastigotes, which is not the case for Leishmune® (Fernandes et al., 2008). In Europe, another vaccine, CaniLeish®, based on excreted-secreted proteins from *Leishmania* was approved in 2011, offering prophylactic action by reducing the transmission of *Leishmania* from vaccinated dogs to sandflies (Oliva et al., 2014). Despite the promising nature of these trials, however, vaccines are used as individual protection for dogs, rather than a public health tool, since there is no evidence that vaccines reduce human infection rate (Manual de Vigilância e Controle da Leishmaniose Visceral Americana do Estado de São Paulo, 2006).

1.3.2 Vector control

Many control and prevention programs have focused on vector control by applying insecticides and repellents in endemic areas and/or on animal reservoirs as vectors are highly zoophilic (Hewitt et al., 1999). The current insecticides include semi-synthetic pyrethroids, such as Deltamethrin, alone or in combination with other insecticides to protect dogs from sandfly bites. However, there is little evidence that the sandfly

population can be reduced effectively by spraying insecticides. As they present a low stability, insecticides have been included in collars, lotions and sprays to facilitate their application by dog owners. Over the years, several field trials provided evidences that insecticide-impregnated collars can prevent dogs from *Leishmania* infection in Europe and in Brazil with an individual protection of 85% towards sandfly bites (Killick-Kendrick et al., 1997; Maroli et al., 2001; Reithinger, 2001; Reithinger et al., 2004; Mondal et al., 2013). The insecticide-impregnated in the collars spread slowly into the skin of the dogs, and is maintained for period up to 6 months, leading to an anti-feeding effect and further death of the sandflies (Killick-Kendrick et al., 1997). Systematic review from Wylie et al. (2014) on the efficacy of insecticide treatments (impregnated collars and spots-on) analysed 8 studies from 2001 to 2012 including 4 papers on collars, 3 papers on spot-on treatments and one paper on both collar and spot-on; all studies reported that deltamethrin-impregnated collars, and spot-on treatments with permethrin (alone or in combination) were significantly reducing the proportion of dogs infected with ZVL. Despite being effective individual preventive methods, the efficacy of the collars at the community level needs to be investigated, widely depends on the coverage of the collars within the population including their application on newly introduced dogs, and the replacement of lost collars (Reithinger et al., 2004; Travi et al., 2018). Moreover, being a high cost intervention for the dog owners, the application of collars, whereas possible in Europe, would require financial support by the government in Brazil (Travi et al., 2018). Finally, the effect of insecticide-treated materials on reducing visceral leishmaniasis is controversial in the literature, with several investigators expressing their concerns over the resistance of sandflies to residual insecticides.

1.3.3 Chemotherapy for humans

Human treatments are readily accessible and available within the National Health System of Brazil. Those drugs currently in use were developed many years ago, and have many disadvantages, such as toxic side effects, relapses, or an incomplete cure due to the development of resistances by parasites. The first line drugs are pentavalent antimonials such as sodium stibogluconate Sb^V compounds (Pentostam®, Wellcome Fundation, UK) and meglumine antimoniate (Glucantime®, Rhone Poulenc, France). In March 2014, the Food and Drug Administration approved the oral agent Miltefosine as a substitute for pentavalent antimonial. Others drugs are also selected as the second

choice for treating selected cases of leishmaniasis, such as the parenteral agents amphotericin B (AmBisome®, Vestar, USA), aminoglycoside paromomycin and pentamidine isethionate, as well as the orally administered "azoles" (ketoconazole, itraconazole, and fluconazole). However, these are not approved by international agencies.

1.3.4 Chemotherapy for dogs

Anti-leishmanial medication used for humans has also been tested on dogs. However, treatment of infected dogs is not curative (Baneth et al., 2002), and the current drugs used for VL do not completely eliminate the parasites (Mancianti et al., 1988). Antimonials reduce most of clinical signs of sick dogs, but require continuous administration, and the incomplete clearance of the parasite leads to extremely high rates of relapse. Drugs such as miltefosine and allopurinol are tested to treat canine infection, as reviewed by Reguera et al. (2016). Gradoni et al. (1987) was the first to report significant reductions in infectiousness to sand fly vectors resulted from treating dogs with antimonials. Afterwards, other studies also demonstrated a considerable reduction of the infectiousness of infected dogs within a few months, and up to 150 days post-treatments (Alvar et al., 1994; Guarga et al., 2002; Ribeiro et al., 2008; Miro et al., 2011 and da Silva et al., 2012). A recent trial published in Brazil (De Mari et al., 2017) concluded similarly the non-infectivity of dogs to sandflies for three months after miltefosine treatment, corresponding to significant reductions in parasite loads in bone marrow. All reported impacts of treatment on infectiousness were significant for limited periods of time, as the treatment is short-lived (Travi et al., 2018). The treatment of infected dogs is controversial, and subject to various politics, as described below. The Ministry of Health in Brazil, on 11 July 2008 (Ministerio de Saude, inter-ministerial 1.426, 2008), voted for the prohibition of the treatment of infected or sick dogs using drugs designed for humans (Ministerio de Saude, Brazil, 2006). However, a court decision from January 2013 (Tribunal Regional Federal da 3ª Região, São Paulo, Brazil) declared this provision to be illegal, based on legislation guaranteeing the right of free exercise of vets. Since 2013, the treatment of dogs with drugs designed for humans has thus been permitted. In November 2017, the Ministry of health website published a factsheet on the consequences of the treatment of infected dogs (Ministerio de Saude, Brazil, 2017). In dogs, treatment might result in improved clinical symptoms, although they are still sources of infection of the vector, and so a risk for the further

transmission. In this case, euthanasia of dogs is recommended as one of the ways to control ZVL.

1.3.5 Dogs culling and consequences

Endemic countries such as Brazil have recommended the identification and the euthanasia of positive dogs by a test-and-slaughter policy. In Brazil, the Visceral Leishmaniasis Control and Surveillance Program (VLCSP) started test-and-slaughter campaigns in 1990, leading to the screening and subsequent slaughter of millions of dogs, but with very little impact on transmission rates (Vieira and Coelho, 1998; Lemos et al., 2008). For example, over 176,000 seropositive dogs were eliminated from 1990 to 1997 (Ministerio da Saude, Brazil, 2006) and more than 160,000 seropositive dogs from 2003 to 2005 (Lemos et al., 2008) without corresponding reductions in human VL cases nationally (online SINAN database). Likewise, at the local level, the removal of seropositive dogs did not result in significant changes in human cases. Despite all efforts to reduce reservoir populations, the dog culling strategy is not therefore sufficiently effective. During 1990-1997, more than 176 000 seropositive dogs were eliminated; however, there is no scientific evidence to indicate that dog culling reduces the incidence of the disease in humans (Romero et al., 2010; Harhay et al., 2011). Ashford et al. (1998) measured the effect of culling seropositive dogs at 12 months intervals, reporting a temporary reduction of seroconversion in one year, but with no change in cumulative incidence after 5 years of study. And, as already mentioned by Evans (1992), there was no change in human transmission rates before and after dog culling. In a more recent study, Grimaldi et al. (2012b) concur with the previous study and demonstrating that removing seropositive dogs with active disease soon after detection may affect the cumulative incidence of seroconversion in dogs temporarily, although it is insufficient as a measure for eradicating of canine VL. The failure of the culling program has been ascribed **(1)** to the low sensitivity of the available serological methods, **(2)** to delays in detecting and eliminating infected dogs, and **(3)** to the tendency to replace infected dogs with susceptible puppies (Courtenay et al., 2002b; Grimaldi et al., 2012b). Indeed, diagnosis of canine infection lacks sensitivity (Braga et al., 1998; Courtenay et al., 2014). The incidence of infection is high in endemic areas, and infectiousness develops on average 6 months after infection in endemic areas. As a result, effective control through culling requires a very high proportion of dogs to be tested, and thus, the use of a highly sensitive diagnostic test (Courtenay et al., 2002b).

Moreover, the time delay between diagnosis and culling can reach 80 to 180 days (Braga et al., 1998). Such a delay leads to a reduction in the sensitivity of the diagnostic test (Courtenay et al., 2002b). Finally, dog owners also have the tendency to replace infected dogs with susceptible puppies (Grimaldi et al., 2012b). At present, Brazil is the only country that euthanizes all seropositive dogs. According to several published papers and as already mentioned, a proportion of infected dogs never become infectious, but these dogs may be replaced by susceptible puppies that do become infectious (Parantos-Silva et al., 1998; Courtenay et al., 2002b; Nunes et al., 2008; Grimaldi et al., 2012b). A model created by Courtenay et al. (2002b) assumes that culled dogs will be rapidly replaced with young susceptible dogs, although this assumption has been criticized. Moreover, the culling programme does lead to greater collaboration on the part of dog owners. Such individuals are not always aware of the danger of transmission that comes with keeping infectious dogs at home. Moreover, owners can oppose the protocol of slaughtering seropositive dogs by hiding or releasing animals in nature. According to Esch et al. (2012), the owners' lack of awareness regarding canine transmission of *Leishmania* is associated with an increased risk of dog infection, whereas educating these dog owners is associated with decreased levels of seropositive dogs. Thus, pet attachment and perception of disease risk are significantly associated with the willingness to voluntarily prevent transmission of canine VL. According to Grimaldi et al. (2012b) during the test-and-slaughter policy, the transmission of *L.infantum* was never curtailed; indeed, newly infected dogs could be detected each month throughout the test-and-slaughter protocol in Brazil. The efficacy of dog culling may be increased if only dogs that were infectious could be identified and removed (Quinnell et al., 2009). However, at present there are no diagnostic methods that can reliably distinguish between infectious and non-infectious dogs (Coura-Vital et al., 2013a). Several published field trials have used more sensitive diagnostic techniques and more efficient dog removal regimes than the official surveillance program, as described below (Braga et al., 1998, Ashford et al., 1998; Dietze et al., 1997).

1.4 Diagnostic tools of VL

1.4.1 Evaluation of current detection tools for Leishmania infection

Various tools based on parasitology, immunology, molecular and cellular biology have been studied and evaluated in order to detect *Leishmania* infection. Diagnosis may be based on direct demonstration of amastigotes by microscopic examination; indirect demonstration by in vivo or in vitro cultures (Schnur et al., 1987); or indirect detection of amastigotes in biopsies using parasite DNA-based techniques, such as polymerase chain reaction (PCR) (Smyth et al., 1992; Ravel et al., 1995; Howard et al., 1991). Parasite observations have a 100% specificity, but their sensitivity depends on the level of parasites in the blood stream. The samples taken are blood, bone marrow, liver, lymph nodes, and spleen, which requires invasive methods. It is estimated that direct parasite detection has an 80% sensitivity in symptomatic dogs, but is lower in asymptomatic dogs. PCR-based assays are among the most successful detection tools, showing a sensitivity range of 94-100% (Quinnell et al., 2013). Among the molecular screening methods, PCR can detect infection before seroconversion (Quinnell et al., 2001; Oliva et al., 2006), but it is important to follow this test up with PCR-positive dogs, so as to monitor seroconversion during the course of *L. infantum* infection. In the same study, a few dogs became PCR-negative for several months before the infection recrudesced and progressed, suggesting that PCR-positive dogs should be monitored throughout the serological testing process (see below). Cultures and microscope analyses are commonly used in research laboratories; however, they require technical skills and specific material, and are therefore less suitable in field settings. For example, PCR requires invasive sample collection, nucleic acid extraction, amplification by a thermocycler, and gel-based analysis. Arguably, these processes have led to a lengthy delay between the sampling, diagnosis and application of the control program (Quinnell et al., 2013). Indirect detection based on immune assays has been developed to make detection strategies more tenable for surveillance programs. Cell-based assays include *Leishmania* skin tests, such as delayed type hypersensitivity (DTH) response and *ex vivo* assays measuring secretion of cytokines in blood (Carrillo et al., 2009; Martinez-Orellana et al., 2017a; Martinez-Orellana et al., 2017b). Compared to cell-based assays, antibody-detection against *Leishmania* is relatively cheap, durable and more practical for screening programs. Most of these assays require only a drop of blood, making them minimally invasive. Such tests include serological analysis such as indirect fluorescent

antibody test (IFAT) (Shaw et al., 1964), direct agglutination test (DAT), (Harith et al., 1986) and the enzyme-linked immunosorbent assay (ELISA) (Hommel et al., 1978).

However, sensitivities and specificities of these tests vary substantially, within the literature, based on the laboratory, the study population, and the threshold titre chosen to define infection. ELISA and DAT have high sensitivity (78-100%), while the sensitivity of IFAT is usually lower. The specificity of serology is more variable (Romero et al., 2010). Overall, the conventional serological test lacks the sensitivity needed to detect asymptomatic dogs. Asymptomatic animals usually present low serological titres (Cardoso et al., 2007; Teixeira et al., 2010), causing borderline and discordant results in different diagnostic tests. Antigen-based immune assays have been refined to improve detection, from the use of crude *Leishmania* antigen (CLA) to the development of purified recombinant antigens. Incorporating them into field-friendly formats, known as rapid diagnosis tests (RDT), has revolutionised diagnosis and screening campaigns.

In an operational setting, a rapid diagnosis test (RDT) allows dogs to be diagnosed in situ. This RDT has several advantages. In addition to being more sensitive and specific in terms of diagnosis, it accelerates the implementation of control measures in endemic areas (only 15 minutes between the sampling and diagnosis) and does not require specialized equipment or supplies (Fraga et al., 2015). Over the last decade, several specific antigens of *Leishmania* have been characterized, allowing a recombinant-based immunoassay to be developed **(**Boarino et al., 2004).

The first RDT field kit for VL diagnosis was based on rK39 RDTs (Kalazar Detect™, InBios). rK39 is a 39 amino acid repetitive immunodominant B-cell epitope in a kinesin-related protein of *L. infantum* and *L. donovani* (Burns et al., 1993; Badaró et al., 1996). In 1996, Badaró and collaborators discovered that during the acute phase of the disease, the host (human or dog) may produce specific antibodies against replicating *Leishmania*, suggested by observing that the sera from symptomatic patients strongly recognized K39, but patients with asymptomatic or self-healing infections had low or undetectable levels of anti-rK39 antibodies. The development of rK39-based rapid diagnostic tests revolutionized the human diagnosis of VL by offering high sensitivity and specificity. In human studies, results from evaluations concluded with a high

sensitivity (95%) and specificity (90%) to detect *Leishmania* infection (Pattabhi et al., 2010). The results of rk39-based RDT in dog assays have been more variable, and it is important to note that test usage has drifted somewhat from its original intent, which was to confirm a clinical suspicion (Guan et al., 2001; Reithinger et al., 2002b). The sensitivity of the test to confirm disease in symptomatic dogs is 77%, although it is not often used to detect infected dogs. According to the evaluation of a cohort of 54 sentinel dogs, the overall sensitivity of the rK39 RDT to detect infected dogs in Brazil is 46%; and concerning the detection of infectiousness, the sensitivity of rK39 RDT is 78% in Brazilian trials (Quinnell et al., 2013). A study of Silva et al. (2014) evaluated several tools for canine VL diagnosis including direct parasitological exam, IFAT, RDT-rK39, ELISA with recombinant-rK39 (ELISA-rK39), and ELISA with soluble extract antigens (ELISA-SE). ELISA-SE was able to detect anti-*Leishmania* antibodies in the serum of the highest number of dogs (71.6%) followed by ELISA-rK39 (65.7%), IFAT (65.7%), RDT-rK39 (55.2%) and finally parasitological exam (40.3%) of positive dogs. It was suggested that RDT-rK39 be used as a complementary method in association with either ELISA-SE or IFAT, particularly in symptomatic dogs. ELISA and RDT using the recombinant antigenic protein (rK39) were the methods that detected the lowest prevalence rates (33.3%) of *Leishmania* infection in asymptomatic dogs. Overall, the sensitivity of tests mainly needs to be improved for asymptomatic dogs (Silva et al., 2014).

In terms of further advancements in diagnosis, a new synthetic polyprotein rK28 has been introduced as a diagnostic tool, named DPP®CVL. A synthetic gene, k28, was generated by fusing multiple tandem repeat sequences of the *L. donovani* haspb1 and k39 kinesin genes to the complete open reading frame of haspb2, thereby increasing antigen epitope density, while providing complementing epitopes in the resulting recombinant protein (Pattabhi et al., 2010). An important note, here, is that the sequence of haspb2 corresponds to k9, and haspb1 is k26. Both related proteins are detailed below; however, for the thesis purpose, the consistent nomenclature of k26/k9 will be used rather than haspb1/haspb2.

Current screening campaigns use antigen K28 in DPP®CVL kits from Bio-Manguinhos/Fiocruz (2005). The sample is considered positive for *Leishmania* infection if the net absorbance value is higher than the cut-off values plus the percentage

of the referent interval in the kit used, which is 20% in Bio-Manguimthos (2005). In the current screening campaigns, confirmation IFAT is used along with ELISA analysis.

While comparing the diagnostic accuracy of rK39 and rK28 in terms of detecting active human VL cases, several studies have observed similar levels of reactivity. Vaish et al. (2012) evaluated sensitivity and specificity of rK28 antigen in a micro-ELISA format in comparison to rk39 antigen in human cases. The rK28 antigen yields 99.6% sensitivity, which was similar to sensitivity of rK39 ELISA (99.6%). The specificity of rK28 antigen and rK39 antigen was 95.4% and 96.6% respectively (Vaish et al., 2012). The results suggest that rK39 and rK28 antigens have similar sensitivity and specificity, and that rK28 can also be used as a serodiagnostic tool in endemic human populations. In dog assays, the RDT based on the chimeric rK28 protein (DPP®CVL) improves diagnosis by reducing the detection of false-positive dogs and minimizing the number of false-negative dogs that are maintained in endemic areas (Fraga et al., 2015). Indeed, the sensitivity of DPP®CVL toward infection is 85.5%, and its specificity reaches 94.3%; however, the test has low sensitivity (47%) in identifying parasite-positive dogs that do not manifest clinical signs of VL (Grimaldi et al., 2012a).

Novel antigens are under development as tool for canine VL diagnostics. For instance, antigens K9 and K26 are two related hydrophilic antigens of *L. chagasi* that differ for the presence of 11 copies of a 14-amino-acid repeat in the open reading frame of K26 (Boarino et al., 2004). While rK39 and rK28 are chimeric proteins containing multiple individual genes fused into one, K9 and K26 are single hydrophilic acylated surface proteins of *L. infantum* (syn. *L. chagasi*). Specific and independent antibody reactivity to the antigens K9 and K26 have been studied and utilised in the serodiagnosis of *Leishmania* (Rosati et al., 2003). The sequences of K9 and K26 are included in recombinant proteins such as rK28 and rK34 (Pattabhi et al., 2010). Specific and independent antibody reactivity to each of the antigens, rK9 and rK26, have been studied and tested in serodiagnosis of VL infection in dogs (Rosati et al., 2003). Antigen rK34 is also a recombinant protein including LdK39, k26, protein A2 and another sequence labelled LinJ32 related to kinesin-proteins.

TR18 and rKR95 are conserved and *Leishmania* specific. TR18 is a B-cell protein containing several tandem repeats (Goto et al., 2007; Vallur et al., 2016) while rKR95 is an *L. donovani* kinesin-related protein (Vallur et al., 2016). Both were both previously identified in a bioinformatics screen and validated as being reactive with VL serum samples (Vallur et al., 2016). Genetic alignment indicates that TR18 is highly conserved across *Leishmania* species, not just in those that cause VL. The other selected antigen, rKR95, is also highly conserved across *Leishmania* species, causing both VL and CL. The high level of conservation of each protein across *Leishmania* species suggests that they serve important functions at a given life stage of the parasite. There are other studies that have sought to set up RDT by using combinations of different antigens, e.g. K26 and K39, but this has been reported to have a lower sensitivity in detecting asymptomatic dogs (Grimaldi et al., 2012a). In similar vein, a multi-epitope recombinant chimeric protein created by Boarino et al. (2005) by fusing the *L. infantum* k9 gene with single repeat units of the k39 and k26 genes (whereas rK28 contains multiple repeats of the same sequences) was evaluated for serodiagnostic of canine and human VL. The ELISA results with this fusion protein provided 96% sensitivity for canine VL with 99% specificity (Boarino et al., 2005). Finally, the antigen rK18 is construct similarly as a recombinant protein that is highly conserved in *Leishmania* (Vallur et al., 2015). The other selected antigens 6H, 8E, Lin14/2, Lin14/4, Lin11/2, and Lin 34/2 are all recombinant protein, highly conversed in *Leishmania*. All antigens were previously identified in a bioinformatic screen and validated as being reactive with VL serum samples. The currently used RDT field kit for VL diagnosis are based on rK39 for the Kalazar Detect™ Rapid Test and rK28 for the DPP® CVL.

In the thesis, recombinant antigens were selected and designed in collaboration with the Infectious Disease Research Institute (IDRI), Seattle, USA. They were all proprietary sequences derived from the *Leishmania* genome, with data suggesting their utility as antigens to detect infection and/or disease. In addition to the currently used and well-characterised rK39 and rK28, novel recombinant antigens were selected, such as rK26, rK9, rK34, rKR95, rK18, TR18, 6H, 8E, Lin14/2, Lin14/4, Lin11/2, and Lin 34/2.

Table 1.1 – List of actual *Leishmania* antigens with the related proteins, the origin species and the references where published at first.

Antigen	Related protein	Species	Reference
rK39	Kinesin-related protein	*L. infantum*	Burns et al., 1993
K9/K26 [1]	Hydrophilic acylated surface proteins	*L. infantum*	Bhatia et al., 1999
LdK39	Kinesin LdK39	*L. donovani*	Gerald et al., 2007
rK28	haspb1/LdK39/ haspb2	*L. donovani*	Pattabhi et al., 2010
rK34	LdK39/A2/ haspb2/LinJ32	*L. donovani*	Not applicable
rK18	Not given	Not given	Vallur et al., 2015
rKR95	Kinesin-related protein	*L. donovani*	Vallur et al., 2016
TR18	Kinesin-related protein	*L. donovani*	Vallur et al., 2016

[1] *K9 and K26 correspond respectively to HASPB2 and HASPB1*
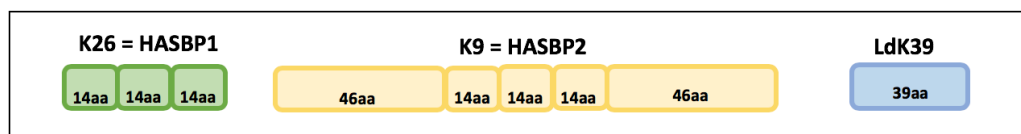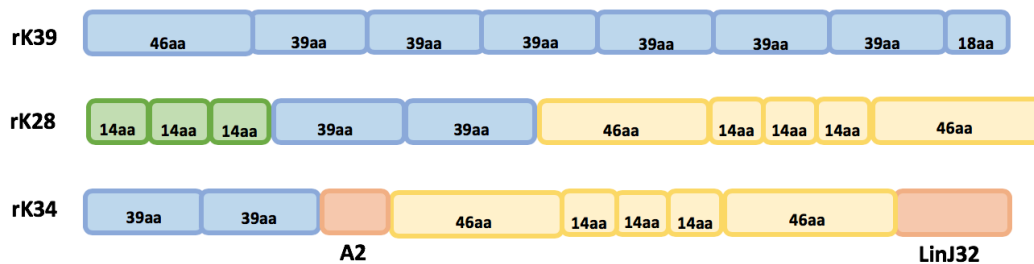
Figure 1.1 – Schematic representation of the structure of single proteins K26 and K9 and of recombinant proteins rK39, rK28 and rK34.

1.4.2 *Diagnostic tools used by the Visceral Leishmaniasis Control and Surveillance Program in Brazil*

Accurate and rapid detection of canine leishmanial infection is crucial due to its role in the transmission of infection to vectors. The Visceral Leishmaniasis Control and Surveillance Program (VLCSP) created by the Brazilian Ministry of Health recommended the setup of screening campaigns in the field to detect dogs infected by *Leishmania* that are based on several diagnostic tools. Before 2011, the dog screening was based on an ELISA screening method followed by Indirect Immunofluorescence Test (IFAT) confirmatory method (Braga et al., 1998). However, these assays were

difficult to setup in the field and offered low results. In an operational setting, a rapid diagnosis test (RDT) was developed to allow dogs to be diagnosed in situ. In addition to being more sensitive and specific in terms of diagnosis, it accelerates the implementation of control measures in endemic areas (only 15 minutes between the sampling and diagnosis) and does not require specialized equipment or supplies (Fraga et al., 2015).

Therefore, in 2012, the VLCSP recommended the use of the field-friendly Dual Path Platform (RDT®DPP) for the screening of infected dogs followed by ELISA to confirm positive results (Ministério de Saudé, 2011). In regards to diagnostic tool, kits are provided by Bio-Manguinhos/Fiocruz (2005). Fraga et al. (2015) compared the previous protocol (ELISA followed by IFAT) to the current protocol (DPP®CVL followed by ELISA), showing the improvements brought by the VLCSP by using the DPP, rather than IFAT. Indeed, the previous protocol had a prevalence of 6.2% while the current protocol had a prevalence of 8.1%. The current protocol showed improved performance with a higher specificity (97.6% versus 94.8%) and a higher positive predictive value (83.3% versus 70.2%) than the previous protocol. The sensitivity of these two protocols was, however, similar (73%), as was the negative predictive value (95%), which means that when a result is negative, it is highly probable that the dog is uninfected. When tested on asymptomatic animals, the new protocol had a much higher positive predictive value (62.5% versus 40%) than the previous protocol. This means that the current protocol provides a greater level of confidence in diagnosing positive dogs (Fraga et al., 2015). Moreover, recent studies have demonstrated that the test sequence used in the current protocol (DPP followed by ELISA) or the reverse order (ELISA followed by DPP) did not lead to a significant change in the final number of infected dogs detected (Coura-Vital et al., 2014b; Almeida et al., 2017). The probability of the two tests in sequence being positive is substantially different than either test used individually. For example, using data from Almeida et al. (2017) where sensitivities for DPP and ELISA were respectively 82.3% and 85%, the probability for both tests to be positive together is 70%, which is considerably lower. However, while specificities for DPP and ELISA reach 92.8% and 92.8% individually, the specificity for both tests used in sequence will be higher 99.5%.

The management of cases is dependent on the final serological test results using these kits. A double negative result for serology does not lead to any intervention. Meanwhile, if one result is negative while the other is positive, the result is not conclusive, and a new test must be performed on a new sample collected within 15 to 30 days after the initial collection. When dogs are positive for both test in sequence, two options are available based on the geographic location: (1) when transmission is known and confirmed in the municipality, euthanasia or treatment of dogs are recommended; (2) in municipalities with no proven transmission, the first recommendation is to perform direct parasitology to identity the species of *Leishmania* in order to inform the Visceral Leishmaniasis Control and Surveillance Program (VLCSP) and further measures include removal or treatment of the double-positive dog.

1.4.3   Improvement strategies for the detection Leishmania infection

All the diagnostic tests described above have focused on the infection. However, not all infected individuals present with clinical symptoms, nor are they infectious to sandflies. The infectiousness and the proportion of asymptomatic dogs strongly impact the success of control strategies (Costa et al., 2013). Nowadays, it is believed that the key goal is transmission, and thus infectiousness. According to Quinnell et al. (2009), the efficacy of dog culling will be increased only if those dogs that are infectious can be identified. Canid transmission must be reduced to prevent human disease. Control targeted toward infectious dogs thus requires a specific diagnostic test for infectious dogs (Courtenay et al., 2002b). At present there are no diagnostic methods that reliably distinguish between infectious and non-infectious dogs. Direct measurement of infectiousness requires xenodiagnostic tests. However, this method is logistically very difficult to set up and is expensive, making it impractical in endemic areas. An alternative could be qPCR, which would require a high cut off titre to find a specific antigen. The sensitivity of serology to detect currently infectious dogs was 96.4%–100% but was much lower in the latent period (62.5%–75.0%). Serology has also detected a high proportion of non-infectious dogs (75.8%–80.6%) and has a low specificity for infectious dogs (19.4%–24.2%). PCR was generally less sensitive than serology, whereas a high clinical score had very low sensitivity but was the most specific test for infectious dogs (91.2%). Use of combined tools did not improve the overall test performance (Courtenay et al., 2002b). Tissue parasite load is also known to have the potential to provide a reliable indirect marker of infectiousness (Travi et al.,

2001; Michalsky et al., 2007; Verçosa et al., 2008). Indeed, infectiousness to the sandfly vector is associated with high parasite numbers. A recent study of Courtenay et al. (2014) has sought to identify infectious individuals by using different tissue samples (skin and bone narrow) and different techniques, such as qPCR and ELISA. Their results suggest that the parasite load in ear skin tissue was the best predictor of being infectious. *L. infantum* amastigotes in skin tissue or skin capillaries are directly accessible to sandflies, which are known to feed abundantly on ear pinnae (Travi et al., 2001; Michalsky et al., 2007; Verçosa et al., 2008). In order to control leishmaniasis, the real focus is on transmission. Canids' transmission must be reduced in order to prevent human disease. However, there are still gaps in our scientific knowledge, including: **(1)** the dynamics of transmission; **(2)** a gold-standard for diagnosis; **(3)** detection of asymptomatic infections and symptomatic infections; **(4)** the distinction in positive serology results between natural infection and vaccine; **(5)** determinants of dog susceptibility to infection; **(6)** determinants of dog infectiousness; and finally, **(7)** efficient evaluation of diagnostic test performance.

1.5   Mathematical modelling

1.5.1   *Modelling infectious diseases*

Elementary transmission models are based on compartmental boxes, where individuals may move from one box to another according to their infection status: susceptible (S), infected (I), recovered (R), or exposed (E), giving rise to the SEIR model (Anderson et May, 1991; Keeling and Pejman, 2008). Compartmental models can be implemented to be *(1)* deterministic, defined by ordinary differential equations (ODE), or *(2)* stochastic which involves random variable for the inputs. In deterministic models (as implemented in the project), assuming that birth and death can be ignored, two parameters are essential: (1) the infection rate which is assumed to be proportional to the prevalence of the infection, and (2) the recovery rate which is often assumed to be a constant rate. From this basic model, 'extensions' can then be considered to increase the precision of the predictions and get closer to the reality. Another key concept to know the intensity of transmission is the basic reproduction number (R_0), defined as the average number of secondary cases arising from a primary case in a susceptible population. Its magnitude correlates with the difficulty of controlling the disease. R_0

can be estimated using the prevalence or the incidence of infection and reservoir life expectancy.

1.5.2 Modelling interventions on visceral leishmaniasis

Mathematical models developed for VL are usually based on the prediction human and canine infection and the understanding the interaction and role with the sandfly vector; or used to evaluate the impact of interventions, such as (1) human diagnostic, treatment and vaccine, (2) control of vectors with insecticides, and (3) dog diagnostic, treatment, culling and vaccination. These models are usually separated based on geographic areas (e.g. Brazil, Africa and Indian Subcontinent) due to the region-specific diversity of *Leishmania*. For example, mathematic models are necessary to evaluate the accuracy of diagnostic tests, as most interventions are depending on them. On humans, the main tools are PCR, ELISA, DAT, LST and RDT based on rK39. The presence of parasites, antigens or antibodies can thus be evaluated. Clinical symptoms and mortality are also considered in these models. They aimed to compare the effectiveness and more specifically the cost-effective relation of the different diagnostic strategies applied in each country. This is also realised for treatment and vaccine strategies. The following section (1.5.3) describes the most recent models of ZVL in canine population, which will be used in Chapter 7 of this thesis. Mathematical models of VL on either humans or animals are considered central to improving the efficacy of control programs. Since the infection involves multiple hosts, control measures must target various populations, as discussed previously. From a theoretical point of view, the best way to control an infectious disease is to change one of the three parameters needed for efficient transmission, namely the vector control (*p*), the proportion of bloodmeals taken from the host (*h)*, and the duration of host infectiousness in days (*d*) (Lloyd-Smith et al., 2005). The most efficient way of doing so is vector control, although this is theoretical, as vectors are difficult to control and insecticide-spreading campaigns have little impact on average transmission, as discussed previously. The blood feeding of the vector is also impossible to change. As a result, the last option is to modify host infectiousness, or at least to reduce it, as is the aim of this study, by developing a novel diagnostic test specific to infectiousness. Nevertheless, even if mathematical models include the heterogenicity for transmission; to the best of my knowledge, not a single model focuses on targeted interventions on super-spreaders. In the paper of Galvani and May (2005), the authors setted as follows: "*Control efforts should aim to identify the highly*

infectious super-spreaders and target interventions at them, {...} even if identifying them before they transmit the infection is easier said than done".

1.5.3 Modelling super-spreaders in other infectious diseases

Super-spreading events should be involved in modelling ZVL transmission. Lloyd-Smith et al. (2005) defined a super-spreader as any infected dog that infects more than $Z^{(n)}$ *others*, where $Z^{(n)}$ is the nth percentile of the Poisson (R) distribution with mean effective reproductive number (R) estimated. Currently, there are no applied models on interventions including super-spreaders in ZVL. Super-spreading events were mathematically modelled for other infectious disease with control settings. Targeting super-spreaders for therapeutic or preventive measures is assumed to increase the efficacy of interventions (Woolhouse et al., 1997; Lloyd-Smith et al., 2005) while failure to target these weakens efforts to achieve herd immunity by vaccination and also severely limits the ability to reduce disease at the population level (Baggaley et al., 2006). For example, a preliminary model of the vaccination of super-spreaders in Influenza virus suggested that this approach would offer a certain benefit (Skene et al., 2014). The authors suggest that targeted vaccination of super-spreaders is more effective to reducing the epidemic size and total cost, and achieves it with fewer vaccine doses than a mass strategy (Skene et al., 2014). Similarly, for helminthic diseases, stochastic models predict that burdens are inversely related to the intensity of the host's immune response; and consequently, it affects interventions such as vaccines, mass or selective chemotherapy (Anderson and Medley, 1985; Galvani, 2003). Concerning the West Nile virus, the heterogenicity in mosquitos biting has been widely demonstrated where birds are hosts of the virus. Recent studies suggested the American Robins (*Turdus migratorius*) have the key role of super-spreaders in the transmission of the virus in America (Kilpatrick et al., 2006). Stress hormones alter the vector's behaviour (such as feeding preferences, success and productivity) but predicts a host super-spreader phenotype (Gervasi et al., 2016; Gervasi et al., 2017). Finally, a retrospective analysis of control interventions in MERS (middle east respiratory syndrome) incorporated and explored the role of super-spreaders in a dynamic compartmental model (Lee et al., 2016). Results suggest that the outbreak duration and size were positively correlated with the number of secondary cases stopping from the super-spreaders. When the control measures containing infections from super-spreaders were implemented, the outbreak duration and size were remarkably reduced. Therefore, focusing in VL canine super-spreaders could offer a better control strategy.

1.5.4   Current models of ZVL in canine population

Understanding the epidemiology of ZVL in dogs is essential to control infection in both humans and canids (Quinnell et al., 2009). Values of R_0 for ZVL infection varies greatly among studies, since previous estimates have led to a large range of 1.44 to 11, mainly due to the poor performance of available diagnostic tests. In Malta and Brazil, it has been estimated that R_0 had values of 11 and 9 respectively (Dye et al. 1992; Dye et al. 1993; Quinnell et al., 1997; Courtenay et al., 2002b; Oliva et al., 2006), suggesting that ZVL will be difficult to control, and requires a minimum reduction of 89% of transmission to be eliminated, whereas others studies have produced much lower R_0 values (Amela et al., 1995; Zaffaroni et al., 1999). Accurate estimates require the use of sensitive methods to detect infection, as well as detailed longitudinal studies (Dye et al., 1992; Hasibeder et al., 1992). In deterministic model of ZVL, the variable R is assumed to be equal to zero as the disease is fatal for dogs. Nevertheless, models have made a number of simplified assumptions. For instance, transmission heterogeneity and infectiousness are not sufficiently taken into consideration. In addition to the vectorial capacity involving parameters of the vector such as species, density, longevity, blood intake, and infective period, ZVL is a multiple-host infection, as is the case with many other vector-borne diseases. Incorporating the contribution of the dog population into the model is a challenge, requiring an increase in the interactions and status of each member. Indeed, the clinical features of infected dogs (symptomatic or asymptomatic) will impact the results when they include innate resistance population, infectiousness capacity, latent period, etc. The impact of intervention programs such as vector control by insecticides or canine removal strategies serve to further complicate the model.

Costa et al. (2013) have offered the most complex model for canine VL. In addition to the basic SEIR model (susceptible (*S*), infected (*I*), recovered (*R*) or exposed (*E*)), this model includes infectiousness, asymptomatic dogs, and the imperfect diagnosis of dogs which is the main aim of this project. To begin with, all dogs are susceptible from birth. But once infected and having passed through the incubation period, only some will develop symptoms. About 30% of infected dogs remain asymptomatic (Sundar et al., 2006b). When applying the diagnostic tools to screen dogs, the parameters include the rate (*r*) with sensitivity (*d*), and correctly diagnosed symptomatic dogs (D_S) and

asymptomatic infection dogs (D_A). Due to the lack of specificity (d_Z), non-infected dogs are incorrectly diagnosed (D_Z). In the bellow figure, the green ellipse shows all dogs categorised as seropositive using the current diagnostic tools. All red boxes correspond to infectious dogs, although asymptomatic dogs are around three-fold less infective to sandflies as those which are symptomatic. The study concludes that the previous models without asymptomatic dogs undoubtedly over-estimate the success of interventions dependent on diagnostic tools (e.g. culling), as they have negative impact on the results. Moreover, the authors conclude that diagnostics need to be improved with high sensitivity identify asymptomatic reservoir and high specificity to reduce the numbers of false positives (and thus, the unnecessary culling of dogs). Modelling will identify the most efficacious test interpretations as criteria for local interventions. Adopting threshold-based diagnostic tests will increase specificity further, thereby increasing dog owner compliance and reducing transmission rates with greater efficacy than is possible with current policy protocols.
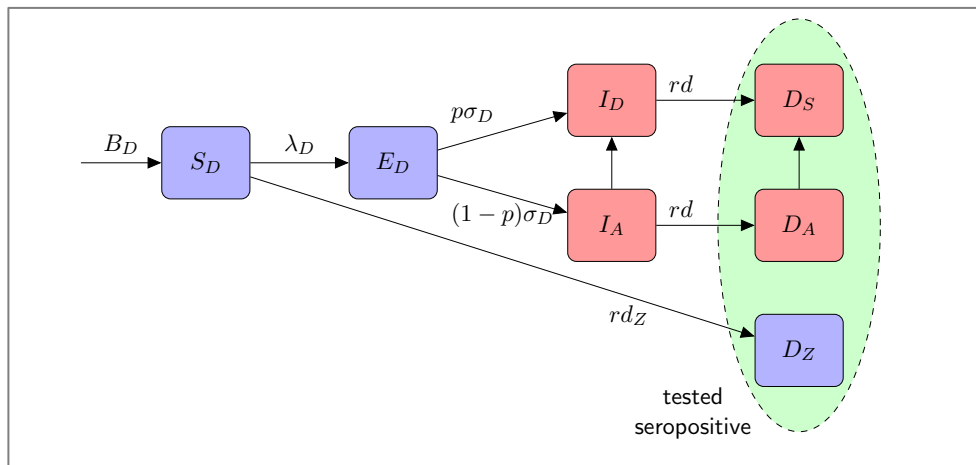
Figure 1.2 – Schematic representation by Rock et al. (2016) of the mathematical model developed in Costa et al. (2013) including canine VL including infectiousness, clinical status of dogs, and current diagnostic methods for infection in the dog population.

Of course, the presence of others animal population that would be able to transmit to humans could completely change the structure of the models; however, Quinnell and Courtenay (2009) have demonstrated that only domestic dogs are reservoirs of ZVL in Brazil. The main weakness of current models is that they fit into a cross-sectional study, rather than in a longitudinal one with dynamic time point data. The next step is therefore

to create a mathematical model from a longitudinal study, and obviously include the super-spreaders.

1.6   Project summary and rational

1.6.1   Summary of the project

In order to control the transmission of leishmaniasis from zoonotic reservoirs, it is necessary to identify the proportion of the population that are infectious to the sandfly vector. Recent xenodiagnosis and quantitative molecular (qPCR) studies of cohorts of naturally infected dogs demonstrate that these super-spreader dogs contributing to over 80% transmission events have significantly higher *Leishmania* burdens in their skin than may be detected with higher specificity and earlier in the infection process by adopting specific qPCR and serum antibody diagnostic test thresholds. The present project aims to test novel antigens or/and antigen combinations to develop a more specific test to identify super-spreaders in the mixed canid population. In conjunction with mathematical modelling, the performance of these tests under varying epidemiological scenarios to reduce transmission will be explored for potential field efficacy. This project seeks to identify efficacious intervention strategies against visceral leishmaniasis in humans, where domestic dogs are the reservoir host. In many endemic regions, current policy against transmission focuses on the blanket culling of *Leishmania* seropositive dogs, and insecticide spraying against vector(s). However, there is little or no evidence that these approaches have had a significant impact on human or canine infection incidence. More specifically, the test-and-slaughter of seropositive dogs uses test antigens that have low specificity for disease and canine infectivity to the vector, i.e. transmission potential. Consequently, the mass slaughter policy is questioned on theoretical, scientific and ethical grounds, and dog-owner compliance with the national program is low.

1.6.2   General aims of the project

This project aims to test novel antigens and/or antigen combinations to develop a more specifics test to identify super-spreaders in the mixed canid population. In conjunction with mathematical modelling, the performance of these tests under varying epidemiological scenarios to reduce transmission will be explored for potential field efficacy.

The project seeks to:

(1) Develop and evaluate a prototype diagnostic assay of novel antigens and antigen combinations to improve the specificity of a potential "roll-out" serological test to target super-spreaders. Serum samples come from endemic cohort dog and fox populations that are well characterised for infectiousness to the vector and clinical and infection outcomes.

(2) Adapt the antigen test to a rapid diagnostic test platform in collaboration with IDRI (Infectious Disease Research Institute) and InBios International, in Seattle, USA.

(3) Mathematically model the performance of these tests under varying epidemiological scenarios to reduce transmission, evaluate potential field efficacy, and use these results in quantitative models to identify optimal testing regimes to impact on transmission to reduce infection. The novel data will be used to help parameterise future mathematical models to predict the potential efficacy of new antigen RDT to reduce transmission.

CHAPTER 2   Validation of canine sera collection from Brazil and cross-reactivity test for *Trypanosoma cruzi* and *Leishmania infantum*

2.1   Introduction

Samples used in the present study were collected in villages of Marajo Island in Brazil, as fully described in the Methods section of this chapter. As the sera were collected several years ago, it is essential to ensure that the samples are still reactive and performing in assays. A previous study carried out on the dog sera in 1996 and 2008 allow a comparative study to guarantee the quality and the veracity of data in the project. For the study, the reactivity of each serum was characterized in standard ELISA conditions using the crude *Leishmania* antigen (CLA). The comparative unpublished data was obtained from the previous researchers on the sera collection (Quinnell, 1996 and Carson, 2008). With the aim to verify once again the reactivity of the samples in 2017, they were tested following in ELISA using the same conditions and compared to the previously collected data. The rationale of this chapter is to validate the performance of samples within the sera collection, prior to any further assays, to ensure the quality of the research.

Moreover, multiple antigens are shared between *Leishmania infantum* and *Trypanosoma cruzi*, the two causative agents of visceral leishmaniasis (VL) and Chagas disease. Antigens common to both agents could lead to cross-reactivity within diagnostics. Serological tools such as ELISA, IFAT and Kalazar Detect™ (RDT based on antigen rK39) designed for the identification of *Leishmania* infection are also able to detect serum samples positive for *Trypanosoma cruzi* (Zanette et al., 2014; Laurenti et al., 2014). In order to develop a novel diagnostic tool for *Leishmania* infection and infectiousness (as documented in Chapters 3 and 4 respectively), the sera collection was tested for cross-reaction to guarantee their specificity for *Leishmania* antigens. Even if there have been no reports of Chagas disease reported for the area Marajo, the prevalence of the infection in others parts of Brazil reaches 15 to 50%. Therefore, diagnostic tool should be tested for cross-reactivity between Chagas disease and VL.

Leishmaniasis and Chagas disease have in common that they are both protozoan parasitic infections, vector-borne, life-threatening, endemic in Latin America and, use the dog population as a reservoir of the infection. Therefore, their discrimination in the canine population is essential to avoid misleading diagnoses and inefficient screening campaigns. To evaluate the potential cross-reactivity of the sera samples, they were assayed against Chagas' antigens. The objective of the chapter is to ensure that the samples are correct to use in the context of the project by (1) validate the reactivity of the samples, and (2) test the potential cross-reactivity of the sera.

2.2 Methods

2.1.1 Description of the sample collection

2.2.1.1 <u>Study design from Brazil field work (April 1993 – July 1995)</u>
Serum samples were selected using archived material from a prospective study. This large-scale longitudinal study was carried out from April 1993 to July 1995, and focused on dogs naturally exposed to *Leishmania* within 24 villages of the municipality of Salvaterra, Marajo Island, Para, Brazil (Quinnell et al., 1997). This area had a high incidence of canine infection (8.66×10^{-3}/day) (Quinnell et al., 1994). The study involved 126 uninfected dogs from 2 sources: 99 were young adults (generally 6–18 months old) obtained in Belem, where there is no leishmaniasis, and 27 were young (6 months old) serologically IFAT-negative animals, born in the study area (Quinnell et al., 1997). These dogs were placed in the field in 8 cohorts and served as sentinels to natural disease transmission. At every 10-week interval, sera, ear tissue and bone marrow samples were collected from each animal. Each dog was sampled for 4–13 sampling rounds during the study. From April 1993 to July 1995, 86 dogs were considered to be infected and 36 dogs were non-infected (Quinnell et al., 2001). Dogs were considered to be infected when testing positive using the following methods: detection of anti-*Leishmania* IgG by ELISA using crude leishmanial antigen (CLA); PCR on bone marrow biopsies using primers specific for kinetoplast DNA (kDNA) and ribosomal RNA (rRNA); and parasitological culture *in vitro* and *in vivo* in hamsters (Quinnell et al., 2001). The exact date of infection was unknown, due to the time lapse between the two sample collections. Rather, the date of patent infection corresponding to the date of the first

positive detection test was used during analyses. From the previously described study, dogs were examined at each time point and classified according to a semi-quantitative method, based on six clinical signs of leishmaniasis: alopecia, dermatitis, chancres, conjunctivitis, onychogryphosis, and lymphadenopathy (Quinnell et al., 2001). The total scoring of the following scale classifies the dogs as asymptomatic (scores 0–2), oligosymptomatic (3–6) and symptomatic (>7) (Courtenay et al., 2002b). Dogs were considered as long-term asymptomatic when the post infection follow-up was over 8 months and all bimonthly clinical scores were above 3.

2.2.1.2   Determination of infection status of dogs

The definition of infection is the same as for the studies performed on the same sample collection and described previously. When testing positive for serology, bone marrow PCR, and parasitological culture, dogs were considered to be infected by *Leishmania*. Similar to previous studies, the exact date of infection was unknown, as collection occurred every 10-weeks. Instead, the date of the first positive detection test was used as the reference day (Quinnell et al., 1997).

2.2.1.3   Xenodiagnosis

From the Brazilian collection, a proportion of the dogs (n=50 dogs in 185 trials) were tested for xenodiagnoses, as described by Courtenay et al. (2002b). Xenodiagnoses was used to determine the infectiousness of the dog to the sand fly vector. Sentinel dogs were placed in individual cages covered by sandfly-proof nets and exposed overnight to females of the laboratory-bred sandfly species *L. longipalpis* for a mean of 3.5 feeds per dog (with a range of 1-12 feeds). Five days after feeding, the flies were dissected and analysed by microscopy for visible parasites in the midgut. The ratio of infected flies to uninfected flies was reported for each dog at each time point. Among these, 15 dogs were male while 34 were female, and 1 was not sexed (Courtenay et al., 2002b).

2.2.1.4   Infectiousness status of dogs

Infectiousness is the potential of each dog to infect sandfly vectors which will then infect more individuals, either other dogs or humans. "Never infectious" dogs are infected by *Leishmania* parasite, but have never become infectious to the sandfly vector (n=36). For this study, the definition was set at six or more successive months of negative results for xenodiagnosis trials during the 1993-1995 study. The category

"ever infectious" regroups naturally infected Brazilian dogs that have become infectious to sandflies at some point during xenodiagnoses trials in the 1993-1995 study (n=180). These dogs were classified as highly infectious (>20% of total flies infected), mildly infectious (0-20% of flies infected), and non-infectious (no flies infected) (Courtenay et al., 2002b; Courtenay et al., 2014). Highly infectious dogs are renamed 'super-spreaders' and are the key target of this study. Another classification includes active and latent dogs. Active dogs became infected and infectious at the same time point, while latency is the period where the dog is infected but not yet infectious. This latency period can take several months, with an average of 94 days (Quinnell et al., 1994).

2.2.1.5 Sample selection from the previously collected sera

From the Brazilian collection, only archived samples with xenodiagnosis data were used as the test benchmark against the antigen candidates. A total of 257 samples from 26 dogs were selected for this study. Among these, 78 samples tested negative for infection, while 180 were positive for infection by *Leishmania*. Dogs were considered to be infected when testing positive for parasite culture, PCR, and serology (Quinnell et al., 2001). Once more, the exact date of infection was unknown, due to a possible prepatent period (Quinnell et al., 1997). Instead, the date of patent infection was used during the present analyses, this being the date of the first positive detection test. During the assays, sera from two negative control groups were tested: (1) non-endemic controls corresponding to unexposed UK dogs' samples from veterinary clinics with no history of foreign travel (n=40); and (2) endemic negative controls corresponding to non-infected Brazilian study dogs (n=36).

2.2.1.6 Storage and conversation of samples

Prior the thesis, the sera samples were stored at -80°C. During the thesis, the quality control of samples was essential. Samples were aliquoted at the start of the thesis, and used one at the time for assays to avoid samples to be thawed and refrozen several times during the thesis.

2.2.2 Enzyme-linked immunosorbent assay with CLA

In order to validate their performance, sera samples were tested in the same conditions as described by Carson et al. (2010a) to allow a comparative study. To this aim, sera

samples were tested using the crude *Leishmania* antigen (CLA). ELISA plates (96-well polystyrene microtitre plates from Maxisorp) were coated overnight at 4°C with bicarbonate buffer containing 0,1μg of CLA. Wells were washed with PBS/0.05%Tween 20. Blocking was performed for 2 hours at 37C with 2% dried milk powder in bicarbonate buffer. After washing, diluted sera samples were added. For the detection of IgG, rabbit anti-dog secondary antibody conjugated with horseradish peroxidase (HRP-IgG, Sigma-Aldrich) was added to the wells for a 1-hour incubation at 37°C. The enzyme reaction was developed with TMB substrate solution for 20 minutes. The reaction was stopped using sulfuric acid. Plates were read using an automated ELISA plate reader set at 450 nm.

2.2.3   Enzyme-linked immunosorbent assay with Chagas antigens

Trypanosome antigens used for the cross-reactivity assays were TCF2, TCF26, TCF43, ICT8.2. They are partially purified sub-fractions of the crude *Trypanosoma* antigen (CTA) on peripheral blood mononuclear cells. These antigens active during the acute phase of *T. cruzi* infection and stimulate the production of cytokines for the progression of infection. Results of the ELISA were considered valid when the negative controls had OD values of less than 0.200 and the positive controls had OD values above 0.800 (pers. comm. IDRI as they manufacture the antigens). Other papers offered the same OD values; for example, in Ferreira et al. (2001). A total of 180 samples positive for *Leishmania* infection (as determined by PCR, parasite culture and serology) were tested by enzyme-linked immunosorbent assays. Briefly, 96-well plates were coated overnight at 4°C with appropriated dilutions of the *Trypanosoma* antigens. The non-specific reactivity on the plate was blocked with 1% BSA in PBS for one hour at room temperature. After washing with PBS and 0.1% Tween 20, sera were added in a serum diluent buffer (PBS, 0.1% BSA, 0.1% Tween 20) and incubated for another 2-hours. A new washing was followed by the preparation of the bound antibody (HRP-IgG, Thermo Fisher Scientific) which was added to the wells for a one-hour incubation at room temperature. The enzyme reaction was developed with 100 μl/well of TMB substrate solution (Tetramethylbenzidine, Fisher) for 30 minutes in the dark. Finally, the reaction was stopped with sulfuric acid. Plates were read using an automated ELISA plate reader set between 450 nm. Their results were compared to the positive control based on the *Leishmania* antigen.

2.3 Results and discussion

2.3.1 Comparative study of sample performance across time

Results of ELISA with the crude antigen from 2008 and 1996 were previously compared, concluding in a correlation of 76.8% (unpublished, Figure 2.1). The yellow dot in that figure indicated an outlier that was removed from analysis of 2008 as it had lost reactivity (personal communication from Rupert Quinnell).
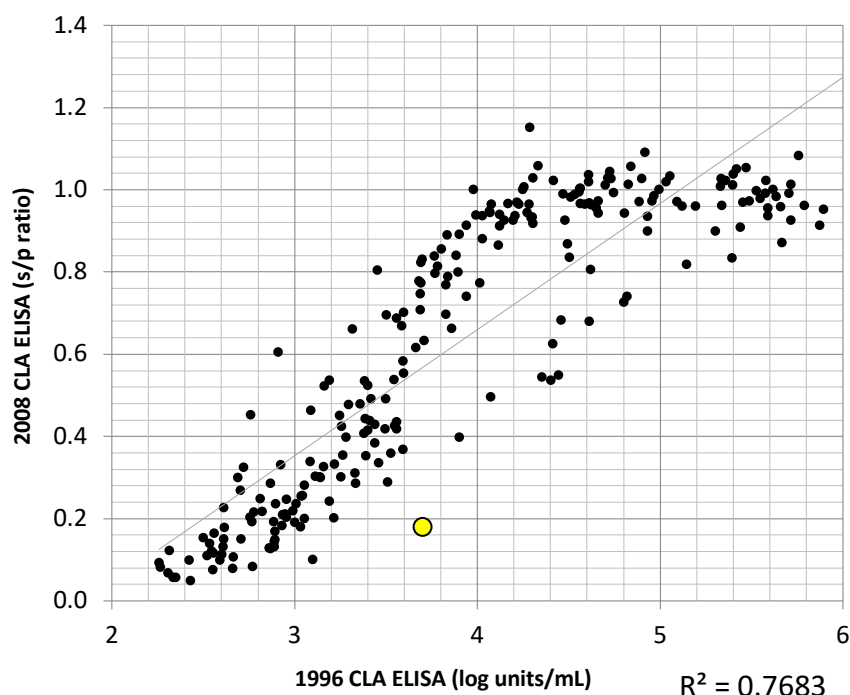
Figure 2.1 The antibodies units detected by the crude *Leishmania* antigen in 1996 and 2008. Linear correlation was reported by the R^2 (0.77).

Data obtained in 2017 using CLA and rK39 were compared to the previously documented data of 1996 and 2008. The units from 2017 was calculated based on the absorbance levels normalised into units with standard curves for each ELISA, as the total antibody content is different for dog. The correlation of CLA results from 1996 with the present study indicates a lower relationship ($R^2=0.55$), compared to the one from 2008 and 1996 ($R^2=0.77$). Therefore, another correlation was realised using the ELISA results from rK39 in 2008 and 2017. The result indicates a correlation of 66%. Despite the variation reported by the coefficient of correlation (R^2), the reactivity of the

sera samples in ELISA using the crude *Leishmania* antigen (CLA) and the recombinant antigen rK39 is sufficient to allow further assays on the sera collection.

(A)
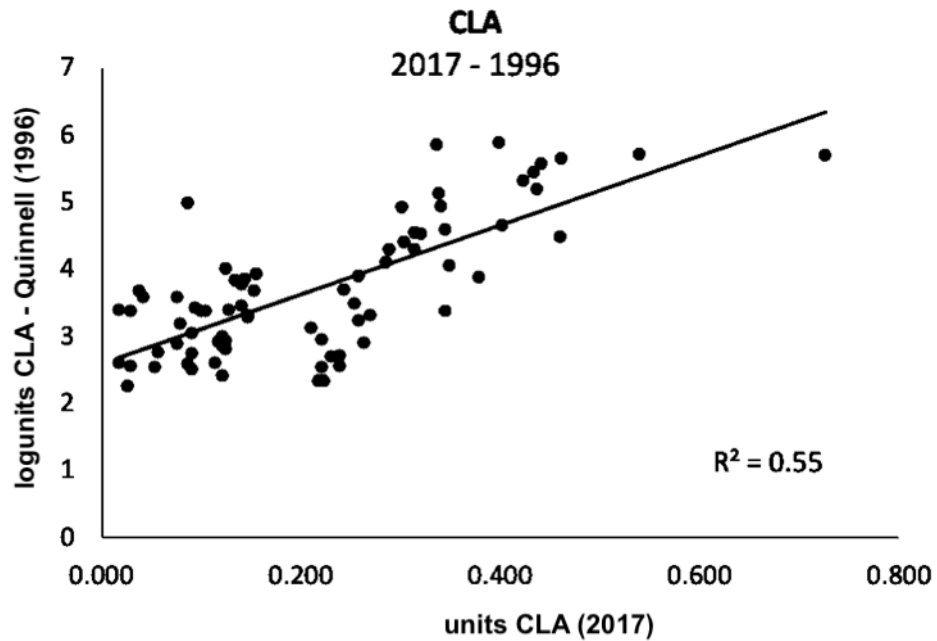
(B)
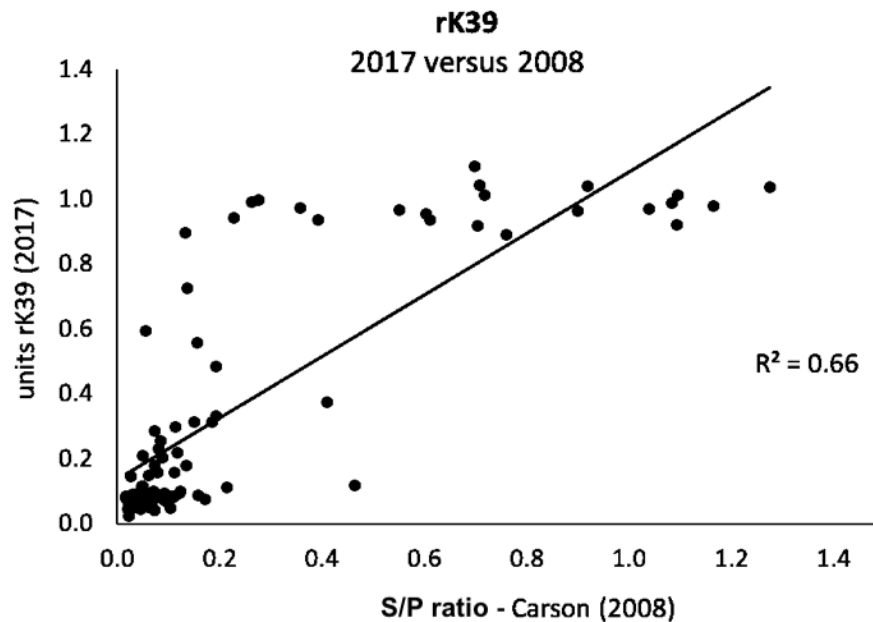
Figure 2.2 Level of antibodies units detected by the crude *Leishmania* antigen in 1996 (A) and by rK39 recombinant antigen in 2008 (B), compared to the related results in 2017. Linear correlations were reported by the coefficient R^2 (0.55 and 0.66, respectively).

2.3.2   Cross-reactivity assays of the samples against Chagas disease

Trypanosome antigens were TCF2, TCF26, TCF43, ICT8.2; all sub-fractions of the crude *Trypanosoma* antigen (CTA) on peripheral blood mononuclear cells. The results were considered positive when they had OD values above 0.800 (personal communication from IDRI as manufacturer of these antigens). Ferreira et al. (2001) offered the same OD values. The threshold was indicated by a dotted line in the below Figure (2.3A). And, indeed, some individual sera samples presented a potential cross-reactivity with *T. cruzi* antigens (2.3B), and were removed from the samples collection. Overall, the samples had no cross-reaction with the Chagas antigens, guaranteeing that the samples are only reacting to the *Leishmania* antigens. While comparing all the *Trypanosoma* antigens (TCF2, TCF26, TCF43, ICT8.2) to the positive control (based on the *Leishmania* antigen), there is a highly significant difference for all of them ($p < 0.0001$, Mann-Whitney test) as reported in Table 2.1. Similarly, the negative control based on the *Leishmania* antigen showed no significant difference compared to the Chagas antigens in multiple comparison tests (Table 2.2). There is no significant difference among the detection levels of *Trypanosoma* antigens (Table 2.2).

Table 2.1 – Results of the statistical tests using a non-parametric Mann-Whitney test, comparing the samples positive for *Leishmania* (n=180) and the results obtained for the Chagas antigens (n=180).

Positive control versus all the other groups in Mann-Whitney test	
	Positive control
TCF2	Mann-Whitney U=3618, $n_1 = n_2 = 180$, $p < 0.0001$ two-tailed
TCF26	Mann-Whitney U=1433, $n_1 = n_2 = 180$, $p < 0.0001$ two-tailed
TCF43	Mann-Whitney U=2906, $n_1 = n_2 = 180$, $p < 0.0001$ two-tailed
ICT8.2	Mann-Whitney U=3925, $n_1 = n_2 = 180$, $p < 0.0001$ two-tailed

(A) Graph using Mean+SD
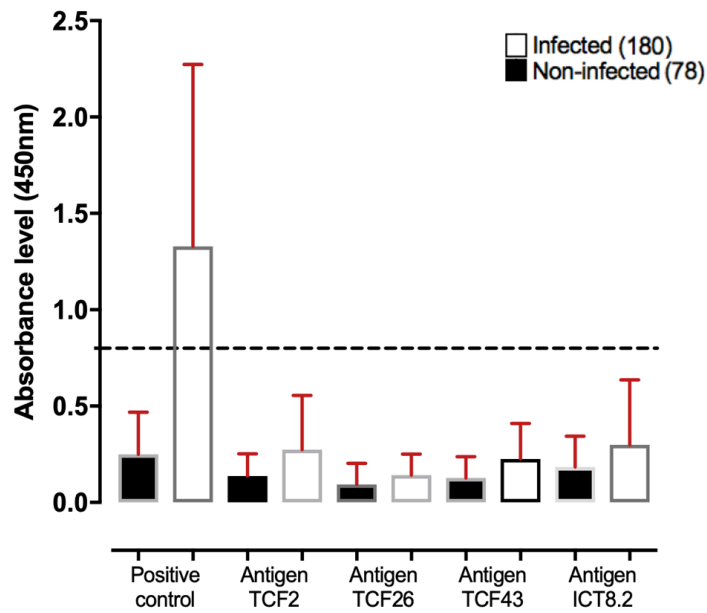
(B) Graph using Mean+SEM
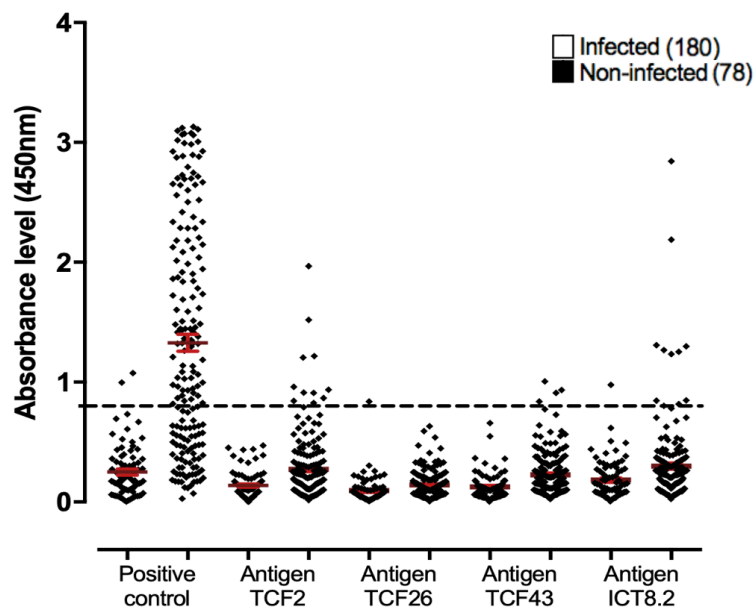
Figure 2.3 – The absorbance level detected against the Chagas antigens (TCF2, TCF26, TCF43, ICT8.2) and compared with a positive detection test (*Leishmania* antigen). The groups are composed of 180 samples positive for *Leishmania* infection and 78 samples negative for *Leishmania* infection. On each graph, the dotted line representds the threshold for the Chagas antigens. Similar, in graph (A) the mean and error bars (SEM) are indicated whereas in graph (B) the mean and the standard deviation (SD) are indicated. on each graph. For both, the statistical test used is a non-parametric Mann-Whitney test which results reported in Table 2.1.

Table 2.2 – Complete table of results of the statistical test, Tukey's multiple comparison test, comparing the mean of each ELISA result for Chagas antigen and the *Leishmania* control.

Multiple comparisons test				
	Mean difference (95%CI)		P Value	
Control Pos vs. TCF2 Neg	1.19	(1.02 to 1.36)	<0.0001	****
Control Pos vs. TCF2 Pos	1.06	(0.92 to 1.19)	<0.0001	****
Control Pos vs. TCF26 Neg	1.23	(1.06 to 1.41)	<0.0001	****
Control Pos vs. TCF26 Pos	1.19	(1.05 to 1.32)	<0.0001	****
Control Pos vs. TCF43 Neg	1.20	(1.03 to 1.38)	<0.0001	****
Control Pos vs. TCF43 Pos	1.10	(0.97 to 1.24)	<0.0001	****
Control Pos vs. ICT8.2 Neg	1.15	(0.97 to 1.32)	<0.0001	****
Control Pos vs. ICT8.2 Pos	1.03	(0.89 to 1.16)	<0.0001	****
Control Pos vs. Control Neg	1.08	(1.25 to 0.91)	<0.0001	****
Control Neg vs. TCF2 Neg	0.11	(-0.09 to 0.32)	0.7830	ns
Control Neg vs. TCF2 Pos	-0.02	(-0.20 to 0.15)	0.9999	ns
Control Neg vs. TCF26 Neg	0.16	(-0.05 to 0.36)	0.3301	ns
Control Neg vs. TCF26 Pos	0.11	(-0.07 to 0.28)	0.6349	ns
Control Neg vs. TCF43 Neg	0.12	(-0.08 to 0.33)	0.6702	ns
Control Neg vs. TCF43 Pos	0.02	(-0.15 to 0.20)	0.9999	ns
Control Neg vs. ICT8.2 Neg	0.07	(-0.14 to 0.27)	0.9909	ns
Control Neg vs. ICT8.2 Pos	-0.05	(-0.22 to 0.13)	0.9967	ns

2.4   Conclusion

This comparative study of the sample collection of their performance in ELISA across time or their cross-reactivity with Chagas disease allowed the validation of further assays on the sera collection. Indeed, no cross-reactivity could be detected, and the samples were still as active due to good conservation conditions. Based on this conclusion, these samples can be used for the next part of this project aim: to develop an improved diagnostic tool in order to limit transmission from the canid reservoir to the human population.

CHAPTER 3   Improved serodiagnosis in *Leishamania*-infected canine population: longitudinal evaluation of novel and current antigens

3.1   Introduction

Zoonotic visceral leishmaniasis (ZVL) remains a major health risk for humans and dogs in endemic regions of *Leishmania infantum* transmission. In Brazil and the Americas, the number of new human cases per year remained stable between 2001 and 2017, with an annual average of 3500 cases reported (WHO, February 2018). The recent expansion into new regions and into peri-urban habitats is particularly challenging for the Visceral Leishmaniasis Control and Surveillance Programme (VLCSP). *Leishmania infantum* is transmitted by the sandfly vector between reservoirs, where domestic dogs are known to be the main source of parasites and where humans are considered to be accidental hosts. Part of the VLCSP, the serological screening of infection in dogs is still a major prevention measure in Brazil, aiming to limit natural transmission. The subsequent culling of dogs tested positive lead to estimations that over 176,000 seropositive dogs were eliminated from 1990 to 1997 (Ministerio da Saude, Brazil, 2006) and more than 160,000 seropositive dogs from 2003 to 2005 (Lemos et al., 2008). However, there is no scientific evidence to indicate that dog culling reduces the incidence of human VL cases (Costa et al., 2008; Romero et al., 2010; Harhay et al., 2011). Indeed, such a mass slaughter policy may be questioned on theoretical and ethical grounds. Several studies have concluded that dog culling did not lead to changes in terms of human transmission. Ashford et al. (1998) measured the effect of culling seropositive dogs at 12 months intervals, reporting a temporary reduction of seroconversion in one year, but with no change in cumulative incidence after 5 years of study and no complete elimination of canine and human infection. Evans et al. (1992) concluded that there was no change in human transmission before versus after dog culling. Grimaldi et al. (2012b) found that removing seropositive dogs with active disease soon after detection may affect the cumulative incidence of seroconversion in dogs, although it is insufficient as a measure for eradicating canine infection. Thus, the ZVL control programme involving dog culling is highly controversial. Control policy in Brazil and related consequences are fully described in Chapter 1.

The lack of sensitivity and specificity of the available screening tools has been ascribed to the failure of the culling programme (Courtenay et al., 2002b; Martinez-Abad et al., 2017; de Mendonca et al., 2017). The serological diagnostic procedure currently recommended by the Brazilian Ministry of Health (Ministério da Saúde, 2006) to detect canine infection consists of a screening using the Dual-Path platform (DPP® CVL; Bio-Manguinhos/Fiocruz, Brazil) based on recombinant antigen rK28 and followed by ELISA for confirmatory. The RDT based on the chimeric rK28 protein (DPP®CVL) improves the diagnosis by reducing the number of false-negative dogs that are maintaining the infection cycle in endemic areas. Its sensitivity and specificity reach 85.5% and 94.3%, respectively, for symptomatic dogs (Fraga et al., 2016); however, DPP®CVL has low sensitivity (47%) in identifying asymptomatic individuals (Grimaldi et al., 2012a). The test performance of this method varies substantially between studies (Santarém et al., 2010; Grimaldi et al., 2012a; Fraga et al., 2016). In absence of a gold standard, studies define infection based on a different number and type of diagnostic test (serological, molecular and parasitological), and clinical sample (sera, whole blood, skin tissue, lymph node, bone marrow), each combination limited by their sensitivity and specificity. Furthermore, most studies are cross-sectional in nature which may fail to identify infected dogs in a pre-seroconversion (pre-patent) infection phase.

This study aims to compare the test performance of an enzyme-linked immunosorbent assays (ELISA) to target a large range of antigens among which rK39, rK28, rK26, rK9, rK34, rKR95, rK18, TR18, and the crude *Leishmania* antigen (CLA) to define infection on longitudinal samples of a naturally infected cohort population of dogs in Brazil (Quinnell et al., 1997). Common criteria to define threshold titres are also examined. An additional novelty of the study is that test performances were assessed longitudinally, up to 20 months after estimated time of infection confirmed by several of tests (PCR, parasite culture, and serology) characterising the infection history of each dog. The strength points of the study are (i) the large range of antigens tested, (ii) the exploration of most efficient thresholds (ii) the well-characterised dog cohort with longitudinal follow-up data (for over two years) and the serological assays which are the most suitable in operational settings. Choice of antigen and manipulation of thresholds would enable better identification of *Leishmania*-infected dogs, aimed to impact VL transmission.

3.2 Materials and methods

3.2.1 Original study collection of dog sera from Brazil

This study used archived dog sera samples generated by longitudinal sampling of naturally infected dogs from 24 endemic villages in the municipality of Salvaterra, Marajo Island, Para, Brazil, where the incidence of canine infection was 8.66x10-3/day. The complete study design has been described in Quinnell et al. (1997). The study involved 126 uninfected dogs from 2 sources: 99 were young adults (generally 6-18 months old) obtained in Belem, where there is no leishmaniasis, and 27 were young (6 months old), serologically IFAT-negative animals born and recruited in the study area. These dogs were placed in the field in 8 cohorts, and served as sentinels to natural transmission. At approximately 10-week intervals between April 1993 and July 1995, sera, ear tissue and bone marrow samples were collected from each animal and clinical signs recorded.

3.2.2 Sample selection

From the full set of 768 archived samples from 125 dogs collected in the original study, samples used in the current study were selected to meet the following inclusion criteria: (i) at least 100µl of sera was available to enable multiple assays; (ii) dogs with confirmed infection data available, and (iii) with longitudinal data available for a minimum of 6 months post recruitment. Based on these criteria, 293 samples from 26 dogs were selected for testing; among which 113 were negative and 180 were confirmed positive for *Leishmania* infection. A table reporting the details for each dog and each time point can be found as supplementary data.

3.2.3 Storage and conversation of samples

Prior the thesis, the sera samples were stored at -80°C. During the thesis, the quality control of samples was essential. Samples were aliquoted at the start of the thesis, and used one at the time for assays to avoid samples to be thawed and refrozen several times during the thesis.

3.2.4 Control group

Control uninfected dogs were composed of healthy sentinel dogs from Brazil (n=36 dogs) (Quinnell et al., 1997) and UK dogs with no history of foreign travel collected

from 3 collaborative UK veterinary clinics with owner consent (n=40). All dogs were shown to be negative for the presence for *Leishmania* parasites in culture, by PCR of bone marrow and ear biopsies and for antibody detection in blood. Moreover, the level of anti-*Leishmania* antibodies in sera samples of the endemic controls had to be under the threshold value to be considered to be negative.

3.2.5 *Definition of confirmed infection*

There is no gold standard test of infection for canine leishmaniasis. Here, dogs were considered infected on first detection of *Leishmania* presence by the following diagnostic methods: parasite culture of bone marrow aspirates and PCR of bone marrow and ear biopsies (Quinnell et al., 2001). Clinical signs were not sufficiently specific to be a reliable marker of infection. The exact date of infection is unknown, due to a possible prepatent period (Quinnell et al., 1997). Rather, the date of patent infection was used during analyses, which was the date of the first positive detection test.

3.2.6 *Leishmanial antigens*

The leishmanial antigens tested comprised of recombinant antigens K26, rK34, rKR95, K9, rK18, and TR18, being proprietary sequences derived from the *Leishmania* genome selected in collaboration with the Infectious Disease Research Institute (Seattle, USA). Their performance to detect infection were compared to rK39 and rK28 which is used for the screening campaigns (Ministerio da Saude, 2006). The candidates are fully described in Chapter 1. Antigen rK39, as used in Kalazar Detect™ Rapid Test, is a 39 amino acid repetitive immunodominant B-cell epitope in a kinesin-related protein highly conserved in different strains of *Leishmania* (*L. infantum, L. donovani* and *L. chagasi*) (Burns et al., 1993). Proteins K9 and K26 are two related hydrophilic antigens of *L. chagasi* that differ for the presence of 11 copies of a 14 amino acid repeat in the open reading frame of K26 (Bhatia et al., 1999; Boarino et al., 2004). The sequence of K9 and K26 are included in recombinant proteins such as rK28 and rK34. Antigen rK28, used in the DPP®CVL, was synthetically generated by fusing multiple tandem repeat sequences of *L. donovani* k26, LdK39 and k9 providing complementing epitopes and increasing the density (Pattabhi et al., 2010). Antigen rK34 is also a recombinant protein including LdK39, k9, A2 and kinesin-related sequence labelled LinJ32. TR18 is a B-cell related protein containing several tandem repeat proteins (Goto et al., 2007), while rKR95 is an *L. donovani* kinesin-related protein (Vallur et al., 2016). Neither

related protein, nor the origin of rK18, were described in detail by the authors, who are however mentioning that the sequence is highly conserved in the genome among the *Leishmania* species (Vallur et al., 2015). The crude *Leishmania* antigen (CLA) made from whole promastigotes or their soluble extracts was produced as described in Stober et al. (2005). Freeze-thawed CLA was prepared from stationary phase promastigotes of *Leishmania infantum* by resuspension in 10 mM Tris-HCl (pH 8.5), 0.5 M NaCl, 1 mM PMSF, and 50 μg/ml leupeptin, with three freeze-thaw cycles over liquid nitrogen (Stober et al., 2005).

3.2.7 *Enzyme-linked immunosorbent assay*

An enzyme-linked immunosorbent assay (ELISA) was used to compare the performance of current and novel *Leishmania* antigens following the protocol below. Initially, each antigen was tested independently. Based on the results of the single antigen testing, combinations of antigens were also tested in order to increase the performance of the test. The best performing antigens were selected to be combined afterwards. *Leishmania* antigens were titrated (25, 50, 100, 200 ng/well) with different dilutions of positive and negative sera (1:100, 1:200, 1:400, 1:800) on 96-well Linbro plates to determine the optimized ELISA conditions. For each assay, the 96-well Linbro plates were coated overnight at 4°C with 1μg/ml (100 ng/well) of the antigens diluted in bicarbonate buffer (0.05 M), except the rKR95, which was diluted in a phosphate-buffered saline solution (PBS). The plate was blocked with 1% BSA in phosphate-buffered saline (PBS) pH 7.2, 0.1% Tween 20 for 2 hours at room temperature to prevent non-specific reactivity. After washing (PBS, 0.1% Tween 20), 50 μl of diluted sera (at 1:400) in diluent buffer (0.1% BSA in phosphate buffered saline pH 7.2, 0.1% Tween 20) were added to the antigen wells and incubated at room temperature for 2 hours. The plates were washed and the bound antibody HRP-IgG at dilution 1:30,000 (Thermo Fisher Scientific) was added to incubate at room temperature for one hour. The enzyme reaction was developed with 100μl/well of TMB substrate solution (Tetramethylbenzidine, Fisher) for 15 minutes. The reaction was stopped using 50 μl/well of sulfuric acid. Plates were read using an automated ELISA plate reader (Wallac Victor2, Perkin Elmer) between 405-410 nm.

3.2.8 Threshold determination

Test-specific threshold values were based on non-infected dogs (n=113 samples). The thresholds determination was based on several methods to define the optimal threshold values to adopt. Conventional thresholds included common practices such as the Mean+2SD or the Mean+3SD. The receiver-operator characteristic (ROC) curves as described by Hanley and McNeil (1982) provided a range of possible threshold values; the threshold value selected represented that with maximised sensitivity and specificity. Finally, a method described by Ruopp et al. (2008) provides a Youden's J Index that summarizes the ROC data, classifying the diagnostic test performance on a scale between -1 and 1. A value of 1 indicates 100% sensitivity and specificity i.e. the perfect test. Post selection of the thresholds, the sensitivity and specificity of tested antigens were calculated.

3.2.9 Statistical analysis

All analyses were carried out using GraphPad Prism 7 and Stata 14.5 (Stata Corporation, College Station, Texas, USA). A comparison of values of optical densities for IgG was performed using the Krustal-Wallis H test with Stata and Mann-Whitney with GraphPad Prism 7 software. The data obtained from ELISA were used to determine receiver-operator characteristic (ROC) curves as described by Hanley and McNeil (1982) that that plot the true-positive rate against the false positive rate. To create these ROC curves, the control group was composed of 113 uninfected samples from Brazil, and the positive group was composed of 180 infected samples from the same Brazilian cohort. Results were reported with the area under the curve (AUC) ranging from 0 to 1, with scores determining the performance of the test. This parameter is widely accepted for evaluating diagnostic accuracy. If the AUC value is between 1-0.9, the test is excellent; it is good between 0.9-0.8; fair between 0.8-0.7; poor between 0.7-0.6; and worthless between 0.6-0.5 (McFall and Treat, 1999; Langlotz, 2003; Tape, 2004). Thresholds were determined as defined below and reported with their related sensitivity and specificity. When comparing the sensitivity and specificity of diagnostic test using different antigen candidates, a pairwise calculation was performed using McNemar's Chi-square (χ^2). Pairwise comparisons of the test performance using different antigen candidates was performed using Pearson's correlation coefficient. Post-hoc pairwise comparisons and Tukey HSD test were performed within the ANOVA one-way analysis, on longitudinal scale.

3.3 Results

3.3.1 Cross-sectional analysis of performance of the antigens to detect infection in dog cohort

The 293 sera samples from 26 dogs were tested among which 113 were negative and 180 were positive for *Leishmania* infection (confirmed by parasite culture, qPCR, and PCR). The antigens tested in ELISA were rK39, rK28, K26, rK34, rKR95, K9, TR18, rK18 and the crude *Leishmania* antigen (CLA). The significant differences between the mean absorbance values for infected (n=180) versus uninfected (n=113) samples suggests that all candidate antigens were potentially able to distinguish infected and uninfected dogs in serum samples (Figure 4.1). However, significant differences in mean absorbance values were observed between the nine antigens (Kruskal-Wallis H test, $\chi^2(9)$=417.8 (P<0.0001). The receiver-operator characteristic (ROC) curves for the antigens were represented in Figure 3.2, offering all the performances of the test calculated for each possible threshold values (***p<0.0001). The high AUC values (>=0.90) estimated by fitting ROC curves to uninfected and confirmed infected samples (Figure 3.2) suggest that rK28, K26, rK34 and CLA have greater higher sensitivity and specificity to distinguish the two groups.
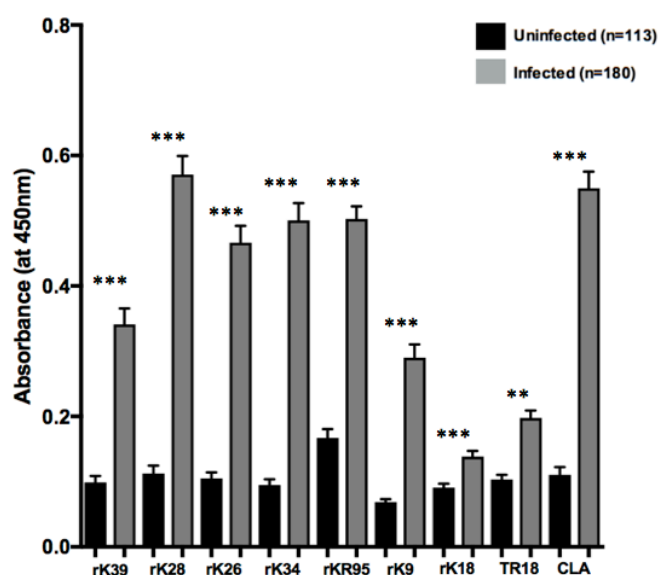
Figure 3.1 – Mean absorbance levels of antibodies detected against the leishmanial antigen candidates (rK39, rK28, rK26, rK9, rK34, rKR95, rK18, TR18, and CLA) for uninfected (n=113) and infected (n=180) dogs of the Brazilian cohort. Mann-Whitney test was performed for each of the antigen (***p<0.0001)

	AUC (CI 95%)	P-value
rK39	0.78 (0.73 − 0.83)	<0.0001
rK28	0.90 (0.86 − 0.93)	<0.0001
K26	0.90 (0.86 − 0.93)	<0.0001
rK34	0.90 (0.87 − 0.94)	<0.0001
rKR95	0.88 (0.84 − 0.92)	<0.0001
K9	0.85 (0.81 − 0.89)	<0.0001
rK18	0.62 (0.55 − 0.68)	0.0006
TR18	0.71 (0.65 − 0.76)	<0.0001
CLA	0.91 (0.88 − 0.95)	<0.0001
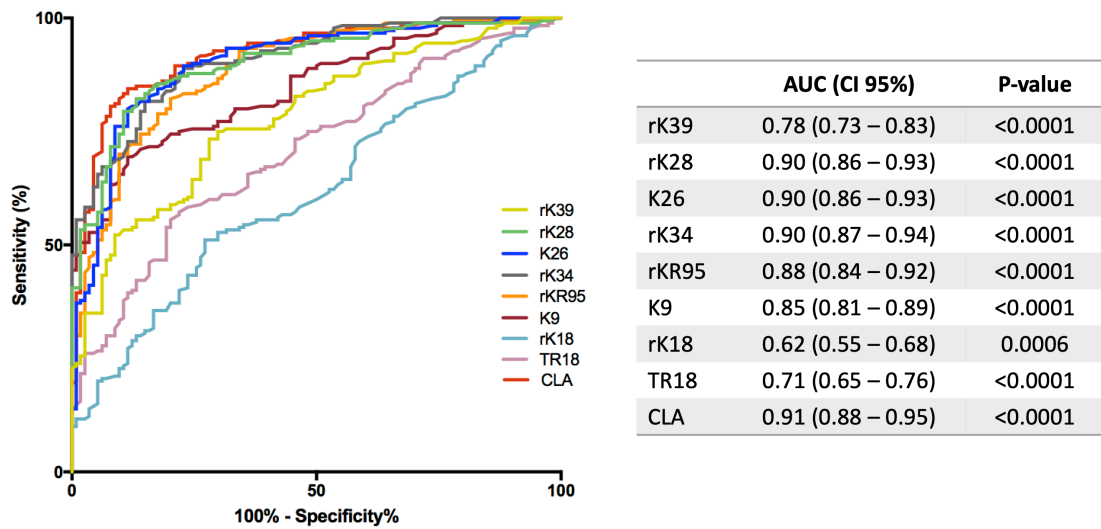
Figure 3.2 – Receiver-Operator Characteristics (ROC) curves determined for antigen candidates. The control group is composed of 113 uninfected samples from Brazil, and the positive group is composed of 180 infected samples from the same Brazilian cohort. The area under the ROC curve (AUC), the 95% confidence interval, and the p-value were reported for the different antigens.

Exploring a range of possible thresholds for each candidate aims to maximise sensitivity and specificity of the diagnosis (as described in the Methods). An illustration of the impact of the thresholds on the antigen detection were represented in the Figure 3.3. In this study, the selection of the threshold was consistent among all antigens, and based on the Youden Index calculated from the ROC curve. The Youden Index was identified to facilitate selection of the possible threshold titre combinations of sensivity and specificity generated by ROC curve analysis (Figure 3.3). Therefore, for all analyses below, the Youden Index interpretation of ROC data were applied. The identified thresholds for each antigen and resulting test performances are shown in Table 3.1 with sensitivity and specificity, false detection (positives and negatives), positive predictive values (PPV) and negative predictive values (NPV). Antigens rK28, K26, and rK34 showed highly significant performances to detect infection compared to rK39 (Table 3.1). The antigen rK28 is currently using in the Dual-Path Platform (DPP-RDT) to detect *Leishmania* infection in canine population; however, K26 offers a similar performance and is an element of rK28 construct.
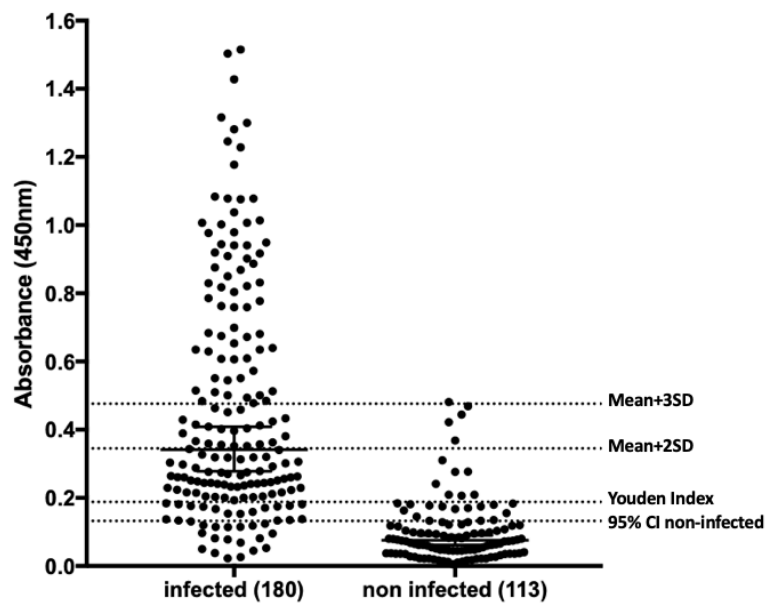
Thresholds options	Sensitivity (95% CI)	Specificity (95% CI)
0.476 (Mean+3SD)	37.8 (30.7 - 45.3)	98.2 (93.8 - 99.8)
0.354 (Mean+2SD)	48.3 (40.8 - 55.9)	94.7 (88.9 - 98.0)
0.188 (Youden Index)	80.0 (73.4 - 85.6)	88.6 (81.3 - 93.8)
0.132 (Upper 95% CI of the mean of the non-infected)	89.4 (84.0 - 93.5)	75.4 (66.5 - 83.0)

Figure 3.3 – Illustration of the impact of threshold values applied on the antigen K26 absorbance values. The median and the 95% CI was indicated separately for uninfected and infected, whereas the thresholds were applied over the complete set of absorbance. Threshold values were 0.476 for Mean+3SD; 0.354 for Mean+2SD; 0.188 for the Youden Index and 0.132 for the values indicating the upper 95% CI of the mean of non-infected dogs.

Table 3.1 – Threshold values determined for each antigen with sensitivity and specificity when applied on the test population. False positives and negatives, as well as the predictive values were reported for each antigen.

Antigen	Threshold[1]	Sensitivity (CI 95%)	Specificity (CI 95%)	False positive	False negative	PPV	NPV
rK39	0.092	75.0 (68.0 – 81.1)	70.2 (60.9 – 78.3)	29.8	25.0	71.6	73.7
rK28	0.201	82.2 (75.8 – 87.5)	86.8 (79.2 – 92.4)	13.2	17.8	86.2	83
K26	0.188	80.0 (73.4 – 85.6)	88.6 (81.3 – 93.8)	11.4	20.0	87.5	81.6
rK34	0.151	81.7 (75.2 – 87.0)	85.1 (77.2 – 91.1)	14.9	18.3	84.6	82.3
rKR95	0.218	82.2 (75.8 – 87.5)	79.8 (71.3 – 86.8)	20.2	17.8	80.3	81.8
K9	0.121	69.5 (62.1 – 76.1)	88.6 (81.3 – 93.8)	11.4	30.5	85.9	74.4
rK18	0.107	51.1 (43.6 – 58.6)	72.8 (63.7 – 80.7)	27.2	48.9	65.3	59.8
TR18	0.133	55.6 (48.0 – 62.9)	79.8 (71.3 - 86.7)	20.2	44.4	73.4	64.3
CLA	0.201	83.3 (77.1 - 88.5)	89.5 (82.3 - 94.4)	10.5	16.7	88.8	84.3

[1] based on the Youden Index

3.3.2 Longitudinal analysis of antigen detection

Analyses of the longitudinal absorbance data accounts for fluctuations in responses over time (Figure 3.4A). All dogs were aligned by the sampling round (month) of confirmed infection (time point labelled 0 in Figure 3.4A). Antibody absorbance was measured an average of 10 months prior to infection confirmation (73 samples), and for this analysis, up to 8 months post infection (144 samples). Prior to confirmed infection, antibody levels against each antigen were similar to those in the uninfected control group (Figure 3.4B). From point of confirmed infection, the longitudinal response profiles showed significant increases for all antigens ($p<0.0001$, Mann-Whitney) reaching a plateau 2 to 8 months post infection (Figure 3.4A). Possible reasons for the apparent decay in absorbance values thereafter (up to 20 months post infection, Figure 3.4A) is discussed (see below). In the Tukey HSD comparison, the absorbance levels before confirmed infection were not significantly different between antigens, except rKR95 which was significantly higher to all other antigens before the point of confirmed infection, which could lead to false positive results. When comparing antigen detection before and after the time of confirmed infection, all

antigen levels increased except rK18 (Table 3.3). Therefore, antigen rK18 is not a good diagnostic candidate for infection in dogs. When comparing the antigen curves after the point of confirmed infection (0 to 8 months post infection) (Table 3.2), antigens rK28, K26, rK34 and CLA presented similar aspect and were not significantly different from each other (p-values between 0.999 and 0.058 in Tukey HSD test). The absorbance levels after infection for antigens rK39, K9, rK18 and TR18 are significantly lower than for the other antigens (p<0.0001 Tukey HSD test; data not shown). However, among the lower performing antigens, antigens rK39 and K9 presented similar rises in detection curves (p=0.05, in Tukey HSD test).

Table 3.2 – Longitudinal evolution of antigens compared for times after confirmed infection as represented in Figure 3.4(A).

	Mean difference (95% CI)	p-value
rK28 after vs. CLA after	0.046 (0.026 - 0.118)	0.9557
K26 after vs. rK34 after	0.038 (0.110 - 0.034)	0.999
K26 after vs. rKR95 after	0.006 (0.078 - 0.066)	>0.999
K26 after vs. CLA after	0.071 (0.143 - 0.001)	0.058
rK34 after vs. rKR95 after	0.031 (0.041 - 0.104)	>0.999
rK34 after vs. CLA after	0.033 (0.106 - 0.038)	>0.999
rK39 after vs. K9 after	0.073 (0.001 - 0.145)	0.050
rK18 after vs. TR18 after	0.079 (0.151 - 0.007)	0.015

Table 3.3 – Comparison on the longitudinal evolution of antigen detection in serum, comparing time before and time after confirmed infection, for 26 naturally infected dogs using the antigens (Mann-Whitney test).

	Mean Difference (95 % CI)	Adjusted P Value	
rK39 before vs. after	0.32 (0.39 - 0.24)	****	<0.0001
rK28 before vs. after	0.55 (0.63 - 0.47)	****	<0.0001
K26 before vs. after	0.45 (0.53 - 0.38)	****	<0.0001
rK34 before vs. after	0.48 (0.56 - 0.41)	****	<0.0001
rKR95 before vs. after	0.36 (0.44 - 0.29)	****	<0.0001
K9 before vs. after	0.27 (0.35 - 0.19)	****	<0.0001
rK18 before vs. after	0.07 (0.15 - 0.00)	ns	0.0768
TR18 before vs. after	0.12 (0.20 - 0.05)	****	<0.0001
CLA before vs. after	0.52 (0.60 - 0.45)	****	<0.0001

(A) Longitudinal evolution of the mean detection of antibodies in serum for 26 naturally infected dogs using the antigens
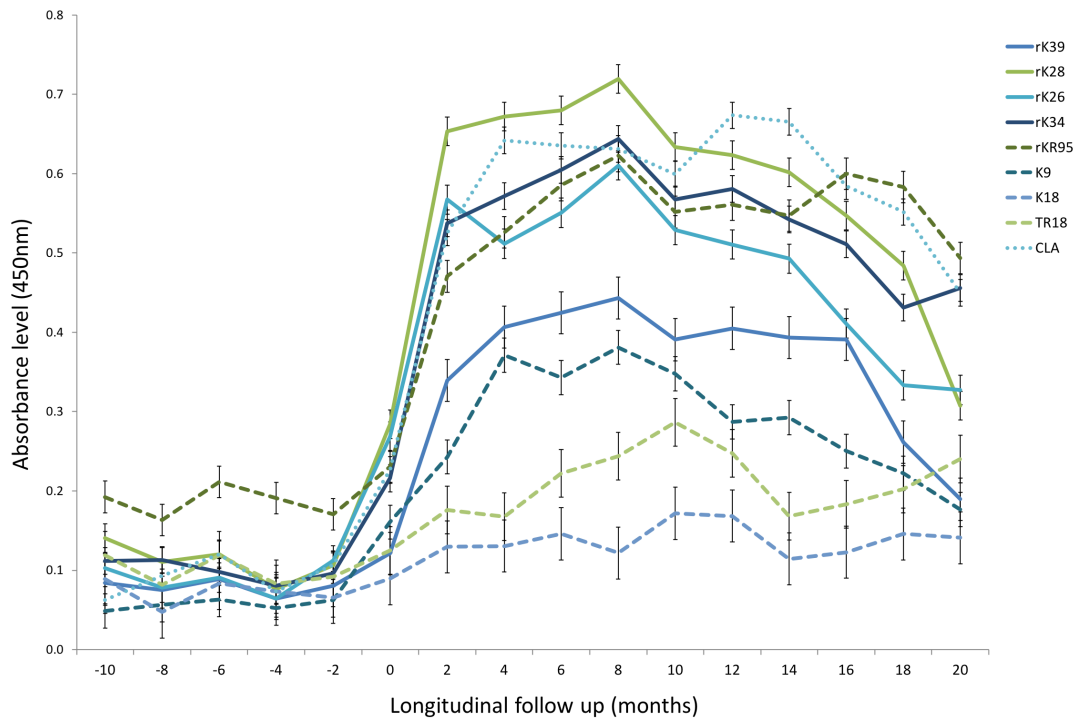
(B) Longitudinal evolution of the mean detection of antibodies in serum for endemic controls (n=36)
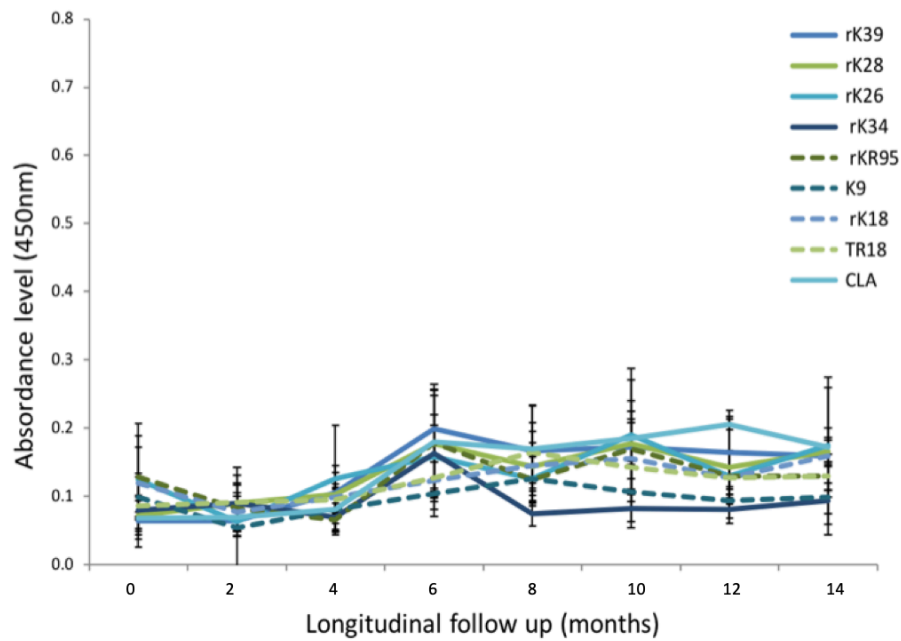
Figure 3.4 – (A) Longitudinal evolution of the mean detection of antibodies in serum for 26 naturally infected dogs using the antigens. From the point of infection, the level of antibodies could be measured up to 20 months. Negative values represent months prior to infection. (B) Longitudinal evolution of the mean detection of antibodies in serum for endemic controls (n=36) using the antigens.

3.3.3   Combined antigens to improve detection of Leishmania infection

To increase sensitivity and specificity of the detection tool, a well-known option in previous studies of diagnostic assays is to combine antigens to increase the performance and the detection rate of infected dogs. Six combinations of 4 antigens were examined: (1) K26/K28; (2) K26/K9; (3) K28/K9; (4) K26/K34; (5) K28/K34 and (6) K9/K34. These were selected from inspection of the individual antigen performances both in ROC analysis and inspection of changes in the longitudinal absorbances. The candidate KR95 was not selected for combination assays based on results for infectiousness potential, as described in Chapter 4. All combinations showed highly significant difference between infected and non-infected sera ($p < 0.0001$, Mann-Whitney test) (Figure 3.5). Thresholds were identified by ROC analysis as described previously, and reported in Table 3.4. Comparing the AUC values of these combinations, no significant superiority among the paired combinations were detected ($p = 0.03$); except for rK28/K26 which showed higher sensivity and specificity compared to the other combinations ($P < 0.0001$). The performance of combined candidates could also be analysed over time, through the longitudinal nature of the study design (Figure 3.6). In the non-parametric test (Mann-Whitney test), all of the combinations present a highly significant difference ($p < 0.0001$) in the level of absorbance detected before and after the point of infection (n°10) Combinations of antigens offer higher sensitivity and specificity than the single antigen assays, and thus improve the performance of the test. Among the combinations, the pair K28/K26 presents the highest performance rate. With the exception of the previously mentioned pair, none of the antigen pairs showed improved performance in sensitivity and specificity compared to the single antigens.
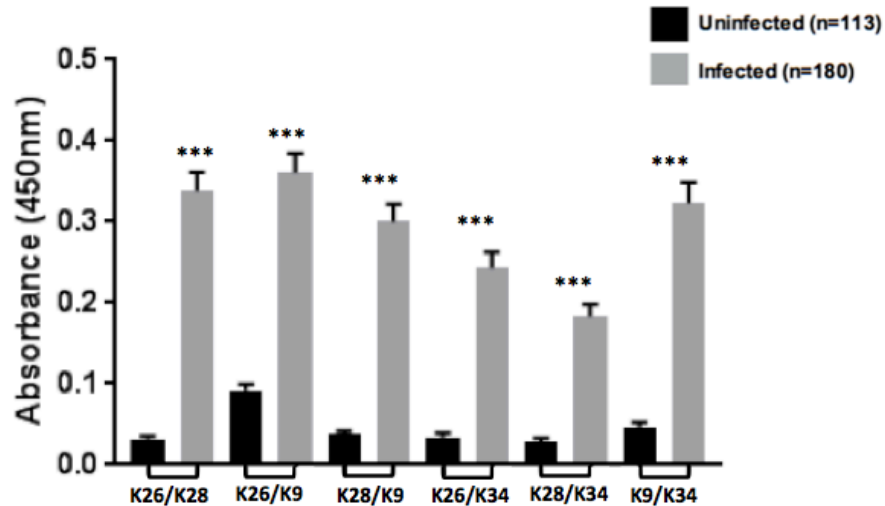
Figure 3.5 – Mean of net absorbance levels and standard errors detected for each combination of antigen candidates for the uninfected group (n=113) and the infected group (n=113) from the same Brazilian cohort.

Table 3.4 – Combined antigens analysis reporting ROC curves with AUC values (and 95% confidence interval), thresholds (based Youden Index), sensitivities and specificities (with 95% confidence interval) when applied on the population. All curves were significant ($p < 0.0001$) based the non-parametric method as described by Hanley and McNeil, 1982.

Antigens	AUC (CI 95%)	Threshold	Sensitivity (CI 95%)	Specificity (CI 95%)
rK28/K26	0.92 (0.89 – 0.95)	0.048	90.6 (85.3 – 94.4)	83.3 (75.2 – 89.7)
K26/K9	0.81 (0.76 – 0.86)	0.137	72.2 (65.1 – 78.6)	75.5 (66.5 – 83.0)
rK28/K9	0.88 (0.85 – 0.92)	0.107	67.2 (59.9 – 74.0)	97.4 (92.5 – 99.4)
K26/rK34	0.87 (0.82 – 0.91)	0.055	74.5 (67.4 – 80.6)	83.3 (75.2 – 89.7)
rK28/rK34	0.86 (0.82 – 0.90)	0.060	67.8 (60.4 – 74.5)	93.0 (86.6 – 96.9)
K9/rK34	0.84 (0.79 – 0.88)	0.103	66.7 (59.3 – 73.5)	92.1 (85.5 – 96.3)
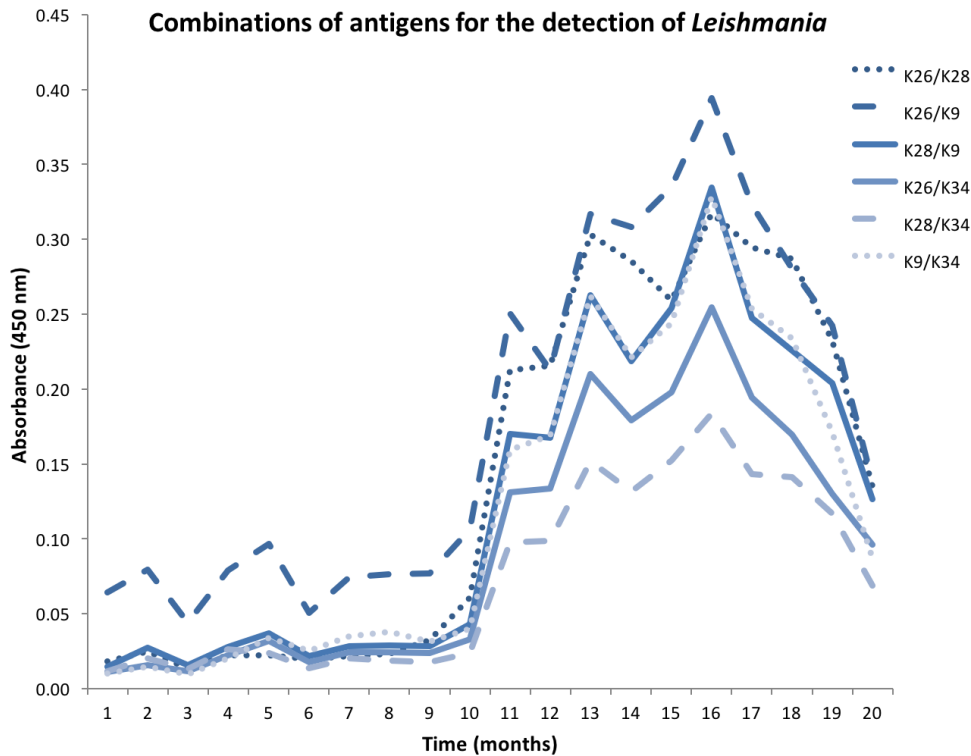
Figure 3.6 – Longitudinal analysis of the detection of antibodies in serum for naturally infected dogs (n=257 samples from 26 dogs) using combinations of antigen candidates. The point of infection is point 10. From points 1 to 9, dogs were not infected and neither were endemic controls. From time point 10, dogs were naturally infected. Measures were taken up to 20 months.

3.4   Discussion

For an equivalent sensitivity, antigens K26 and rK34 surpass antigens rK39 and rK28, with respective specificities of 89%, 89%, 70% and 87% towards detecting canine infection. Test performances were improved by applying thresholds identified using the Youden Index within the ROC curves analysis. The results demonstrate the potential of select novel antigens in their performance relative to currently available tests to detect canine infection. The Kalazar Detect™ Rapid Test based on rK39 was used for screening of canine infection (Ministério de Saùde, 2006). Previous studies of rK39 RDT and rK39 ELISA reported generally high but variable sensitivity (77% to 88%) to detect infection in the symptomatic dog population, but a lack of sensitivity (46% to 56%) to detect infection in asymptomatic dogs (parasite-positive dogs that do not manifest clinical signs of VL) (Guan et al., 2001; Reithinger et al., 2002b; Lemos et al.,

2008; Santarém et al., 2010; Quinnell et al., 2013). Since all dogs in the current study were symptomatic to varying extents, and clinical signs are not generally specific to canine *Leishmania* infection, it was not possible to differentiate test performances between these two clinical categories of dogs. But following published results, it may be that the symptomatology of the current study dogs contributed to the high-test sensitivities in this study. Nonetheless, the results here study suggest that antigens rK28, K26, rK34 have higher specificity than rK39 in reducing the number of false-positive dogs. This is important as this should increase dog owner compliance by reducing the numbers of uninfected (often asymptomatic) dogs that are likely to be culled in a test-and-slaughter control program, such as practised by the Visceral Leishmaniasis Control and Surveillance Programme in Brazil. The VLCSP currently recommends testing dogs using the Dual-Path Platform (DPP®CVL) which is based on the chimeric rK28 protein (Ministério de Saùde, 2011). This is an improvement to rK39; its overall sensitivity in the mixed population ranges from 86% to 90.6% (Grimaldi et al., 2012a; Laurenti et al., 2014; Fraga et al., 2016). The test also shows high specificity when tested on symptomatic dogs from 94% to 96% (Grimaldi et al., 2012a; Laurenti et al., 2014; Fraga et al., 2016), but poor specificity in asymptomatic dogs (47%) (Grimaldi et al., 2012a). A report from Venturin et al. (2015) compared antigens rK39 and rK28 in enzyme-linked immunosorbent assays, showing that the performance of rK28 is higher than rK39 in detecting infected dogs, even if there was no signification difference. In their study, rK39 and rK28 had sensitivities of 96.25% and 100%, and specificities of 97.6% and 100%, respectively (Venturin et al., 2015). Human trials have already shown that antigens rK39 and rK28 have similar sensitivity and specificity in detecting infection (Vaish et al., 2012).

Towards further advancement in canine infection diagnosis, two related hydrophilic antigens of *L. infantum* (K26 and K9) have been proposed for implementation (Rosati et al., 2003). Applying ROC derived thresholds, this study shows antigens K26 and rK34 and rK28 to out-performed antigens rK39 with respective specificities of 89%, 87% and 89%, versus 70% at equivalent levels of sensitivity derived from ROC analysis. Comparing the gene construct of the antigens, K26 is a component of many other proteins including antigen rK28. When comparing the performance of antigen K26 in the present study we showed similar test performance to that reported by Martinez-Abad et al., (2017) with sensitivities of 80% and 77%, and specificities of

88.6% and 91% respectively. These results implicate K26 to be a promising candidate for canine infection screening. Contrary to previous studies that consider rK28 to be more performant than K26 in detecting canine infection (Venturin et al., 2015; Martinez-Abad et al., 2017), our results show no significant differences between antigens rK28, K26, and rK34 overall, though it is clearly a better performing antigen than rK39 (Rosario et al., 2005; Venturin et al., 2015; Martinez-Abad et al., 2017). The important of the threshold determination was demonstrated here.

As this study used total IgG detection, Martinez-Abad et al. (2017) also performed assays on the different IgG subclasses. Using antigen K26 for detection, the AUC for IgG1 and IgG2 were, respectively, 0.64 (0.569-0.706) and 0.89 (0.847–0.936). The amount of IgG2 antibodies is thus higher than IgG1 in seropositive dogs. Indeed, the protective response against *Leishmania* are specifically associated with high level levels of IgG2 (de Freitas et al., 2012; Iniesta et al., 2005; Day, 2007; Reis et al., 2014; Rodriguez-Cortes et al., 2007, Laranjeira et al., 2014). Moreover, correlations between asymptomatic dogs and high levels of IgG1 were also reported by several studies (Deplazes et al., 1995; Iniesta et al., 2005; Asl et al., 2013). Indeed, Th1 responses are characterized by a predominant IgG2 antibody response associated with the control of leishmaniasis and maintenance of asymptomatic infection (Asl et al., 2013; Cruz-Chan et al., 2014). Previous studies have shown that detection of infection in asymptomatic dogs has lower sensitivity than in symptomatic dogs, as described in Chapter 1. While comparing detection of infection in symptomatic vs. asymptomatic dogs, rK39 in ELISA has high sensitivities, from 93% to 100% for symptomatic dogs, but only 53% to 65% in asymptomatic dogs (Badaro et al., 1999; Rosario et al., 2005; Mettler et al., 2005). A comparative study of enzyme-linked immunosorbent assays was done using the antigens rK39 and K26 on infected dogs demonstrated specificities of 85% and 90% (Porrozzi et al., 2007). Sensitivities were 100% and 94%, respectively, for symptomatic dogs, and both 66% for asymptomatic dogs (Porrozzi et al., 2007) supporting the need to develop more efficient diagnostic tool based on K26. The obvious correlations between symptomatic/asymptomatic dogs, IgG subclasses and detection antigens should be the next target of research to develop diagnostic tool efficient for all infected dogs.

Antigen combinations have proven to improve test performance to detect canine infection (Boarino et al., 2005; Grimaldi et al., 2012a; Martinez-Abad et al., 2017). In this study, K28/K26 was superior to all the other combined antigens, with a sensitivity of 91% and a specificity of 83%. As such, in its gene construct it represents a double-sequence of k26, which might explain the improved detection. Moreover, results indicate that the K26 antigen performed even better in combination with K9 or K28 than either single antigen alone. This supports the case for inclusion of the K28/K26 combination (and K26) as novel candidate serological tools. According to previous studies, the combination K26/K39 showed 96% sensitivity and 99% specificity (Boarino et al., 2005), though another study reported a lower sensitivity in detecting asymptomatic infections (Grimaldi et al., 2012a). More recently, a novel antigen of Sudanese *L. donovani* (rKLO8) in combination with K26 was tested, and showed improved diagnostic accuracy for canine infection (Martinez-Abad et al., 2017).

With respect to the longitudinal antibody profiles in the current study dogs, all antigen responses increased from the time of confirmed infection reaching a plateau within 6 months (Figure 3A). Comparison of the rises in antigen absorbances were analysed for this phase (from 2 to 8 months). The decay in absorbance titres for most antigens thereafter i.e. from 10 months post infection, is most likely related to the reduced number of dogs for which samples were available after 10 months post infection. Sero-recovery to negative against CLA in this cohort was rare (Quinnell 2007) and decays rates in anti-*Leishmania* titres are slow (Bhattacharyya et al., 2014). The loss of signal in the profiles observed here is more likely due to the loss of the sickest dogs of the cohort, thus those with the highest antibody titres. Moreover, as observed in the longitudinal data, there is a delay of 2 to 4 months in antibody rise post confirmed infection, depending on the antigen, which is in line with the expected pre-patent period between infection and seroconversion, previously estimated for this population as a median 94 (95% 82 – 111) days (Quinnell et al., 1997).

Previous diagnostic papers used a threshold of "Mean+2SD" as most convenient. Exploring threshold possibilities is time-consuming, but essential to reach optimal performance of tools. Whether the current test could be upgraded by adapting their threshold, this study suggested that would be an option for improvement, as for antigen rK28. However, defined antigens have higher specificity to detect infection, as

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demonstrated for K26, predictably due to the antigenicity of the molecules. Indeed, rK28 was generated by fusing multiple tandem repeat sequences including K26, K39 and K9, resulting in the recombinant protein (Pattabhi et al., 2010). The change in polymorphism, as the charge change in K26 compared to rK28, potentially affected the antigenicity. Therefore, we recommend the develop of diagnostic assays based on K26 with optimisation of thresholds.

The choice of high-performance antigens, and threshold values to define infection, enables more precise identification of infected (seropositive) dogs in the mixed canine population.VL is a health risk for both humans and canids, where canine infection maintains zoonotic transmission to humans (Quinnell & Courtenay, 2009). With the ultimate aim to impact on *Leishmania* transmission, the pursuit of cost-effective diagnostic tools also needs to consider implementation and follow-up actions with regard to identified seropositive dogs, with few currently acceptable or affordable options. Improved test specificity, as shown in this study, is paramount in winning the consent of dog owners for effective implementation of reservoir control (Courtenay et al., 2002). Furthermore, the heterogeneity in transmission potential as demonstrated by Courtenay et al. (2002b), suggest that focusing on the infectiousness for dogs is likely to improve the control program in Brazil. The second part of this project concerns the transmission potential of dogs and how this transmission potential, known as infectiousness, may be detected.

CHAPTER 4 Serodetection of infectiousness : identification of *Leishmania* super-spreaders in mixed canine population. Comparative study of current and novel antigen-based tools.

4.1 Introduction

Being an essential component of the national (albeit decentralised) leishmaniasis control program, dog culling remains highly controversial. While current intervention programs focus on eliminating VL disease as a public health problem by removal of seropositive dogs, breaking the transmission cycle requires understanding transmission dynamics and which portion of the population is most important for onward transmission (Rock et al., 2016). A published field study of Ashford et al. (1998) suggested that canine culling in Brazil led to a temporary and incomplete reduction in canine incidence; whereas other studies showed no reduction of infection rates in humans or dogs (Evans et al., 1992; Grimaldi et al., 2012b). However, published field studies tend to use more efficient detection method than the current control program applied in Brazil (Ashford et al., 1998; Evans et al., 1992; Grimaldi et al., 2012b). Likely reasons for the low efficacy are discussed in Chapter 1. In this project, novel *Leishmania* antigens were compared to the currently used antigens in order to identify potentially more sensitive and specific serodiagnostic tests for canine infection. Some of the candidates, such as K26, out-performed the antigens currently used, as demonstrated in Chapter 3. However, these diagnostic tools detect the presence of canine anti-*Leishmania* antibodies, which can be variably interpreted as current infection, previous exposure to *Leishmania*, latent infection, or residual antibody following cure. Indeed, IgG antibody levels do not decay rapidly following interruption of exposure or cure (Bhattacharyya et al., 2014). Moreover, it is established that not all infected dogs become equally infectious (Courtenay et al., 2002b; Courtenay et al., 2014). In longitudinal xenodiagnosis studies of dogs naturally infected with *L. infantum* in Brazil, Courtenay et al. (2002b) observed 42.9% of infected dogs became infectious post infection, whereas a much smaller proportion of infected dogs (17%) were considered highly infectious. The latter dogs, referred to as super-spreaders, accounted for over 80% of all transmission events (Courtenay et al., 2002b). Such heterogeneities in transmission potential is seen across many infectious diseases (Graham et al., 2007;

Clay et al., 2009; Stein, 2011; Enriquez et al., 2016; Castillo-Neyra et al., 2017; Hodo et al., 2017). As only a minority of infected dogs are super-spreaders, it is reasonable to propose that these offer a potential focal target for control. It is still unclear why certain dogs disproportionately infect secondary contacts; however, canine infectiousness is associated with high parasite loads in tissues, skin and blood, severe clinical signs of disease, and high IgG antibody titers (Courtenay et al., 2002b; Courtenay et al., 2014; da Costa-Val et al., 2007; Guarga et al., 2000; Magalhães-Junior et al., 2016; Borja et al., 2016; de Sousa Gonçalves et al. 2016). As only a minority of infected dogs are super-spreaders, it is reasonable to propose that they offer a potential focal target for control (Courtenay et al., 2014). To date, xenodiagnosis is the only method that provides conclusive data to discern infectious from non-infectious dogs. Despite these potential surrogate markers, there are no field friendly diagnostic tools to detect infectiousness. Due to the need for identifying highly infectious dogs, a newly proposed diagnostic test was developed for the specific detection of super-spreaders in a mixed canine population. Novel and current *Leishmania* antigens were evaluated in enzyme-linked immunosorbent assays on archived sera collected from naturally infected cohort of dogs in Brazil. The antigens tested included rK39 and rK28, known and used for infection screening in Brazil, (Ministério da Saúde, 2011; Almeida et al., 2017), as well as antigens K26, rK9, rK34, rKR95, rK18, TR18, 6H, 8e, Lin14/2, Lin14/4, Lin11/2 and Lin34/2. Another novelty of this study is the longitudinal analysis of antigenic responses over 20 months in relation to canine infectiousness measured by longitudinal xenodiagnosis (Quinnell et al., 1997). Importantly, these data allow estimation of the assays' ability to detect infectious classes of dogs from the point of seroconversion relative to the time of the onset of infectiousness.

4.2   Materials and methods

4.2.1   *Original study design (April 1993 to July 1995)*

Dog sera were collected during a longitudinal study from April 1993 to July 1995 in 24 endemic villages in the municipality of Salvaterra, Marajo Island, in Brazil. The complete study design has been described in the method section (Chapter 2). Briefly, the study involved 126 uninfected dogs from 2 sources: 99 were young adults (generally 6–18 months old) obtained in Belem, where there is no leishmaniasis, and 27 were young (6 months old), serologically IFAT-negative animals born in the study

area (Quinnell et al., 1997). Dogs were placed in the field and served as sentinels to natural disease transmission. At every 10-week interval, sera, ear tissue and bone marrow samples were collected from each animal. Each dog was sampled for 4 to 13 sampling rounds during the study. These samples were stored at -80°C. These samples were stored at -80°C. Despite the long storage of these samples until the current study, their continued immunoreactivity was shown as described in Chapter 2. Serial xenodiagnoses were performed on a proportion of these original cohort dogs (n=50 dogs, in 185 xenodiagnosis experiments) (Courtenay et al., 2002b) for which sera samples were available, and were the baseline against which the antigens were tested. In brief, female laboratory-bred sandflies, *L. longipalpis*, were exposed to infected dogs on a mean of 3.5 occasions per dog (range of 1–12 feeds) over 12 months (range 8.9-15.6) after being placed into the endemic study site. The sandflies were observed by microscopy for the presence of promastigotes in their midgut at 4-5 days post exposure. At any single timepoint (point xenodiagnosis), the number of infected flies of the total number of flies dissected was recorded for each dog.

4.2.2   Sample selection

From the archived 345 sera samples for 58 infected dogs with xenodiagnosis data, 145 samples from 26 dogs were used in this study. Sample selected was realised to meet the inclusion criteria: (i) the availability of sera samples in sufficient quantity (at least 100μl) for repeat assays, (ii) the quality of sera (tested prior to experiments with the crude antigen as described thereafter), (iii) the longest follow-up period after infection with an average of 6 repeat samples per dogs, (iv) accompanying xenodiagnosis data, and (v) the dog was considered infected at the time of sample. A total of 145 sera samples for 26 infected dogs were selected.

4.2.3   Definition of confirmed infection

Dogs were considered infected on first detection of *Leishmania* presence by the following diagnostic methods: parasite culture of bone marrow aspirates, and PCR of bone marrow and ear biopsies (Quinnell et al., 2001). Clinical signs were not sufficiently specific to be a reliable marker of infection. The exact date of infection is unknown, due to the prepatent period, so the date of patent infection defined above was used here in analyses.

4.2.4 Measures of infectiousness (the ability to transmit the infection)

Dogs were classified according to their infectious status at point xenodiagnosis, and longitudinally by calculating the proportion of dissected flies infected measured across all point xenodiagnosis trials on the dog. The latter measures the intensity of an individual dog's transmission potential referred to here also as the number of transmission events resulting from each dog. These classifications resulted in 3 groups of individuals: never infectious, mildly infectious, or highly infectious (syn. super-spreaders). "Never infectious" dogs were naturally infected with *Leishmania* but never became infectious to the sand fly vector (n=72 samples from 9 dogs) for six or more consecutive months of xenodiagnoses. The "ever infectious" group comprises dogs that become infectious to sandflies at some point during longitudinal xenodiagnosis follow-up (n=73 samples from 17 dogs). The "ever infectious" dogs were further classified as "highly infectious" when the proportion of infected sandflies was >= 20% (n=29 samples from 7 dogs), or as "mildly infectious" when the percentage was >0% and <20% of flies infected (n=44 samples from 10 dogs). The highly infectious group (syn. super-spreaders) was a key target for investigation as they were shown to contribute 80% of all transmission events in the larger study (Courtenay et al., 2002b).

4.2.5 Leishmania antigens candidates

The originals antigen candidates, rK39, rK28, rK26, rK9, rK34, rKR95, rK18, TR18 and CLA (used in Chapter 3), and an additional six novel antigens candidates known as 6H, 8e, Lin14/2, Lin14/4, Lin11/2 and Lin34/2 were tested for their performance to detect infectiousness and super-spreaders. The latter were identified from proprietary sequences derived from a *Leishmania* genome (Infectious Diseases Research Institute). Protein rK39 is a 39 amino acid repetitive immunodominant B-cell epitope in a kinesin-related protein highly conserved in different strains of *Leishmania* (*L. infantum, L. donovani* and *L. chagasi*) (Burns et al., 1993). K9 and K26 are two related hydrophilic antigens of *L. chagasi* that differ for the presence of 11 copies of a 14 amino acid repeat in the open reading frame of rK26 (Bhatia et al., 1999). Antigen rK28 is synthetically generated by fusing repeated sequences of *L. donovani* such as k26, LdK39 and k9 providing complementing epitopes and increasing the density (Pattabhi et al., 2010). Antigen rK34 is also a recombinant protein including LdK39, k9, A2 and kinesin-related sequence labelled LinJ32. TR18 is a B-cell related protein containing several

tandem repeat proteins (Goto et al., 2007), while rKR95 is an *L. donovani* kinesin-related protein (Vallur et al., 2016). Neither related protein, nor the origin of rK18 was given by the authors but this recombinant antigen is made of highly conserved among the *Leishmania* species (Vallur et al., 2015). The crude *Leishmania* antigen (CLA) made from whole promastigotes or their soluble extracts was produced as described in Stober et al. (2005). Freeze-thawed CLA was prepared from stationary phase promastigotes of *Leishmania infantum* by resuspension in 10 mM Tris-HCl (pH 8.5), 0.5 M NaCl, 1 mM PMSF, and 50 μg/ml leupeptin, with three freeze-thaw cycles over liquid nitrogen (Stober et al., 2005).

4.2.6   *Enzyme-linked immunosorbent assay (ELISA)*

An enzyme-linked immunosorbent assay was used to quantify *Leishmania* specific IgG antibody responses to the candidate antigens in the test samples. Initially, each antigen was tested independently. Based on the results of the single antigen testing, combinations of antigens were also tested in order to increase the performance of the test. The best performing antigens were selected to be combined afterwards. As mentioned in chapter 3, the optimisation of the ELISA conditions was realised by titrating the antigens (25, 50, 100, 200 ng/well) and different dilutions of sera (1:100, 1:200, 1:400, 1:800). For each assay, 96-well Limbro plates were coated with 1μg/ml (100 ng/well) of antigen diluted in bicarbonate buffer (0.05 M) overnight at 4°C. The non-specific reactivity on the plate was blocked with 1% BSA in phosphate-buffered saline for 2 hours at room temperature. After washing (PBS, 0.1% Tween-20), 50 μl of diluted sera at 1:400 in serum diluent buffer (PBS, 0.1% BSA, 0.1% Tween-20) was added to the antigen wells and incubated at room temperature for a further 2 hours. The plates were then washed and the diluted (1:30,000) bound antibody (HRP-IgG, Thermo Fisher Scientific) was added at room temperature and left for 1 hour. The enzyme TMB substrate solution (Tetramethylbenzidine, Fisher) was added 100μl/well for 15 minutes, and stopped using 50 μl/well of sulfuric acid. Optical absorbance values were then read using an automated ELISA plate reader (Wallace Victor2, Perkin Elmer) at 405-410 nm.

4.2.7   *Defining threshold values*

All analyses were carried out in Graph Pad Prism 7 and Stata 14.5 (Stata Corporation, College Station, Texas, USA). Test-specific threshold values were identified for each

antigen using the ELISA absorbance values to generate receiver-operator characteristic (ROC). The area under the curve (AUC) values ranged from 0 to 1; following the classification described by Tape (2004), values between 1-0.9 for an excellent test; 0.9-0.8 as "good"; 0.8-0.7 as "fair" 0.7-0.6 as "poor", and 0.6-0.5 as "worthless". The ROC analysis offered a range of possible threshold values associated with sensitivity and specificity values, from which the threshold values with concurrent maximum sensitivity and specificity to detect super-spreader dogs the mixed population were identified. Sensitivity and specificity for each of the threshold was reported according to the ROC list with the related proportion of 95% confidence interval. These were reported for the threshold list in Appendix II. The Youden's J Index, as described by Ruopp et al. (2008), was also applied to the optional ROC threshold values, providing an objective interpretation of the test's performance, whereby each potential threshold value was scored on a semi-quantitative scale between -1 and 1 where a value of 1 indicates that there are no false positives or negatives i.e. the perfect diagnostic test.

4.2.8 Statistical analyses

All analyses were carried out in Graph Pad Prism 7 and Stata 14.5 (Stata Corporation, Texas, USA). Comparison of mean absorbance values were performed using a non-parametric Mann-Whitney U test. Pairwise comparisons of the performance of the diagnostic tests was examined by inspection of Pearson's correlation coefficients. The degree of agreement of the detection with the infectiousness data was measured by Cohen's method (κ) and McNemar's Chi-square test (χ^2). Kaplan-Meier curves were generated to identify the mean time that each infectious class of dog was detected for each candidate antigen candidate. The onset of infectiousness was calculated from the introduction of the dogs in the field.

4.3 Results

4.3.1 Performance of antigens to detect infectiousness in ELISA

Ten of the fifteen candidate antigens (rK39, rK28, K26, rK34, rKR95, K9, CLA, 6H, 8e, and LinJ14) significantly differentiated non-infectious (n=72 samples) and infectious (n=73 samples) dogs at point xenodiagnosis (Figure 4.1). The antigens that failed to differentiate infectious and non-infectious dogs were dropped from further analysis. The remaining antigens were then tested to differentiate two subclasses of

infectious dogs: mildly infectious (n=44) and highly infectious (n=29). Significant differences in mean absorbance values between the two groups were observed using 7 of the antigens (rK39, rK28, K26 and rK34, 6H, 8e and LinJ14); among these, rK28 was the most significant ($p<0.0001$) (Figure 4.2). Candidates rKR95, K9, the crude antigen and LinJ14 did not demonstrate differential potential for highly and mildly infectious dogs ($p>0.05$) and were thus dropped from further analyses.
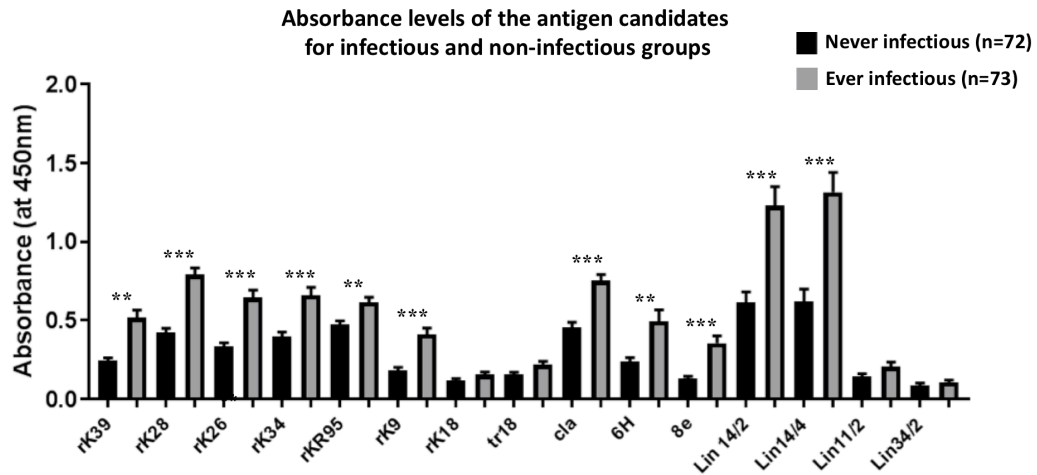
Figure 4.1 – Mean absorbance level of antibodies detected against infectious and non-infectious samples (*Leishmania* antigen) reported with the related standard error (SEM). The never infectious group is composed of 72 samples negative for xenodiagnosis while the infectious group is composed of 73 samples positive for xenodiagnosis (*$p<0.05$, **$p<0.001$, ***$p<0.0001$ Mann-Whitney test).
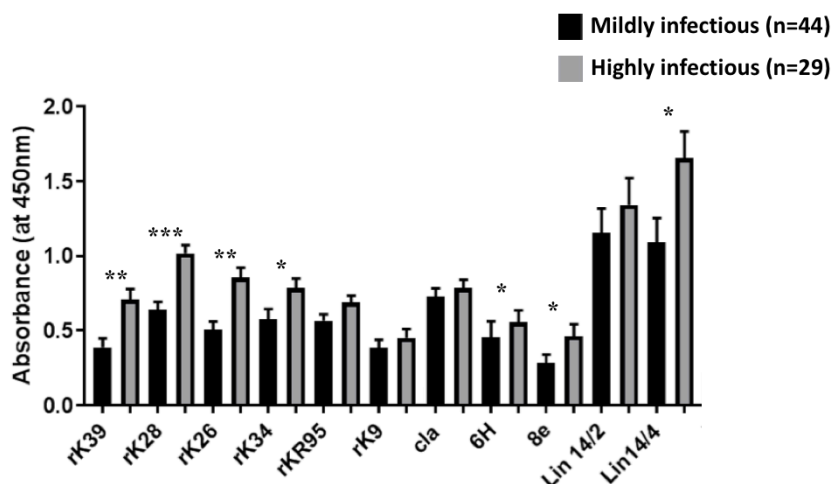
Figure 4.2 – Mean absorbance level of antibodies detected against mildly infectious and highly infectious samples reported with the related standard error (SEM). The mildly infectious group is composed of 44 samples while the highly infectious group is composed of 29 samples (*$p<0.05$, **$p<0.001$, ***$p<0.0001$ Mann-Whitney test).

Potential threshold values were explored for each antigen to differentiate super-spreaders from the rest of the infected population (results in Appendix II). The threshold method providing the greatest test performance in all cases was the defined by the Youden Index and applied throughout. From the ROC analyses, antigens rK39, rK28, and K26 were shown to have AUC values between 1 and 0.9, indicating an excellent test performance, while other candidates were shown to have lower AUC values, suggesting either a good performance (0.9–0.8) or a fair performance (0.8–0.7); the degree of agreement of the test with the infectiousness data measured by the Cohen's coefficient of agreement were also high (Table 4.1). Candidate rK28 offers the best threshold-based test performance with complete detection of the super-spreader samples (29/29) with a sensitivity of 100% (95% CI: 88.1–100). The specificity of rK28 was 93% (95%CI: 84.5-97.7) with only 5/72 (6.9%) never infectious samples detected, and 14/44 (31%) mildly infectious samples detected. Moreover, candidate rK28 showed the highest degree of agreement to the infectiousness data (93.1%) and no systematic differences between the antigen detection and the xenodiagnosis data methods. The Cohen's kappa value κ equals 0.81 suggests an almost perfect strength of agreement (Table 4.1). Pairwise comparison between antigenic performances indicated positive correlations between all of them (Pearson's correlation, p<0.0001 in each case), however, the degree of agreement with the xenodiagnostic data were lower for the other antigens than for rK28 (κ=0.65) (Table 4.2). rK39, K26, rK34 and K9 showed sensitivities of 97%, 90%, 93% and 83%, and specificities of 70%, 85, 75% and 81% respectively (Table 4.1).

	ROC curves			Detection of samples			Performance[2] (%)						Degree of agreement[3]		
	AUC (95% CI)	p-value	Threshold[1]	Never (n=72)	Mildly (n=44)	Highly (n=29)	Sensitivity (95% CI)	Specificity (95% CI)	False positive	False negative	PPV	NPV	%	κ	p-value
rK39	0.923 (0.872 – 0.975)	<0.0001	0.272	26.4% [19/72]	45.5% [20/44]	96.6% [28/29]	96.6 (82.2 – 99.9)	70.8 (59.0 – 81.0)	29.2	3.4	76.8	95.4	82.2	0.57	Z=7.54 P<0.0001
rK28	0.995 (0.987 – 1.000)	<0.0001	0.720	6.9% [5/72]	31.8% [14/44]	100% [29/29]	100 (88.1 – 100)	93.1 (84.5 – 97.7)	6.9	0	93.5	100	93.1	0.81	Z=9.96 P<0.0001
K26	0.928 (0.876 – 0.980)	<0.0001	0.531	15.3% [11/72]	34.1% [15/44]	90% [26/29]	89.7 (72.6 – 97.8)	84.7 (74.2 – 92.1)	15.3	10.3	85.4	89.2	87.0	0.65	Z=8.09 P<0.0001
rK34	0.897 (0.835 – 0.959)	<0.0001	0.522	25.0% [18/72]	40.9% [18/44]	93% [27/29]	93.1 (77.2 – 99.2)	75.0 (63.4 – 84.5)	25	6.9	78.8	91.6	81.5	0.55	Z=7.22 P<0.0001
K9	0.856 (0.778 – 0.935)	<0.0001	0.235	19.4% [14/72]	59.1% [26/44]	83% [24/29]	82.7 (64.2 – 94.2)	80.6 (69.5 – 88.9)	19.4	17.3	81.0	82.3	82.2	0.54	Z=6.77 P<0.0001
6H	0.835 (0.751 – 0.919)	<0.0001	0.223	27.8% [20/72]	50.0% [22/44]	82.7% [24/29]	82.7 (64.2 – 94.2)	72.2 (60.4 – 82.1)	27.8	17.3	74.8	80.7	77.4	0.45	Z=5.92 P<0.0001
8e	0.890 (0.823 – 0.957)	<0.0001	0.156	23.6% [17/72]	45.5% [20/44]	86% [25/29]	86.2 (68.3 – 96.1)	76.4 (64.9 – 85.6)	23.6	13.8	78.5	79.2	79.5	0.50	Z=6.47 P<0.0001
Lin14	0.888 (0.823 – 0.952)	<0.0001	0.802	23.6% [17/72]	43.2% [19/44]	90% [26/29]	89.7 (72.7 – 97.8)	76.4 (64.9 – 85.6)	23.6	10.3	84.7	88.1	81.5	0.54	Z=7.02 P<0.0001

[1] Threshold selected based on the ROC curves and Youden Index

[2] Towards detection of super-spreaders

[3] Compared to infectiousness data determined by xenodiagnosis analysis

Table 4.1 – Table summarising the ROC curves analysis, threshold selection and performance of each antigen. Receiver-Operator Characteristics (ROC) curve analysis, determined by the boundary between the control group composed of 72 never infectious samples and the positive group composed of 29 highly infectious samples from the Brazilian cohort. Thresholds were determined based on point xenodiagnosis, selected to be the best performing using ROC curves and applied to samples selected for longitudinally classified dogs within the xenodiagnoses study. The proportion with related percentage of samples detected in each group (never, mildly, and highly) by each possible cut-off value for indicating the performance of the diagnostic tool. Respective sensitivity and specificity towards detecting super-spreaders is reported with the 95% confidence interval (CI); and the degree of agreement compared to the infectiousness data was reported with the kappa coefficient (κ).

4.3.2 Times of antibody detection relative to the onset of infectiousness

The mean time of antigen detection from exposure relative to the onset of infectiousness was illustrated as Kaplan-Meier survival curves, and the estimates compared using the log rank test (Figure 4.3, Table 4.2). Antigens K28, K26 and rK34 detected super-spreaders significantly earlier than they detected mildly infectious and never infectious dogs (Figure 4.3). The mean time to detection of mildly and non-infectious dogs were not statistically different for all antigens. The P values are indicated in each graph (in green, for the comparisons between highly and never infectious; and in blue, for the comparisons between mildly and never infectious). All super-spreaders were detected before the mean of onset of infectiousness using K26 and rK34, and rK28 threshold-based assays, the latter detect super-spreaders slightly later than the other two antigens (Table 4.2). Threshold-based rK9 curves for highly and mildly infectious intersect which does not offer distinct detection. For rK39, the detection time of the never infectious is earlier than the mildly infectious, which do not offer a significant difference. The onset of infectiousness was calculated from the introduction of the dogs in the field.

Table 4.2 – Mean of detection time in days for each group of dogs (never, mildly, and highly infectious dogs) using threshold-based antigen assays and analysis by Kaplan-Meier based longitudinal analysis, compared to the onset of infectiousness determined during xenodiagnoses follow-up from the moment of introduction in the field.

Mean detection times and onset of infectiousness (in days)			
	Never infectious (n=9)	Mildly infectious (n=10)	Highly infectious (n=7)
rK39	251 (117 – 386)	282 (130 – 433)	97 (69 – 124)
rK28	488 (336 – 641)	371 (193 – 549)	83 (55 – 110)
rK26	421 (261 – 581)	278 (120 – 435)	80 (57 – 103)
rK34	320 (176 – 464)	288 (134 – 442)	80 (57 –103)
rK9	412 (251 – 574)	180 (98 – 262)	147 (96 – 198)
6H	338 (159 – 517)	257 (155 – 358)	123 (63 – 182)
8e	261 (120 – 402)	260 (136 – 384)	98 (52 – 143)
Lin14/4	439 (263 – 614)	288 (159 – 416)	107 (70 – 144)
Onset	–	408 (255 – 561)	298 (218 – 377)
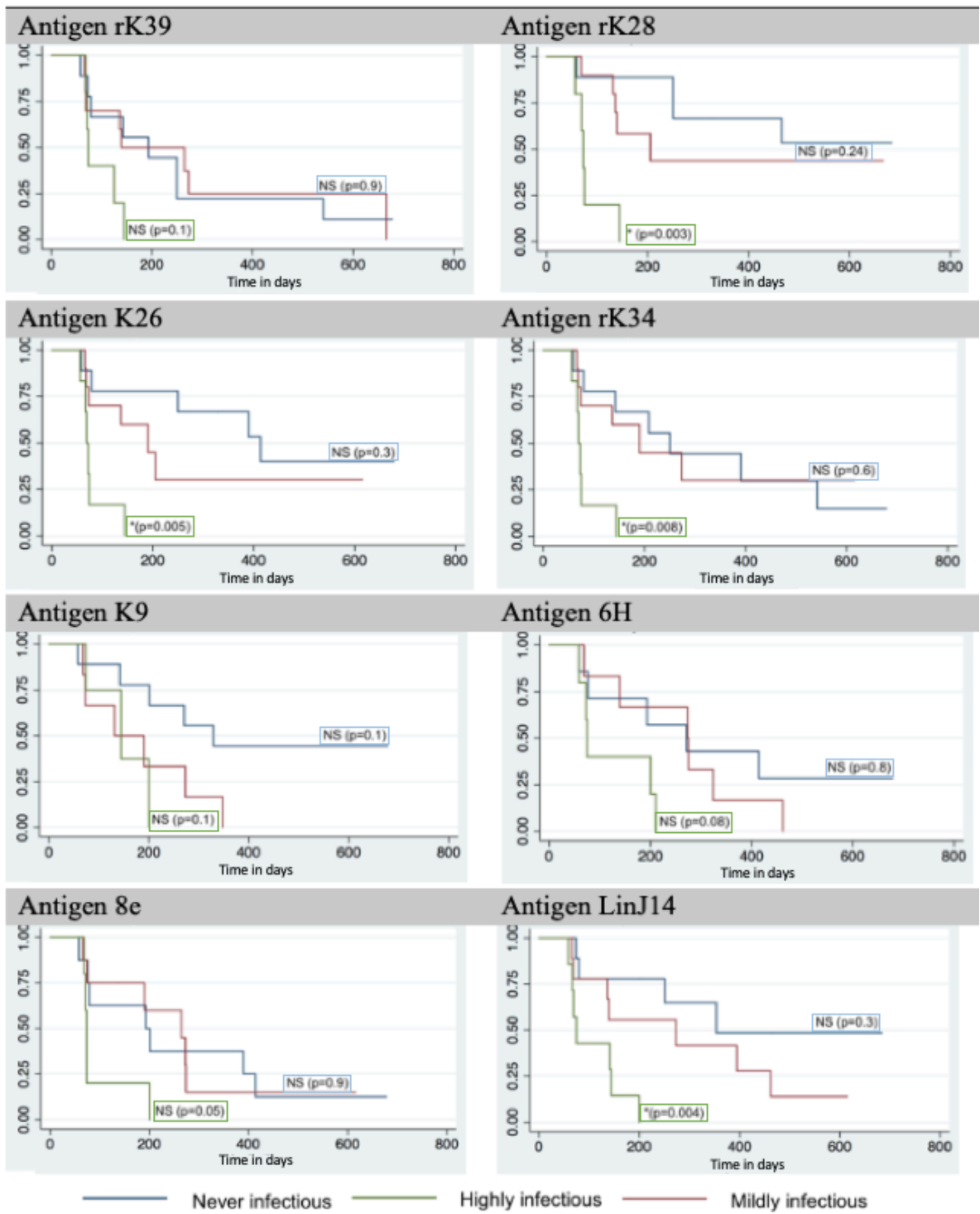
Figure 4.3 – Kaplan-Meier based longitudinal analysis of detection time of the xenodiagnoses-classified dogs by threshold-based antigen assays. Different curves and time estimates were compared using the log rank test. The P values are indicated in each graph (in green, for the comparisons between highly and never infectious; and in blue, for the comparisons between mildly and never infectious).

When the threshold-based antigen was applied to the dog population, the actual number of infected flies detected at the seroconversion time point was reported for each group of dogs, with the proportion of positive flies detected to the total positive flies reported (Table 4.3). In relation to the time of detection, the proportion of transmission events that would be avoided by removal of dogs based on detection, here estimated as the proportion of total positive flies infected by individual dogs, was 98.3% (467/475) applying antigen rK28; this included 422 transmission events by highly infectious dogs and 45 events by mildly infectious dogs. The other antigens ranged between 51% and 98% overall detection (Table 4.3).

Table 4.3 – For each threshold-based antigen, number of infected flies detected at the seroconversion time point was reported for each group of dogs. The ratio is based on the positive flies detected to the total positive flies for a group of dogs (never, mildly, and highly infectious dogs) using threshold-based antigen assays and analysis by Kaplan-Meier based longitudinal analysis.

Proportion of all transmission events detected by threshold-based antigens					
	Overall detection		Highly infectious		Mildly infectious
rK39	93.7%	(445/475)	93.8%	(396/422)	92.4%	(49/53)
rK28	98.3%	(467/475)	100.0%	(422/422)	84.9%	(45/53)
rK26	98.1%	(466/475)	100.0%	(422/422)	83.0%	(44/53)
rK34	98.5%	(468/475)	100.0%	(422/422)	86.9%	(46/53)
rK9	51.2%	(243/475)	45.7%	(193/422)	94.3%	(50/53)
6H	56.8%	(270/475)	54.0%	(228/422)	79.2%	(42/53)
8e	58.3%	(277/475)	66.7%	(235/422)	79.2%	(42/53)
Lin14/4	51.4%	(244/475)	46.2%	(195/422)	92.4%	(49/53)

4.3.3 Combining antigens

Combined antigens were examined for potential improvement relative to the single antigens. Four antigens were used in different combinations: (1) K26/rK28; (2) K26/K9; (3) rK28/K9; (4) K26/rK34; (5) rK28/rK34 and (6) K9/rK34. These were selected from inspection of the individual antigen performances and inspection of changes in the longitudinal absorbances. The equivalent ROC and Kaplan-Meier analyses showed that in each case, the antigen combinations showed a lower performance compared to the respective single antigens (as showed in the below figures and table).
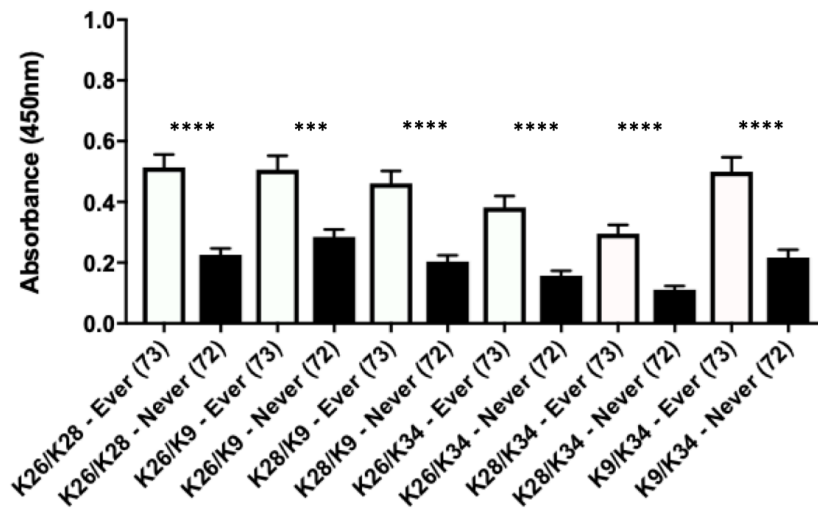
Figure 4.4 - Absorbance level of antibodies detected against ever infectious and never infectious samples (*Leishmania* antigen). The never infectious group is composed of 72 samples negative for xenodiagnosis while the infectious group is composed of 73 samples positive for xenodiagnosis. All p-values are <0.0001 (Mann-Whitney test).
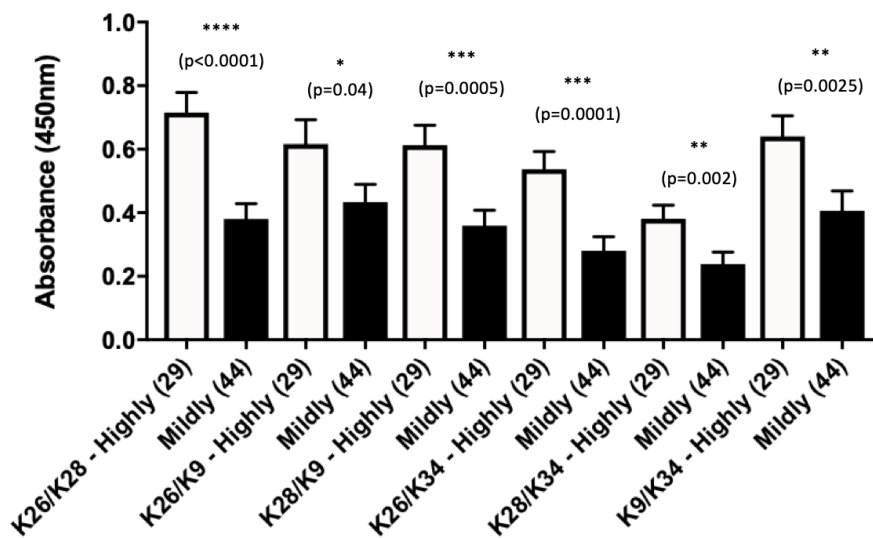
Figure 4.5 - Absorbance level of antibodies detected against mildly infectious and highly infectious samples. The mildly infectious group is composed of 44 samples while the highly infectious group is composed of 29 samples. Analysis was performed using Mann-Whitney U test (p-value **** for <0.0001).
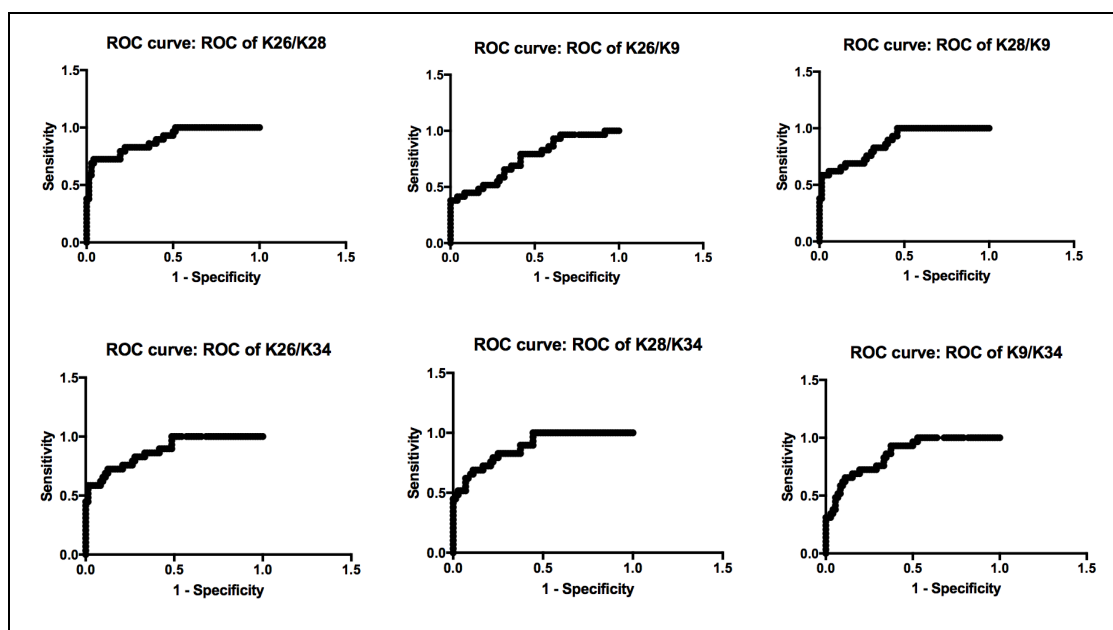
Figure 4.6 - Receiver-Operator Characteristics (ROC) curves for each of the six combinations of antigens. The control group is composed of never infectious dogs (n=72) while the positive group is composed of the super-spreaders (n=29).

Combined antigens	ROC curves			Performance based on threshold[2]			Detection of samples		
	AUC (95%CI)	Error	p-value	Threshold[1]	Sensitivity (95%CI)	Specificity (95%CI)	Detection of Never	Detection of Mildly	Detection of Highly
K28/K26	0.89 (0.82–0.96)	0.035	<0.0001	0.539	72.4% (52.5 – 87.3)	95.8% (88.3 – 99.1)	2.78% (2/72)	27.27% (12/44)	72.41% (21/29)
K26/K9	0.75 (0.64–0.85)	0.055	<0.0001	0.263	79.31% (60.3 – 92.0)	58.3% (46.1 – 69.8)	41.67% (30/72)	56.82% (25/44)	79.31% (23/29)
K28/K9	0.87 (0.79–0.94)	0.037	<0.0001	0.576	58.62% (38.9 – 76.5)	98.6% (92.5 – 99.9)	1.39% (1/72)	27.27% (12/44)	58.62% (17/29)
K26/K34	0.88 (0.81–0.95)	0.036	<0.0001	0.340	72.41% (52.8 – 87.3)	87.5% (77.6 – 94.2)	12.50% (9/72)	29.55% (13/44)	72.41% (21/29)
K28/K34	0.88 (0.81–0.95)	0.035	<0.0001	0.164	82.8% (64.2 – 94.1)	75% (63.4 – 84.5)	26.39% (19/72)	38.64% (17/44)	82.76% (24/29)
K9/K34	0.86 (0.78–0.93)	0.038	<0.0001	0.207	93.10% (77.2 – 99.2)	62.4% (50.3 – 73.6)	38.89% (28/72)	50.00% (22/44)	93.10% (27/29)

[1] Threshold selected based on the Youden Index
[2] Towards the detection of super-spreaders

Table 4.4 – Summary table of ROC curves, threshold and performance for combined antigens. Receiver-Operator Characteristics (ROC) curves were based on the control group is composed of never infectious dogs (n=72) while the positive group was made of 29 super-spreaders. The threshold was selected based on the Youden Index, and the performance calculated towards the detection of super-spreaders (as sensitivity, specificity and detection of samples).

4.4 Discussion

The present study demonstrates for the first time the possibility to differentiate highly infectious dogs within a mixed population by serological assay. The results suggest that antigens rK28, rK26 and rK34 are highly sensitive with respect to super-spreaders when using carefully selected thresholds. Antigen rK28 had a sensivity and specificity of 100% (95%CI: 88.1–100) and 93% (95%CI: 84.5–97.7) respectively.

Current intervention programs in Brazil focus on eliminating canine reservoirs and hence human VL disease. This assumes that infection is synonymous with transmission potential, which are quite different infection states (Rock et al., 2016). Detection and removal of infectious hosts is the underlying aim of control strategies to reduce the basic reproduction number of VL infection. Targeting super-spreaders would concentrate intervention effort on a smaller number of dogs, potentially reducing costs to the health system, and lowering the number of unnecessary sacrificed dogs (Courtenay et al., 2002b; Moreno and Alvar, 2002). However, this could be possible only with the availability of a practical differential test to identify super-spreaders.

Based on carefully selected thresholds, we demonstrated high performances of a select number of antigens to detect super-spreaders. Antigens such as rK39, K26, rK34 and K9 had sensitivities of 97%, 90%, 93% and 83% respectively, as well as specificities of 70%, 85, 75% and 81% respectively; however, their degree of agreement with the xenodiagnostic data was lower than for rK28 (Cohen's kappa values were below 0.65). Among all the antigens, rK28 out-performed with a sensitivity and specificity of 100% (95%CI: 88.1–100) and 93% (95%CI: 84.5–97.7) respectively. Antigen rK28 is already used in the field under the RDT tool, known as the Dual-Path Platform (DPP; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), used for the current screening campaigns for canine infection, serological screening of humans' infection, and as confirmatory test of infection or confirmatory test of disease in humans as well as in dogs. The test was initially developed for a detection purpose: its current multiple use can confound interpretation and case management.

During this study, one of the first concerns was the ability of serological tests to differentiate infectious and non-infectious dogs, as another study concluded that

serological tests fail to detect dogs that transmit *Leishmania* to the sandfly vector (de Mendonca et al., 2017). That study tested current diagnostic tools ELISA (no antigen specified), immunofluorescence (IFAT), direct agglutination tests, and immunochromatographic assays with the recombinant antigen rK39, and Dual-Path platform (DPP®RDT) based on the chimeric protein rK28. They attempted to differentiate non-infectious dogs from infectious dogs with resulting specificities under 13%, but with high sensitivities (>85%). In that study, the antigens showed poorer test performances to differentiate all infectious dogs from non-infectious dogs, which assumes all infectious dogs have similar transmission potential. The published study does not differentiate dogs based on their infectious status i.e. super-spreaders or mildly infectious. However, the authors admitted that their study is not conclusive due to the small number of xenodiagnosis tests performed. Indeed, in their field trials, sandfly blood feeding was limited, and the threshold used for xenodiagnosis (which was not specified) may bias the results of performance. Indeed, it is known that, under natural conditions, infectious dogs may be exposed to higher number of flies, thus leading to different degrees of infectiousness and at different periods of the dogs' lives.

So, whereas our results show that the threshold-based antigen rK28 offered a high performance with 100% sensitivity and up to 98% specificity in detecting super-spreaders, the study of de Mendonca et al. (2017) suggested failure of antigens rK39 and rK28. This trial has two major advantages: (1) dogs were naturally infected and (2) the longitudinal follow-up, over two years, of the sentinel dogs (Quinnell et al., 1997). And as demonstrated, detection of infectious dogs was possible using enzyme-linked immunosorbent assay with novel antigen candidates. No other studies done on the development of serological assays to detect infectiousness could be found to date. A carefully selected threshold is particularly important to optimise diagnostic tools. Indeed, various other threshold options tested in this study showed lower performances (data not showed) indicating the crucial importance of thresholds selection.

The timing of detection is also important for limiting transmission of *Leishmania*. Dogs that become infectious before seroconversion would not be detected; also, delays in testing and follow-up action could permit dogs to become infectious prior to their removal or treatment (Courtenay et al., 2002b). Using the longitudinal xenodiagnoses data, the ability of the antigen assays to detect super-spreaders from the time of

observed onset of infectiousness could be calculated. The threshold-based antigen rK28 detected all the super-spreaders in the mixed population an average of 83 (55-110) days from exposure, whereas the onset of infectiousness was observed to be 298 (218-377) days from the introduction of dogs in the field. There is thus an early discrimination of the super-spreaders while using the threshold-based antigens, never infectious and mildly infectious dogs were detected later, 488 (336-641) days, and 371 (193-549) days, respectively. The impact of the rK28 threshold-based antigen was clear: 100% of transmission events from highly infectious dogs and 85% from mildly infectious dogs, were detectable and could be avoided with the early timing of testing. In the comparative analysis with the xenodiagnostic data, antigen rK28 offer the highest degree of agreement (93.1%) with no systematic difference and a Cohen's coefficient of 0.81 suggesting an elevated strength of agreement. Furthermore, the Logit model indicates a perfect success in the detection of super-spreaders; it also offers a perfect prediction of failure in the case of never-infectious dogs (data not showed). Thirty percent of mildly infectious dogs were also detectable by the threshold-based antigen, reducing the proportion of transmission events.

Concerning the relationship between parasite load and canine infectiousness, previous studies have demonstrated their positive correlation (Courtenay et al., 2014; de Sousa Gonçalves et al., 2016; Borja et al., 2016). Highly infectious dogs seemed to have higher parasite loads in skin, in bone marrow and in hair, where the ear skin was the strongest predictor of being infectious (Courtenay et al., 2014; de Sousa Gonçalves et al. 2016). A recent study compared the intensity of parasite loads in skin and bone marrow dogs and the parasite loads detected in sandflies after feeding on those dogs, demonstrating a strong positive correlation (Borja et al., 2016). We predict that the threshold-based antigens could also predict tissue parasite loads, however very few skin biopsy PCR data were available in the current sample to test this hypothesis.

Several studies indicated that infectiousness is higher in symptomatic than asymptomatic infection (Quinnell and Courtenay 2009; da Costa-Val et al., 2007; Guarga et al., 2000; Magalhães-Junior et al., 2016), though an individual study show that asymptomatic dogs may be equally infectious as symptomatic dogs (Laurenti et al., 2013); and some other studies reported that infectiousness is independent of clinical symptomology (Molina et al., 1994; Guarga et al., 2000). However, longitudinal studies

in Brazil demonstrated that dogs classified as asymptomatic at a single time point, as in cross-sectional studies, usually go on to develop progressive disease, and so should be more appropriately described as pre-symptomatic. Thus, it is assumed that asymptomatic dogs contribute very little to transmission compared to (pre-) symptomatic dogs (Courtenay et al., 2002b).

In Brazil, despite control measures applied, the incidence of *Leishmania infantum* infection has remained high and unchanged over the years in Brazil (WHO report, February 2018). Culling campaigns that removed asymptomatic seropositive dogs from the population have led to dog-owner low compliance with the program. Dog owners usually replace the "lost" dog with a new and often young dog (Nunes et al., 2008). As a result, removal of seropositive dogs that do not transmit the infection may be replaced with susceptible potentially infectious dogs, which will contribute to sustained transmission through maintaining the infectious dog population turnover.

The euthanasia of dogs in Brazil is not well accepted by locals. In developed countries, an alternative for dog culling is the treatment of dogs with drugs designed for human, recently legalized in Brazil (Ministerio de Saude, 2016). According to several studies, treatment of dogs can reduce infectiousness but do not prevent it as the cure is short-lived with incomplete clearance of tissue parasite (reviewed by Travi et al., 2018). Moreover, those treatments are mainly applied in developed countries as their costs are very high.

The preliminary step to a potential different approach to current blanket control operations is provided by the results of this study. Focusing on the transmission potential of dogs rather than infection *per se* could improve the efficacy of reservoir control program. What is required now is the application of the test results using mathematical models to define the coverage and the frequency of testing required to enable efficient control of transmission. Simulations should include variable epidemiological scenarios accounting for transmission rates, canine turnover rates, and sandfly biometrics, as shown by previous model sensitivity analyses (Rock et al., 2016; Buckingham-Jeffery et al., 2018).

Finally, some limitations of a potential novel diagnostic tool for super-spreaders using threshold-based rK28 were considered. The main concerns regarding a diagnostic test based on the rK28 is that the test is already used in the field for different purposes, such as the current screening campaigns for canine infection, serological screening of human's infection, and as confirmatory test of infection or confirmatory test of disease in humans as well as in dogs. The use of these similar antigens can be confounding in the field with regard to interpretation of the results in case management. If this antigen is also used to identify super-spreader dogs, as we have proposed, it will add to the confusion. We offer an alternative by developing a novel tool with a specific antigen designed for infectiousness in Chapter 5.

CHAPTER 5　　　Development of novel recombinant antigen KL914 to identify VL super-spreaders in the canine reservoir population

5.1　Introduction

The presence of *Leishmania* super-spreaders in the field currently hinders the implementation of successful prevention strategies. Identifying super-spreaders would offer a more efficient Visceral Leishmaniasis Control and Surveillance Programme (VLCSP). To develop a tool that is able to detect super-spreaders, existing antigens derived from the *Leishmania* genome, were tested on archived dog sera (as described in Chapter 4). The best performing antigen to detect super-spreaders in a mixed canine population was rK28, a recombinant protein synthetized to increase the B-cell epitope in a kinesin-related protein of *Leishmania infantum*. This finding, however, leads to a further discussion concerning the potential field application of rK28. The protein is already used in the Dual-Path Platform (DPP®RDT) as a screening tool for infected dogs were a positive result triggers the recommendation of culling (Almeida et al., 2017). However, the use of an identical antigen for different purposes (detection of infection, disease, relapses, reinfection, and even after-cure controls) could lead to mis-interpretation by the field-workers in VL case management. Hence, having a clear and stated goal of the diagnostic tool is essential for field use.

The development of an antigen-based diagnostic tool, designed specifically for the detection of super-spreaders in a mixed canid population, seems essential for both improved understanding of transmission dynamics and as a preventive method against VL. In collaboration with the Infectious Diseases Research Institute (IDRI, Seattle, USA), the design of the synthetic recombinant KL914 protein was made following an analysis of various fusion proteins and mixtures of proteins. The adaptation of the recombinant KL914 protein within a rapid diagnostic test (RDT) prototype was made with InBios International, Inc. (Seattle, USA), and will be described in Chapter 6. Both the recombinant protein within immunoassays and the RDT prototype were evaluated using archived sera collected from prospective studies carried out from 1993 to 1995 in the Marajo Island in Brazil (Quinnell et al., 1992). An analysis of the performance of the novel protein was conducted by monitoring infectiousness longitudinally, along with the correlations to the parasite loads in ear skin or bone marrow biopsies and the

clinical signs observed. The development of the novel KL914-based rapid diagnostic tool will enable *Leishmania* super-spreaders to be diagnosed more easily within the canine population, thereby improving the efficiency of the VLCSP.

5.2 Materials and methods

5.2.1 Gene design and protein expression

The gene construct design, as well as protein expression and purification, was performed in collaboration with the Infectious Disease Research Institute (IDRI), Seattle, USA. Targeted gene sequences were previously identified based on the bioinformatic screening of the genome of *Leishmania infantum* (as described in details below). The design of KL914 was made following analyses of various fusions and mixtures of proteins, as well as from the previous analysis of their performance. The synthetic gene for KL914 was designed by fusing two nucleotide sequences: 261 nucleotides of gene k9, and 477 nucleotides of gene *LinJ14.1160r4*. Gene k9 correspond to HASBP2, a hydrophilic acylated surface protein (Alce et al.,1999) that is an essential key component of other fusion proteins such as rK28 and rK34. The gene product of *LinJ14.1160r4* is a kinesin-related protein containing four tandem repeats of LdK39. The multiple repeat is responsible for varying the number of repetitive regions typically impacting signal intensity in assays. In attempts at further improvement, more sequence was added to the initial construct of KL914 but these did not generate any significant improvement in the performance to detect super-spreaders. Therefore, the focus was placed on the original recombinant antigen to develop a novel tool. In regard to the protein expression and purification, the complete protocol has been described below. Each step of the production of recombinant proteins is described below.

5.2.1.1 Bioinformatic screening and identification of candidates

The identification of antigen candidates was based on the bioinformatic screening of Tandem Repeat (TR) genes of the *Leishmania donovani* Complex. Using a program (Tandem Repeats Finder), tandem repeats were located and displayed in DNA sequences of *L. major* and *L. infantum*. A scoring system based on characteristics of the TR genes (period size of the repeat, number of copies aligned with the consensus pattern, and the percentage of matches between adjacent copies overall) was used as a scale for the possession of larger TR sequences with highly conserved repeats among

copies. Only the highest scoring TRs were used for analyses and protein production.

5.2.1.2   Polymerase Chain Reaction to amplify DNA

Using Expand High Fidelity^{PLUS} PCR System (Roche #3300242), the DNA was amplified. The protocol was complete as follows. Diluted DNA (1-50ng Template DNA in water) was added to the Master Mix Cocktail, which was composed of solution buffer, dNTPs, 5' Oligonucleotide, 3' Oligonucleotide, Expands Enzyme and distilled water is the concentrations and volumes detailed below. To inhibit secondary structure formation in G/C rich sequence, dimethyl sulfoxide (100% DMSO) was added.

Master Mix Cocktail
5x Buffer (final concentration 1x)
10mM dNTPs (final concentration 0.2mM)
10x conc. 5' Oligonucleotide (final concentration 0.4 μM)
10x conc. 3' Oligonucleotide (final concentration 0.4 μM)
Expands Enzyme (concentration of 2.5 U per 50μl reaction)
Mg2+ (final concentration of 1.5 mM to 4 mM)
DMSO (final concentration at 10%)
Distilled H$_2$O

The total volume of the PCR reaction was variable to specific needs. The annealing temperature was calculated based on amount of A/T and G/C using the below formula.

$$4(G+C) + 2(A+T) = melting\ temp\ of\ the\ primers$$

The program was run as following **(1)** 1 cycle at 5°C for 5 min; **(2)** 0.45 sec at 95°C; **(3)** 0.45 sec at 55°C; **(4)** 35 cycles at 72°C, 1 min per kb of product; **(5)** 1 cycle at 72°C for 5 min. The PCR was run for appropriate time intervals based on the sequence being amplified. PCR products were run on an agarose gel to ensure a quality product, proceeding with only the best products.

5.2.1.3   Purification of PCR product

The PCR products obtained previously were purified using mini-elute PCR Purification Kit using a micro centrifuge (Qiagen #28004). Five volumes of Buffer PB were added for every 1 volume of PCR sample. Using the mini-elute spin column, PCR samples were applied to the column and spun for 30-60 seconds at 1300 RPM. The flow-through was discarded and Buffer PE, containing ethanol, was added to the column. The column was spun for 30-60 sec. Again, the flow-through was discarded and spun to remove

excess ethanol. Buffer EB was added to the column, incubated for 1 minute, and then spun again.

5.2.1.4   Restriction digest of DNA Insert

The digest of the DNA insert was mixed with React Buffer (Invitrogen) and two restriction enzymes. For optimal restriction, the enzyme concentration was kept under 10% of the total volume. The DNA digest was incubated in a bath at 37°C for 1 hour. Sample buffer was added to digest the samples. The digests were run on a 1% agarose gel. The percentage of agarose varied depending on the size of insert being run. The product was visualised using UV light.

5.2.1.5   Gel Extraction Protocol

For the extraction, a mini-elute Gel Extraction Kit and a micro-centrifuge (Qiagen #28604) were used. The desired DNA bands were cut from the gel with a scalpel and put into a 1.5ml tube. Based on the weight of the gel slice, three volumes of Buffer QG were mixed with 1 volume of gel (usually 100 mg of slice for 300 μl of buffer) and incubated at 50°C for 10 minutes. A gel volume of isopropanol was added to each sample. To bind the DNA, the samples were added to QIAquick columns and spun at 1300 RPM for 1 minute (each column holds 750ul and one can split the sample between 2 spins if more than that is present). The flow-through was discarded, and Buffer QG was added to the column and spun for 1 minute. Again, the flow-through was discarded and Buffer PE was added to the sample and spun for 1 minute. The column was then placed into a new 1.5 ml tube and buffer EB was added to the column, being sure to wet all of the filter. The column then stood for 1 minute before being spun for 1 minute.

5.2.1.6   Ligation of Insert into Plasmid Vector

Using the Rapid DNA Ligation Kit (Roche, #1635379), the insert was ligated into the plasmid vector. Both insert and vector were run on a gel before ligation to estimate the intensity of them to determine amounts present of both. If both intensities were similar and neither was degraded, then one was selected for further use. Ligations were conducted with 3x the molar amount of insert as the vector, and since the insert was much smaller than the vector, the intensities being the same meaning that the insert was at about 3x the molar amount. A tube was set up to be a control that received no DNA

insert and one tube was set up per desired insert. First, the vector was prepared. The restriction digest begun with 5-10 μg of plasmid DNA in 100 μl total volume of the React Buffer, and was mixed with the two enzymes. When using two restriction enzymes, the most compatible React Buffer were determined. Again, the concentration of enzyme was kept at <10%. The total volume was brought to 100 μl with dH_20. The incubation took place in a 37°C bath for 90 minutes. The product was then cleaned using the mini-elute PCR Purification Kit using a micro centrifuge (Qiagen, #28004). The same protocol was followed as listed above, but plasmid sample was split into two columns as the mini-elute columns hold only 5 μg of DNA each. The elutions were combined to finish. For the sample ligation, the prepared vector was mixed with the prepared insert and DNA dilution buffer. 2x DNA Ligation Buffer was added to the preparation, and then immediately after, the ligase was added. The reaction took 5-10 minutes. The vector was dephosphorylated to prevent the cut ends of plasmid from religating. The dephosphorylation buffer (Roche # 1243284) was mixed with the plasmid DNA and Alkaline Phosphatase (Roche #71302) and incubated at 37°C for 30 minutes. A 1% agarose gel was prepared by adding 10x dye to the sample, which was then placed into wells for the electrophoresis. The gel was then visualized with UV light and the plasmid band was excised. DNA concentration was read at 260-280 nm. If the DNA concentration was above 50 ng/μl, it was diluted appropriately to 50 ng/μl. The same mixes and procedures were realized for the control ligation, but with no DNA insert. Therefore, the water must be adjusted accordingly.

5.2.1.7 Transformation into competent *E. coli* cells

Competent XL10 *E. coli* cells were obtained from Stratagene (#200315) and thawed on ice for 10 minutes. Cells had to be kept on ice throughout the whole protocol. Beta-mercapto-ethanol was added to the cells as soon as they thawed and left on ice for 10 minutes. Always on ice, the ligated sample and XL10 cells were mixed and incubated for 10 minutes. Cells received a heat shock at 42°C for 30 seconds. To allow the cells to recover, they were incubated with high nutrient broth (2x YT) and incubated for 30 minutes at 37°C with shaking. Cells were finally plated on corresponding antibiotic plates and incubated at 37°C overnight.

5.2.1.8 Plasmid DNA Preps.

One colony from the transformed cell plate was picked up using a toothpick and dropped into a tube containing nutrient broth and the appropriated antibiotic (Kanamycine in this case). After an overnight incubation at 37°C with shaking, cells were spun down and the supernatant was discarded. The pellet was then resuspended in buffer containing RNAse A from the Mini-Prep protocol using the QIAprep Spin Miniprep Kit using a microcentrifuge (Qiagen #27106). The resuspended pellet was transferred into a new tube and Buffer P2 (lysis buffer) was added and mixed gently by inverting the tubes. Violent shakes cause shearing of genomic DNA and thus contamination of the sample. Buffer N3, a salt solution that binds the DNA, was then added and mixed by inverting the tubes. After a quick spin, the supernant was transferred into a QAIprep spin column from the QIAprep Spin Miniprep Kit using a microcentrifuge (Qiagen #27106). A new spin allowed the flow-through to be discarded, and Buffer PE was added. This step was repeated twice to remove excess ethanol. The QIAprep column was then placed into a new tube and Buffer EB (10 mM Tris and dH20) was added for elution. The eluted DNA was digested and run on an agarose gel to verify the presence of the insert. This step allowed the selection of the samples that had the greatest and clearest amount of DNA insert and sequence, to ensure that the DNA insert was precise and non-mutated. DNA Star software was used for sequence analysis.

5.2.1.9 Transformation into Expression Cells

E. coli (BL-21plys-E, BL-21plys-S, and Rosetta plys-S) cells were obtained from Invitrogen and left to thaw on ice for 10 minutes. The previously prepared plasmid was added to the cells and incubated on ice. A heat shock at 42°C was followed by one minute on ice to permeabilize the cell to the plasmid and de-permeabilize after it was entered. To let the cells recover, they were incubated with high nutrient broth (2x YT) and incubated for 30 minutes at 37°C with shake. Cells were finally plated onto appropriated antibiotic plates and incubate at 37°C overnight.

5.2.1.10 Large-Induction

A mini-induction was carried out before the large induction to ensure that the cells had grown well and that proteins were present on gel in the required amounts. Picking one colony from the transformed cells plate, it was shacked it into a flask of nutrient broth

along with the appropriated antibiotic for an overnight incubation with shaking at 220 RPM at 37°C. Cells were transferred to 1L of broth/antibiotic once the 50 ml culture had reached an optimal density (OD) of ~1.00. After, the culture was transferred to 1L of media and incubated at 37°C with shaking. Optimal density (OD) of the culture was taken every hour until it had reached an OD of 0.4. The culture was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 3 hours at 37°C with shaking. Optimal density (OD) of the culture was recorded at T=0 (pre-binding) and T=3 (post-binding). Samples were run on SDS-PAGE later. T0 and T3 were spun down and resuspended into sample buffer according to the formula below:

150 x OD μl of 2X sample buffer

The sample buffer was composed of Tris at pH 6.8, 4% SDS, 20% glycerol, 10% Beta-mercaptoethanol, and $1x10^{-3}$% Bromophenol Blue. After T3, the culture was poured into centrifuge bottles and spun for 15 minutes at 3,500 RPM. Discarding the supernatant, pellet was resuspended in Lysis Buffer, which was composed of 0.1M PMSF, Tris pH 8, NaCl and H_2O. Cells were frozen at -60°C overnight.

5.2.1.11 Cell Lysis

Frozen cells were placed into a warm water bath to thaw. Once thawed, cells were sonicated until they had the consistency of water. Cells were transferred into polycarbonate centrifuge tubes and spun down at 10,000 RPM for 15 minutes. Supernatant was transferred to a new tube and a sample of 15 μl was taken for an SDS-PAGE gel. The pellet was resuspended in 15 ml of Chaps solution (0.25 g Chaps for 1% final, 1 M Tris at pH 8 for 10 mM final and 30 ml with dH_2O). After a new spin, the supernatant was transferred into a new tube, labelled "Chaps." Finally, the pellet was resuspended in the Chaps solution and label "pellet." Samples of "Chaps" supernatant and pellet were also taken to run on the gel later. All tubes were placed onto rotation for at least 4 hours to a night. The samples in aliquots (T=0, T=3, Sup, Chaps, and Pellet) were kept for SDS-PAGE to determine where the largest quantity of protein resides.

5.2.1.12 Nickel-NTA affinity chromatography under denaturing conditions

The sample was briefly centrifuged to discard the supernatant and the protein product was added to Nickel-NAT agarose bind tubes. After 1 hour of rotation, tubes were centrifuged. The supernatant was poured into a new tube, labelled "post-bind soluble." A DOC solution, composed of 0.5 M Naphosphate at pH 6.3, 1 M Tris at pH 6.3, urea, and H_2O, was added and mixed to the pellet. After another rotation and centrifugation, the sample was labelled "wash 1 – soluble." The pellet was then washed with TrisWash and the supernatant was discarded before a new rotation with DOC solution, and the new sample was labelled "wash 2 – soluble." The samples in aliquots (Wash 1 and 2) were kept for SDS-PAGE to determine where the largest quantity of protein resided.

5.2.1.13 Elution of supernatants

Using elution columns, the supernatant remaining after the second DOC wash was added to a column and washed with Elution Buffer (1 M Tris at pH 8, 0.5 M Naphosphate at pH 8, 0.8 g Imadazole, urea and H_2O). The elution was kept in a new tube labelled "Elute 1." Samples of each elution were taken for a later gel run. After the first elution, the column was placed under a new tube, "Elute 2," and washed again with the same amount of Elution Buffer. This protocol was repeated once more and labelled "Elute 3." The samples in aliquots (Elute 1, 2, and 3) were kept for SDS-PAGE to determine where the largest quantity of protein resides.

5.2.1.14 SDS-PAGE Gel run

All samples in aliquots taken since the beginning (T0, T3, Pellet, Pre- and Post- Bind, Wash 1 and 2, Elute 1, 2 and 3, and the last nickels) were kept for SDS-PAGE to determine where the largest quantity of protein resides. 1x Sample Buffer was added to each of these and ran on SDS-PAGE. Finally, elution containing the largest amount of proteins were combined.

5.2.1.15 Protein Dialysis

Using Pierce SnakeSkin® Pleated Dialysis Tubing, the elutions (Elution 1, 2, and 3) were placed into dialysis tubing, leaving enough space to allow expansion inside the pouch. After wetting one end of the tube and squeezing out the air, the tube was closed by fold-over and clamped tightly together. The elution was poured into the tubing using the open end and carefully closed, as described previously. The tubes were placed into

a large bucket of H_2O and Tris at pH 8 and incubated overnight. This solution was renewed twice, allowing it to incubate for at least ~12 hours in between. After dialysis, the solution was filtered at 20 microns, and placed in a tube at 4°C for storage.

5.2.1.16 Protein quantification

A Bio-Rad Protein Assay Kit was used for quantification. This dye-binding assay results in a colour change when the solution of dye, phosphoric acid, and methanol is in contact with various concentrations of protein. The degree of colour change was measured by a spectrophotometer (595 nm).

5.2.2 Characteristics of sera collection

Sera from archived dogs were collected in villages of Marajo Island in Brazil during a two-year follow up trial (Quinnell et al., 1992; Courtenay et al., 1994; Quinnell et al., 1994; Quinnell et al., 1997). The complete study has been described in the previous chapters (Methods, Chapters 4 and 5) and elsewhere (Courtenay et al., 1994; Quinnell et al., 1994; Quinnell et al., 1997). The samples collected were tested for reactivity, and their performance was confirmed in Chapter 3. In this study, only infected dogs with matching data for infectiousness were used to focus on the transmission potential (n=145). Dogs were considered to be infected when testing positive for parasite culture, PCR and serology (Quinnell et al., 2001). Xenodiagnoses was used to investigate infectiousness to the sand fly vector, as described previously (chapter 5) and elsewhere (Courtenay et al., 2002b). Dogs were classified according to **(1)** their infectious status (yes or no) at point xenodiagnosis, and **(2)** longitudinally by calculating the proportion of dissected flies infected measured across all point xenodiagnosis trials on the dog. The latter measures the intensity of an individual dog's transmission potential, referred to here also as the number of transmission events resulting from the dog. These classifications resulted in 3 groups of individuals: never infectious, mildly infectious, or highly infectious (syn. super-spreaders). "Never infectious" dogs were naturally infected with *Leishmania* but never became infectious to the sand fly vector (n=72 samples from 9 dogs) for six or more consecutive months of xenodiagnoses. The "ever infectious" group comprises dogs that become infectious to sandflies at some point during longitudinal xenodiagnosis follow-up (n=73 samples from 17 dogs). The "ever infectious" dogs were further classified as "highly infectious" when the proportion of infected sandflies was >= 20% (n=29 samples from 7 dogs), or as "mildly infectious"

when the percentage was >0% and <20% of flies infected (n=44 samples from 10 dogs) (Courtenay et al., 2002b; Courtenay et al., 2014). The highly infectious group (syn. super-spreaders) was a key target for investigation in this study. Moreover, the proportion of transmission events (individual blood meal from sandfly) was calculated based on the ratio of positive flies (for the presence of *Leishmania* parasite into sandfly gut) detected by the antigen-based tool, onto the total positive flies as collected from the xenodiagnosis study (Brazil, 1993–1995) from to the onset of infectiousness.

5.2.3 *Enzyme-linked immunosorbent assay*

For each assay, the 96-well Linbro plates were coated overnight at 4°C with 1μg/ml of the antigen diluted in bicarbonate buffer (0.05 M). The non-specific reactivity on the plate was blocked with 1% BSA in phosphate-buffered saline (PBS, pH 7.2) 0.1% Tween 20 for 2 hours at room temperature. After washing (PBS, 0.1% Tween 20), 50μl of diluted sera at1:400 in serum diluent buffer (PBS, 0.1% BSA, 0.1% Tween 20) were added to the antigen wells and incubated at room temperature for 2 hours. The plates were washed and the diluted (1/30.000) bound antibody (HRP-IgG, Thermo Fisher Scientific) was added at room temperature for 1 hour. The enzyme reaction was developed with 100μl/well of TMB substrate solution (Tetramethylbenzidine, Fisher) for 15 minutes. The reaction was stopped using 50μl/well of sulfuric acid. Plates were read using an automated plate reader (Wallac Victor2, Perkin Elmer) set between 405-410 nm.

5.2.4 *Statistical analyses*

All analyses were carried out in Graph Pad Prism 7 and Stata 14.5 (Stata Corporation, College Station, Texas, USA). A comparison of mean absorbance values and antibody titres were performed using a non-parametric Mann-Whitney test. To maximize sensitivity and specificity, several threshold values were explored based on the receiver-operator characteristic (ROC) curve that plots the true positive rate (sensitivity) against the false positive rate (1–specificity). The control group was composed of 58 samples testing negative for xenodiagnosis follow-up, while the positive group was composed of 29 highly infectious samples from the Brazilian cohort. Results are reported with the AUC value (area under the curve) ranging from 0 to 1, so as to classify the performance of the test. If the AUC value is between 1-0.9, the test is

excellent; good (0.9-0.8), fair (0.8-0.7), poor (0.7-0.6), worthless (0.6-0.5). For every possible boundary in the two variables, the ROC plot shows the trade-off between sensitivity and specificity. Performance of the test was also compared to the xenodiagnosis data, which is the gold standard for measuring true infectiousness. Cohen's pairwise method determined the percentage of agreement between both methods (ELISA and xenodiagnosis) with the kappa coefficient defining the strength of this agreement. Furthermore, a pairwise correlation was realised by Pearson's *r* correlation test, with Bonferroni adjustment. Kaplan-Meier analysis and survival curve were generated to determine the median time of detection, based on the stated threshold value.

5.3   Results

5.3.1   *Characteristic of recombinant antigen KL914*

The synthetic protein contains a k9 sequence in alignment with four tandem repeats of LdK39 (*LinJ14.1160r4*). The 738-bp nucleotide sequence was expressed and purified to give a final protein of 245 amino acids, among which 57 were strongly acidic (Aspartic acid [D], Glutamic acid [E]); 28 strongly basic (Lysine [K], Arginine [R]); 64 hydrophobic or apolar (Alanine [A], Isoleucine [I], Leucine [L], Phenylalanine [F], Tryptophan [W], Valine [V]) and 71 hydrophilic or polar (Asparagine [N], Cysteine [C], Glutamine [Q], Serine [S], Threonine [T], Tyrosine [Y]). The complete sequence, related to amino acids listed above, is reported in Figure 1B. The length of gene KL914 (738 bp) is similar to the sequence's length of other proteins such as rK28 (795 bp) (Bhatia et al., 1999). The recombinant protein KL914 is highly acidic (pI 4.486) and has a molecular mass of 26.9 kDa (26 914.44 Da). By comparison, the molecular mass of protein rK39 is at 35.3 kDa, protein rK28 at 28.33 kDa and protein rK26 at 26 kDa (Pattabhi et al., 2010). The mobility of KL914 is slightly faster than rK26, due to the high acidity and the high lysine content included in K9. As previously mentioned, K9 and K26 are highly hydrophilic and show aberrant migration, but their sequence differs in the presence of 11 copies of a 14 amino acid repeat for rK26, which is significant. Indeed, it has been demonstrated that K9 is a highly acidic protein (pI 4.04) of 80 amino acids with a molecular weight of 8.54 kDa (Bhatia et al., 1999; Alce et al., 1999) whereas K26 is an acidic protein (pI 4.59) of 247 amino acids with a molecular mass

of 26.1 kDa. The presence of the gene coding for K9 in the sequence of the recombinant protein KL914 allows for a faster migration on the membrane.

Figure 5.1 – Novel protein description. Complete sequence of the recombinant protein KL914 containing the 261-bp sequence of gene K9 and the 477-bp sequence of Lin14 (*Linj14.1160r4*). The final sequence of KL914 is 738 bp length and encompasses for 245 amino acids.

5.3.2 *Expression of the protein KL914*

In the mini-induction, expressed protein KL914 run on 4-12% Bis-tris Gel (NuPAGE® Novex), with SeeBlue2 pre-stained standard (Thermo Fisher). The proteins were expressed using *E.Coli* BL21 plyS cells and Rosetta cells, where only BL21 cells showed induction as observed in the gel (Figure 5.2A). After completed expression protocol, the final product was run on gels at different volume, which good expression levels at 2.5 µg and 5 µg (Figure 5.2.B). The last gel was performed to verify the effect of the lyophilisation followed by resolubilisation of the protein on the final expression levels (Figure 5.2.C). As they were similar, the lyophilisation process does not affect the expression of the protein.

(5.2A) Mini-induction

1. SeeBlue Plus2 ladder
2. BL21 plysS t=0
3. BL21 plysS t=3
4. Rosetta DE3 t=0
5. Rosetta DE3 t=3

(5.2B) Final product

Wells contain
1. SeeBlue Plus2 ladder
2. t=0
3. t=3 post-induction
4. 2.5ug
5. 5.0ug

(5.2C) Final product with lyophilisation check

Wells contain
1. SeeBlue Plus2 ladder
2. 5.0ug pre-lyophilisation
3. 2.5ug pre-lyophilisation
4. 5.0ug post-lyophilization /resolubilise (1mg/ml)
5. 2.5ug post-lyophilization /resolubilise (1mg/ml)
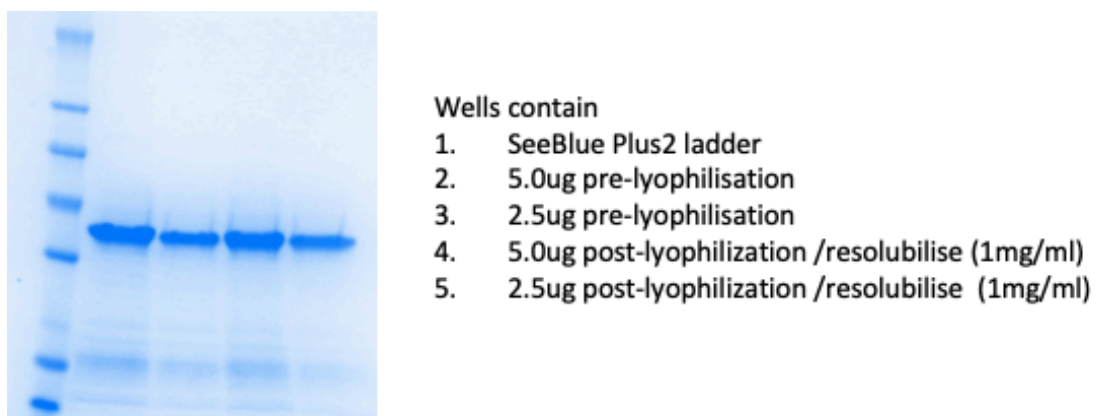
Figure 5.2 – (A) Mini-inductions with protein KL914 expressed in *E.Coli* BL21 plyS cells and Rosetta cells; only BL21 showed induction. (B) The final product and the lyophilised version were run on another gels. (C) Gel run with protein before and after lyophilisation to compare expression levels. All gels were 4-12% Bis-tris Gel (NuPAGE® Novex) with SeeBlue2 pre-stained standard (Thermo Fisher).

5.3.3 Detection of infectiousness in assays using protein KL914

Serum samples previously selected as infected were tested in enzyme-linked immunosorbent assays (ELISA). Absorbance levels obtained for never infectious samples (n=58) were compared to samples classified as ever infectious (n=73) from the Brazilian dog population, showing a significant difference (Mann-Whitney test, U=328, p<0.0001) (Figure 5.3A). Amongst the ever infectious dogs, samples classified as mildly infectious (n=44) and highly infectious (n=29) were also evaluated in ELISA, presenting a highly significant difference (Mann-Whitney test, U=339.5, p=0.0006) (Figure 5.3B).

(5.3A)   **(5.3B)**
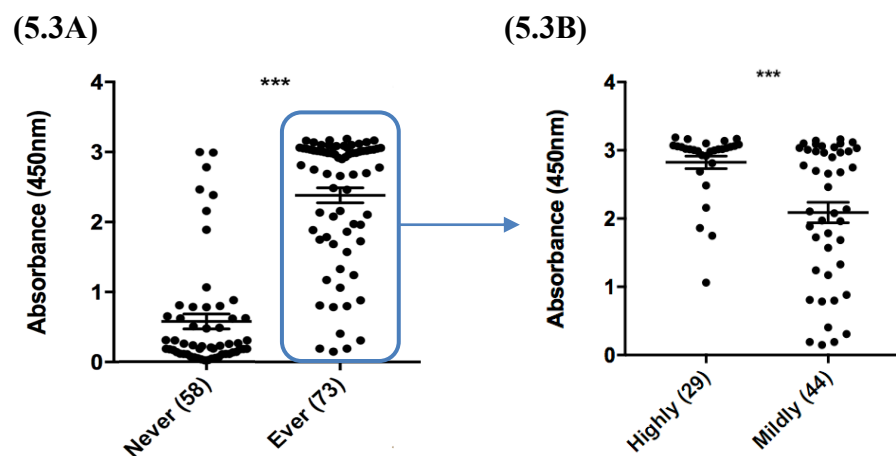
Figure 5.3 – Cross sectional analysis of absorbance level in immune assays (A) Absorbance level detected against ever-infectious (n=73) and never-infectious (n=58) samples. (B) Absorbance level of antibodies detected against mildly infectious (n=44) and highly infectious (n=29) samples (p<0.0001 Mann-Whitney U test).

5.3.4 Thresholds determination and perfromance

The values obtained from ELISA were used to construct a receiver-operator characteristics (ROC) curve, with the control group composed of 58 samples negative for the xenodiagnosis follow-up and the positive group composed of 29 highly infectious samples from the Brazilian cohort. Whereas the maximal value is 1, the area under the curve for KL914 has a value of 0.975 with confidence limits from 0.95 to 1 (p<0.0001), which indicates an almost perfect discrimination ability of the antigen (Figure 5.4). Threshold values were explored to maximize the sensitivity and specificity of the antigen assay and were reported with their related detection performance for longitudinally-classified dogs (never, mildly and highly infectious) (Table 5.1). The

selected threshold was 1.06, offering a performance of 100% sensitivity for the detection of super-spreaders and the lowest detection of never infectious: only 7/58 samples, which gives a specificity of 87.5%. In other words, no false-negative was detected using the threshold-based antigen, and only 12.5% of false-positives were detected using the threshold-based antigen. While comparing the ELISA results to xenodiagnosis data used to determine the infectiousness of dogs, Cohen's method gave a degree of agreement of 91.1% with no systematic difference between the ELISA and the xenodiagnosis (z=7.23, p<0.0001) and a kappa coefficient of 0.80 suggesting an almost perfect strength of agreement between the two methods.
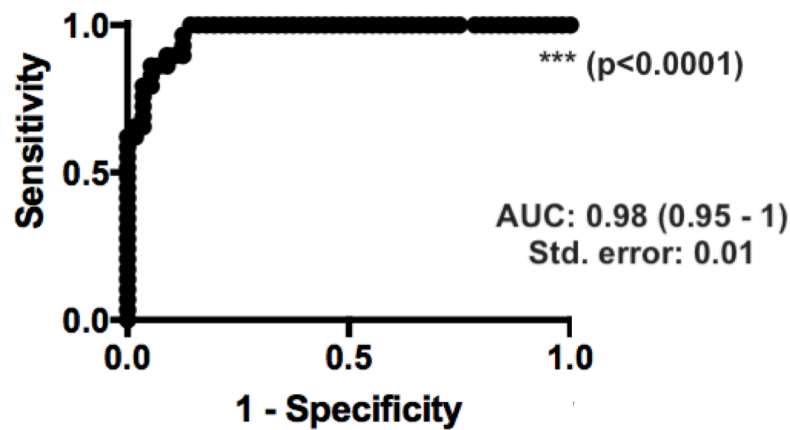
Figure 5.4 – Receiver-Operator Characteristics curve of recombinant KL914 protein based on the control group of 58 samples from never infectious dogs and the positive group of 29 samples of highly infectious dogs from the Brazilian cohort.

Performance and proportion of samples detected (n/total)					
Threshold	Sensitivity (95% CI)	Specificity (95% CI)	Never infectious	Mildly infectious	Highly infectious
2.99	62.1 (42.3-79.3)	98.2 (90.4-99.9)	(0/58)	(11/44)	(18/29)
2.16	86.2 (72.6-97.8)	91.1 (80.4-97.0)	(5/58)	(22/44)	(25/29)
1.15	96.6 (82.2-99.9)	91.1 (80.4-97.0)	(5/58)	(35/44)	(28/29)
1.06	100 (88.0-100)	87.5 (80.0-95.0)	(7/58)	(35/44)	(29/29)
0.97	100 (88.0-100)	85.7 (73.8-93.6)	(8/58)	(35/44)	(29/29)

Table 5.1 – Based on each threshold value, proportion and the related percentage of samples detected in groups of dogs (never, mildly, highly) by each possible values of cut-offs for indicating the performance of the diagnostic tool. Thresholds were determined based on point xenodiagnostic and applied on samples selected for longitudinally classified dogs within the xenodiagnoses study.

5.3.5 Detection times by threshold-based antigen

As a result of the longitudinal follow-up, the time (in days) needed for the complete detection of infectious dogs (mildly and highly) was measured using the Kaplan-Meier curves. Never infectious dogs were excluded, as this category is not the intended target of the assay. The difference between the curves offers a significant difference between mildly and highly infectious dogs (χ^2= 5.73, p<0.01). From this longitudinal analysis, 50% of the super-spreaders were detected after 67 days (Figure 5.5). Complete detection of super-spreaders was achieved after 72 days. The mean time of the onset of infectiousness determined during xenodiagnoses follow-up for super-spreaders was 298 (218–377) days, while the mean detection time for threshold-based antigen is 67 days (62–72) as determined by Kaplan-Meier longitudinal analysis. For mildly infectious dogs, the mean of onset of infectiousness is 203 (58–349) days while the mean of detection time for threshold-based antigen is 172 (100–243) days (Figure 5.5). Therefore, the KL914 antigen offers an early detection of the super-spreaders in the mixed population.
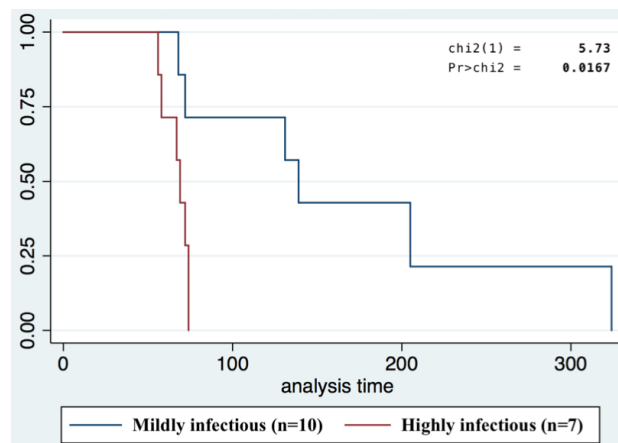
	First *and* Last detection	Mean detection (in days)	Standard error	Onset infectiousness (in days)
Mildly infectious	at day 68 *and* 324	172 (100–243)	3.93	408 (255–561)
Highly infectious	at day 56 *and* 74	67 (62–72)	2.61	298 (218–377)

Figure 5.5 – Kaplan-Meier curves and longitudinal analysis on the detection time (in days) of the xenodiagnoses-classified dogs. The statistical difference in curves and time estimates were compared using the log rank test (χ^2= 5.73, p<0.01). Mean of detection time in days for dogs (mildly and highly) using threshold-based antigen is compared to the onset of infectiousness determined during xenodiagnoses follow-up.

5.3.6   *Detection of individual transmission events*

Besides the importance of super-spreader detection, it is also essential to determine if a single transmission event can be detected. As the previous analysis used dogs at some points of their infectiousness period, the proportion of transmission events for early detection by each threshold-based antigen was also explored. The biological question here was to know if the KL914 protein could also detect single time points of transmission (i.e. at point xenodiagnosis) The proportion of transmission events detected was calculated based on the ratio of positive flies (for the presence of *Leishmania* parasite into sandfly gut) detected by the antigen-based tool, to the total positive flies as collected from the xenodiagnosis study (Brazil, 1993-1995) from to the onset of infectiousness. Threshold-based protein detects 99.2% of transmission events overall (471/475) in a longitudinal analysis, of which 100% (422/422) within super-spreaders dogs and 92.5% (49/53) within mildly infectious group of dogs, demonstrating that the novel protein is able to detect individual events of VL transmission.

5.4   Discussion

The development of the novel recombinant protein KL914 aimed to offer an alternative to the multiple use of existing antigens within diagnostic tests misleading the field work and the case management. The purpose of a diagnostic tool should always be clearly defined; in this case, the tool was designed to detect super-spreaders within the canine population as responsible for the largest fraction of VL transmission.

Protein KL914 is a fusion of hydrophilic acylated surface protein (HASBP2) and kinesin-related protein, as the gene construct was designed by fusing a sequence of gene k9 in alignment with four repeats of gene *LinJ14.1160r4*. The multiple repeat is responsible for varying the number of repetitive regions typically impacting signal intensity in assays. In enzyme-linked immunosorbent assays, protein KL914 offered a sensitivity of 100% and a specificity of 88% in identifying super-spreaders. As no other papers have been published on the detection of super-spreaders dogs, KL914 was compared to the recombinant antigen rK28, described above and analysed in Chapter 5. Both KL914 and rK28 are highly performing to detect super-spreaders with 100% sensitivity. The specificity is slightly lower for KL914 (87%) compared to rK28 (93%),

without being substantially different. Moreover, concerning the detection of single transmission events (as infected flies) in the longitudinal analysis, both antigens offered similar performances with 99.16% detection for KL914 and 98.3% detection for rK28. From a longitudinal perspective, the mean detection time, in days, towards super-spreaders for rK28 and KL914 are, 83% (55–110) and 69% (56–74), respectively. Overall, data suggests that KL914 and rK28 are performing in very similar ways, as KL914 was designed to build upon previous data rather than discovery of new target. However, KL914 still represents the first rationally designed for screening super-spreaders. Data indicates that rK28 detects super-spreaders, but the antigen is currently used in the Dual-Path Platform for the screening of canine infection, and confirmatory test of disease in humans as well as in dogs (Almeida et al., 2017). The distinctions in the use of different antigens are important in clarifying the purpose of test, especially since the outcome of the result under a test-and-slaughter regime is extreme.

As a further step from bench research to bedside, the recombinant protein developed here was adapted into a rapid diagnostic test (RDT) prototype. This opportunity was offered through a collaboration with InBios International, Inc. (Seattle, USA) and described in Chapter 6. The prototype of RDT based on protein KL914 was created in the hope of offering a suitable point-of-care tool for field application.

CHAPTER 6   Evaluation of the novel protein KL914 in dipstick format, a newly porposed rapid diagnostic tool for the detection of *Leishmania* super-spreaders

6.1   Introduction

The development of a novel diagnostic tool designed specifically for the detection of VL super-spreaders is an important key point in the prevention of transmission. Rapid diagnostic tests (RDT) have improved the control and the management in many infections, for example in malaria (WHO report, March 2018). Performing RDT is effortless as they do not require much laboratory knowledge or skills; moreover, they give results within minutes and their interpretation is instinctive. These field-friendly tools allow diagnosis at the community level, even in the more remote areas. Chapter 5 aimed to answer this conundrum by developing a novel protein named KL914. Tested in enzyme-linked immunosorbent assays, it offered sensitivity and specificity of 99% (CI95%: 88-100%) and 87.5% (CI95%: 80-95%), respectively, in the detection of super-spreaders in a mixed canid population of Brazil (Chapter 5). While the recombinant protein ended up being quite similar to known recombinant protein rK28, due to its design being built upon previous data, rather than the discovery of new target, KL914 represents the first rationally designed to detect super-spreaders. The translational aspect of this project, the recombinant protein KL914 was adapted into a rapid diagnostic test (RDT) prototype in collaboration with InBios International, Inc. (Seattle, USA), offering a potential point-of-care field assay. The rational for RDT development is that despite the good performance of the ELISA based assay, it is lab-based and requires time and specific equipment, whereas the RDT is field-based and allows real time decision making. The recombinant protein both within the ELISA and the RDT prototype were put to the test using the archived sera collection from the prospective study carried out from 1993 to 1995 in the Marajo Island in Brazil (Quinnell et al., 1992). Performance of the RDT (labelled KL914-RDT) were also compared to the performance of the antigen in enzyme-linked immunosorbent assays (KL914-ELISA). The prototype was compared to the Kalazar Detect™ Canine kit (RDT based on rK39) from InBios International, Inc.

6.2 Materials and methods

6.2.1 Production of protein KL914

The gene construct was designed by fusing a sequence of gene k9 in alignment with four repeats of gene *LinJ14.1160r4*. The gene product is, therefore, a fusion of a hydrophilic acylated surface protein (HASBP2) and kinesin-related proteins. The multiple repeat is responsible for varying the number of repetitive regions typically impacting signal intensity in assays. In brief, to express and purify the protein, the DNA sequence (738 bp) was cloned into a plasmid (expression vector pET-15b) containing LACK and TRYP promoters, and transformed in *Escherichia* coli XL 10 cells (from Stratagene) for selection and verification of the DNA insert. Transformation into *Escherichia* coli expression cells BL21plyS and Rosetta (from Invitrogen) was then performed to express recombinant protein. Proteins were purified by Nickel-NTA agarose affinity chromatography under denaturing conditions, and were quantified using the Bio-Rad protein assay. The final protein KL914 is composed of 245 amino-acid, is highly acidic (pI 4.486) and has a molecular mass of 26.9 kDa.

6.2.2 Performance of protein in immuno-assays (KL914-ELISA)

The recombinant protein was tested in ELISA using the archived sera collection from the prospective study carried out from 1993 to 1995 in the Marajo Island in Brazil. Complete analyses of the performance of the antigen is available in Chapter 5. The area under the ROC curve for KL914 had a value of 0.975, whereas the maximal value was 1, indicating a high discrimination potential of the antigen. In ELISA assays, the threshold-based antigen of 1.06 offered a sensitivity and specificity of 100% (29/29) and 87.5% (CI95%: 80-95%), respectively, towards the detection of super-spreaders. From the Kaplan-Meier analysis, complete detection of super-spreaders was achieved after 72 days, while the mean of the onset of infectiousness was 298 (218–377) days.

6.2.3 Development, principle and interpretation of rapid test format (RDT)

A prototype of RDT based on the novel recombinant antigen KL914 was developed with InBios International, Inc. (Seattle, USA). Although the complete features and precise details of the recombinant antigen KL914 RDT prototype are under a non-disclosure agreement with InBios International, Inc. (Seattle, USA), a schematic representation of the general composition of the dipstick is represented in Figure 6.1.

The prototype has the format of a dipstick which is field-friendly, does not require lab experience or equipment, and offers results within minutes. The nitrocellulose membrane of the dipstick was coated with the recombinant protein KL914 on the test line. A separate control line, based on protein G, was also present on the membrane and captures all IgG demonstrating that both the sample was appropriate and the membrane had allowed a proper flow in migration. The principle is represented in Figure 6.1B. The sample pad received the drop of serum (10μl) to be tested, as well as a stable solution of A-colloidal gold conjugate which facilitates the migration on the membrane through capillary action. Another drop of Chase buffer (10μl) was added to the pad a few minutes later to ensure the development of the assay. The interpretation of the results is highly intuitive. To test reliability of interpretation, the results were scored twice independently by the operator and blind by a second person. If the sera contained antibodies for the associated antigen, the conjugate would react with the complex on the test line which will make the band visible. A positive test was indicated by two visible bands on the membrane, while a negative test had only one band (the control line). The dipstick assay is invalid when the band for the test line is visible without the band for the control line. Similarly, if after the migration, no visible band appears, the test is considered to be invalid. In these cases, samples must be retested on other strips.

Principle and interpretation of rapid test format
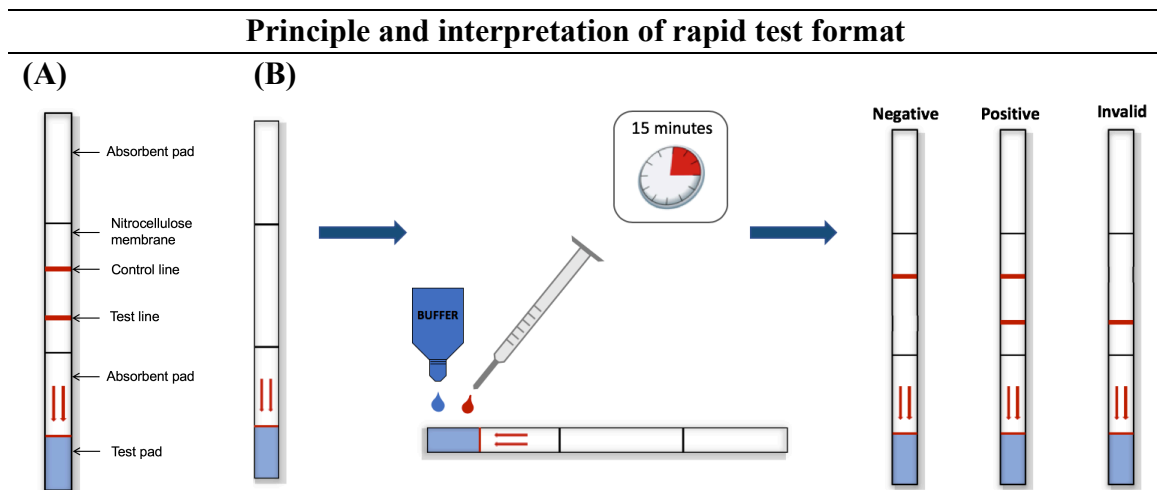
Figure 6.1 – Development of rapid test format (A) Schematic representation of the dipstick; (B) The dipstick-format of the prototype includes a test pad (blue square) where the blood sample and the migration buffer are dropped. After 15 minutes migration, results are indicated by one visible line if negative, and two visible lines if positive. In other cases, the test is invalid and must be redone.

6.2.4 Kalazar Detect™ Canine

The Kalazar Detect™ Canine (lot #XA1089) was provided by InBios International, Inc. in Seattle, USA) and is based on the recombinant protein rK39. In a previous study, a set of the same sera collection was tested on the Kalazar Detect™ strips (personal communication from Rupert Quinnell), and these results were compared to (i) the xenodiagnosis data and (ii) the performance of KL914-RDT.

6.2.5 Sera selection for RDT evaluation

Sera samples used for the RDT evaluation were selected among the archived collection from the prospective study carried out from 1993 to 1995 in the Marajo Island in Brazil (Quinnell et al., 1994). A total of 109 samples could be included in these preliminary assays, due to the limited number of dipsticks available for this project. Selection was made so that there were matching data for xenodiagnoses and ELISA. All the samples selected came from *Leishmania*-infected dogs (positive for parasite culture, PCR and serology), as the focus is infectiousness. For the purposes of this study, preliminary assays included 23 samples from highly infectious dogs, 30 samples from mildly infectious dogs and 56 samples from never infectious dogs, as based on the xenodiagnoses study used to investigate infectiousness to the sand fly vector as described previously (Chapter 5) and elsewhere (Courtenay et al., 2002b). Never infectious dogs were naturally infected, but never became infectious for six or more consecutive months of xenodiagnoses. Highly infectious dogs had a proportion of infected sandfly over 20% of the total sandflies collected, and mildly infectious dogs had between >0% and <20% infected flies (Courtenay et al., 2002b; Courtenay et al., 2014).

6.2.6 Statistical analyses

All analyses were carried out using Graph Pad Prism 7 and Stata 14.5 (Stata Corporation, College Station, Texas, USA). The results of the KL914-RDT were classified according to the intensity of the observed band: (0) for negative test result and (1) for positive test result. Performance (sensitivity and specificity) of the rapid diagnostic test (labelled KL914-RDT) were compared to those of the antigen in enzyme-linked immunosorbent assays (KL914-ELISA). The percentage of agreement between the results of the two KL914-based tools (RDT and ELISA) compared to the

xenodiagnosis (which is the gold standard for measuring the true infectiousness) was measured using Cohen's method and the kappa coefficient defining the strength of this agreement.

6.3 Results

6.3.1 *Validation of KL914-RDT dipstick*

Several attempts were needed to obtain an operational dipstick. When lyophilising the protein to apply on the stick, the sugars contained in the excipient used for the lyophilisation interfered with the strip-coating. After revising to use protein in liquid form (i.e. suspended in Tris buffer), the adaptation was successful. A first comparative assay used the sera from a highly infectious dog (dog A78, sample n°8) on three strips of KL914-RDT, tested as triplicates, and one strip of Kalazar Detect™ Canine kit as control. All three KL914-RDT were positive in detecting the sera sample, and showed no significant difference among them, while the Kalazar Detect™ Canine strip had no visible band on the test line (Figure 6.3A). To compare with Kalazar Detect™ Canine, three different samples from super-spreaders dogs (A37, A78 and A79) were tested, all of which appeared to be negative (Figure 6.3B).

(A)

Comparative assay – Highly infectious sample (Dog A78- Sample 8)

KL914-RDT (1)
KL914-RDT (2)
KL914-RDT (3)
Control Kalazar Detect ™

CTRL   TEST

(B)

Kalazar Detect ™ Canine
Highly infectious samples (Dog A37 and A79)

CTRL   TEST
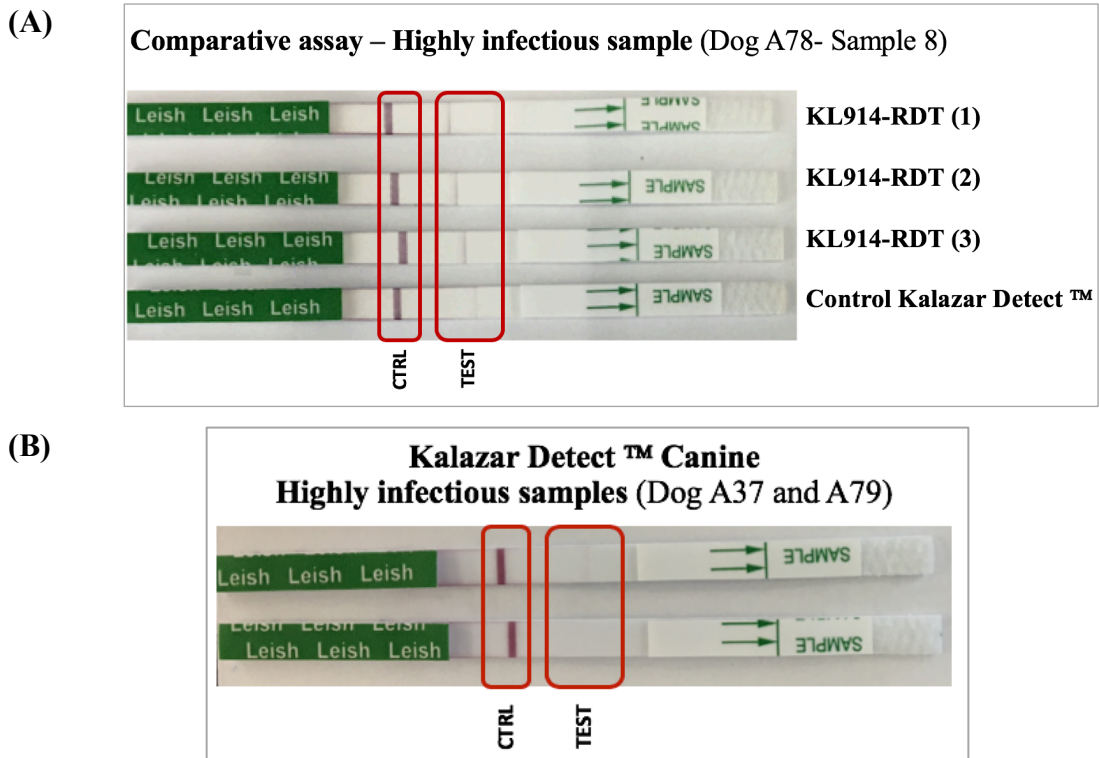
Figure 6.2 – (A) Rapid diagnostic test prototype comparing three positive strips on KL914 and the control based on Kalazar Detect™. The antigen reactivity for the three prototype strips is stronger than on the control strip. (B) Kalazar Detect™ Canine negative for the detection of super-spreaders.

In this study, three sera samples only were tested on Kalazar Detect™ (as described above) due to the limited number of dipsticks and sera available. While Kalazar Detect™ is well-known to detect infection, its performance towards super-spreaders is not established yet. In a previous study, a set of the sera collection was tested on the Kalazar Detect™ strips (personal communication from Rupert Quinnell), and these were compared to the xenodiagnosis data of this project. The number of samples reporting results for both tools (KL914-RDT and Kalazar Detect™) were too low (n=20) for statistical analysis. The only comparison that could be realised in this project was by using different sera samples to establish the performance towards super-spreaders independently of each other. The results for Kalazar Detect™ are described as follows: 7 out of 9 were correctly identified as highly infectious (e.g. super-spreaders) and 3 of 6 were correctly identified as mildly infectious. This sample size is too low as well to be able tp confirm the lower performance of Kalazar Detect™ strips

on super-spreaders. However, the rate of false positive was high (32.75%): 19 out of 58 samples where positive on the strip whereas the sample was not infectious according to the xenodiagnosis classification. These results appear to be coherent as the aim of the tool is to detect all infected dogs regardless of their infectiousness. The false positive samples were infected but not infectious. By opposite, the novel KL914-RDT was designed to detect infectious dogs only. The results for KL914-RDT are reported below, in Table 6.2. with a false positive rate lower than 4%. Therefore, it seems that the novel KL914-RDT is more specific towards super-spreaders. Furthermore, the tool currently used for screening infected dogs in the field is the Dual-Path Platform; however, no strips were available for testing during this PhD. So far, this initial project only confirmed that the novel KL914-RDT can detect the infectiousness of super-spreaders, without being able to confirm or disprove the performance of other rapid tests such as Kalazar Detect™ or Dual-Path Platform (DPP®RDT).

6.3.2 KL914-RDT on longitudinally collected sera

An example of the RDT assay results from testing a *Leishmania* super-spreader dog (A78) with eight samples collected in longitudinal follow-up with the description of each condition (Table 6.1). This dog was classified as highly infectious from sample n°5 to n°8 for over 200 days. Determination of the transmission potential was tested by xenodiagnoses, with more than 20% of *Leishmania*-infected sandflies collected; in this case, the proportion of positive sandflies varied between 35% and 45% during the infectiousness period. Results of the protein KL914 in ELISA (as described in Chapter 5) were compared to the xenodiagnoses results, confirming the ability of KL914 to detect super-spreaders. Finally, rapid tests based on KL914 were run with samples of the longitudinal collection of sera (Figure 6.4). As mentioned in the method section, the tests were confirmed twice by the operator and a blinded person to avoid bias. Two visible bands were observed for strips n°5 to n°8, meaning a positive test (Figure 6.4), whist tests were negative for samples tested on strip prior to that point. These results corresponded to those in xenodiagnoses and ELISA (Table 6.1). In this example, the RDT worked as well as the ELISA and the xenodiagnoses to identify sera sample of dogs with high infectiousness potential.

Table 6.1 – Super-spreaders dog (A78) and related data collection, comparing xenodiagnosis detection, KL914-ELISA detection and KL914-RDT detection.

Sera sample	Days in the field	Infection[1]	Highly infectious[2]	KL914-ELISA (absorbance value)		KL914-RDT (Figure 4)
n°1	0	0	0	0	(A=0.262)	0
n°2	73	0	0	0	(A=0.038)	0
n°3	129	0	0	0	(A=0.058)	0
n°4	198	1	0	0	(A=0.115)	0
n°5	272	1	1	1	(A=3.006)	1
n°6	341	1	1	1	(A=3.065)	1
n°7	408	1	1	1	(A=2.998)	1
n°8	477	1	1	1	(A=2.982)	1

[1] Determined by parasite culture, PCR and serology.
[2] Determined by xenodiagnosis with over 20% of infected sandflies on the total collected.
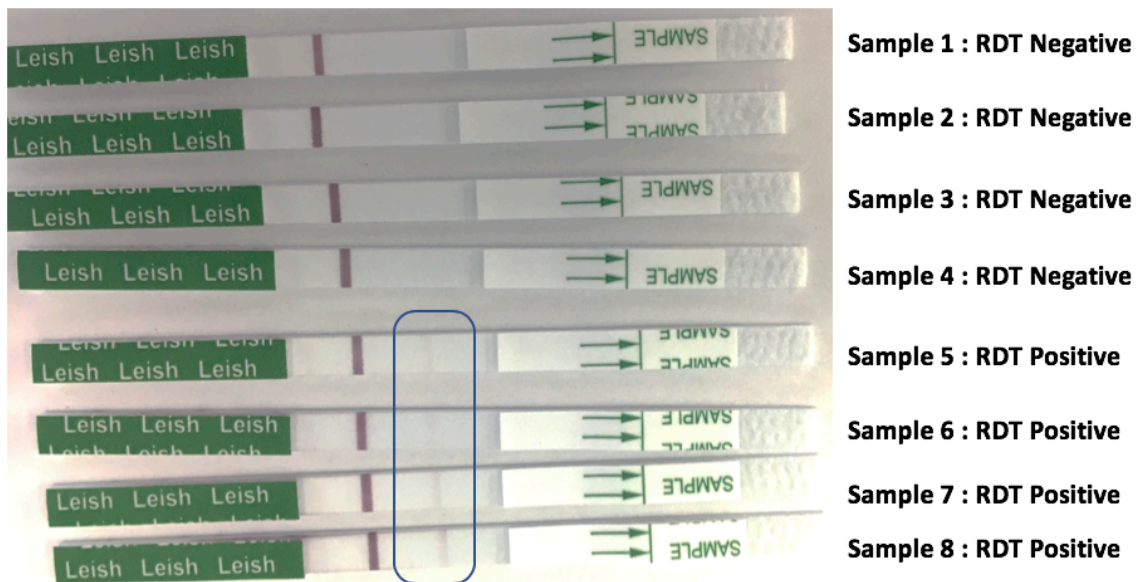
Figure 6.3 – Example of longitudinal follow up in rapid diagnostic test on dog A78 classified as super-spreader from sample n°5 to n°8 in xenodiagnoses, which also corresponds to the positive results of the RDT.

6.3.3 Comparative study of performance of KL914 in RDT and ELISA

Preliminary trials were performed on a proportion of the sera collection (n=109) due to the limited number of dipsticks created. The KL914-RDT detected 60% (14/23) of the samples from super-spreaders, which corresponds to the sensitivity, and 20% (6/30) of the sample of mildly infectious dogs (Table 6.2). Moreover, only 3.6% of samples were detected as false positive by the RDT (2/56 of never infectious). Hence, KL914-RDT has a higher specificity to detect super-spreaders (96.4%) compared to KL914-ELISA (87.5%). Unfortunately, the sensitivity of KL914-RDT is substantially lower than in the ELISA, reaching 60% and 100% respectively (Table 6.2). While comparing these methods to the xenodiagnosis, Cohen's method was applied. A percentage of agreement of 86.08% for RDT was measured with no systematic difference ($z=5.76$, $p<0.0001$) and the kappa coefficient was at 0.63, suggesting a substantial strength of agreement between the infectiousness and the rapid detect test. For K914-ELISA, the degree of agreement was 91.14% ($z=7.23$, $p<0.0001$) and the kappa coefficient was 0.80, suggesting a highly strength of agreement with the xenodiagnosis. The degree of agreement between the two serological methods (KL914-ELISA and KL914-RDT) was also measured, giving a Cohen's kappa coefficient of 0.30.

Table 6.2 – Comparative study of the detection of samples from highly, mildly and never infectious dogs using the rapid diagnostic prototype based on the novel protein (KL914-RDT) and immunoassays (KL914-ELISA) in preliminary assays. Performance of KL914-RDT and KL914-ELISA to detect super-spreaders in a mixed population. The degree of agreement between the test and the reality (as measured by xenodiagnosis) data was measure using Cohen's method with the percent and the kappa coefficient.

	Performance of KL914-ELISA and KL914-RDT to detect super-spreaders								
	Highly	Mildly	Never	Sensitivity	Specificity	False positive	False negative	Degree of agreement[1]	Kappa
RDT	14/23 (60%)	6/30 (20%)	2/56 (3.6%)	60.1%	96.4%	3.6%	39%	86.1% $z=5.76$ $p<0.001$	0.63
ELISA	23/23 (100%)	27/30 (90%)	7/56 (12.5%)	100%	87.5%	12.5%	0%	91.1% $z=7.23$ $p<0.001$	0.80

[1] Compared with xenodiagnosis data

Finally, while comparing the results of ELISA and RDT, the degree of agreement was 61.16% (Table 6.3) which suggests improvement is needed, and a kappa coefficient of 0.26 (z=3.87, p<0.0001). Individuals results were also compared in the Table 6.3 for each group of infectious (super-spreaders, mildly and never-infectious)

Comparison of the individual detection between RDT and ELISA and degree of agreement using Cohen's method
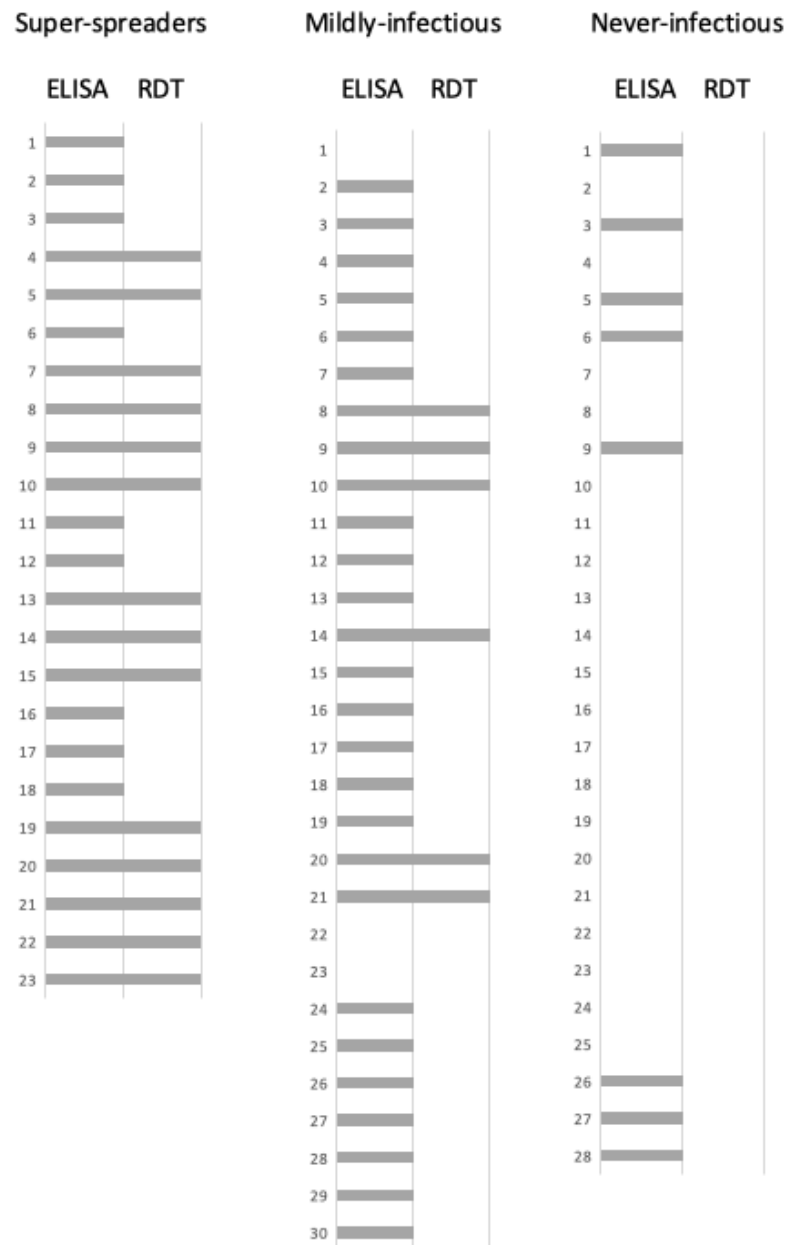
Agreement=61.16%; z=3.87 ; p<0.0001 and kappa=0.26

Table 6.3 – Comparison of the detection between KL914-RDT and KL914-ELISA with degree of agreement measured using Cohen's method and the kappa coefficient.

6.4 Discussion

The development of the first prototype RDT for detecting super-spreaders was achieved. Due to the limited number of tests available for the experiment, the results presented here are considered to be preliminary; however, the project generated a proof-of-concept that the RDT can be developed. Further development to improve its sensitivity is needed (discussed below), before it can be evaluated under field conditions.

In the example of longitudinal follow-up of a super-spreader dog, the rapid diagnostic tests based on KL914 were run with all samples of the sera collection. The RDT used as screening method matched at all times points with the xenodiagnosis and the ELISA results, giving a 100 percent of agreement between these three methods. Hence, the RDT worked perfectly for this dog to identify sera sample of dogs with high infectiousness potential. However, due to individual variability, not all dogs offered a perfect longitudinal detection. After analysing all the dogs tested in the assays, the overall performance of KL914-RDT showed a strong degree of agreement (86.1%) with the xenodiagnoses used to determine the infectiousness of the dog (κ=0.63), suggesting a high diagnostic potential for super-spreaders. However, the degree of agreement was higher for the KL914-ELISA and xenodiagnosis (91.1%) without being significant difference compared to the RDT and the correlation between ELISA and RDT was also lower by Pearson's' correlation. While comparing the sensitivity and specificity of the KL914-rapid test to its equivalent in immune-assays, the results highlight that ELISA is the more sensitive method, but that the RDT has a higher specificity in detecting super-spreaders. The specificity of RDT to identify super-spreaders increased compared to the immune-assays, to 96.4% and 87.5 respectively. The RDT had a low detection of mildly infectious dogs (20%); this is not considered a major issue as mildly infectious dogs are believed not to contribute to most transmission events. Moreover, only 3.6% of the samples were detected as false positives by the RDT which is an advantage to improve dog-owner compliance.

The reduced sensitivity of the RDT is likely the result of the conversion of the antigen into a rapid diagnostic tool, as observed in other studies (Pattabhi et al., 2010; de Silva Solcà et al., 2014). Similar discrepancies were observed between other antigens and

their respective RDTs for the detection infection in canine population. For example, the crude *Leishmania* antigen (CLA) was tested in ELISA and RDT, offering sensitivities of 78,3 % and 72,5%, and specificities of 90% and 84%, respectively, in detecting *Leishmania* infection (de Silva Solcà et al., 2014). This shows a decrease in detection when the antigen is converted to RDT. Moreover, the adaptation of rK28 into a dipstick was only measured for human VL infection: the RDT showed a lower sensitivity compared to the ELISA format, without being significantly different, in the detection of human VL infection (Pattabhi et al., 2010). No publications comparing rK28 in ELISA and in RDT could be found on canine trials. Very limited studies involve the detection of canine infectiousness; indeed, only one could be found in the literature comparing the performance of tests towards infectiousness. The performance of rK39 was compared in ELISA and in the format of Kalazar Detect™ Rapid Test, showing that the detection of highly infectious dogs dropped from 97% to 79% (Courtenay et al., 2014). The reason why antigens perform weaker in rapid diagnostic tools than in ELISA formats remains unclear. Several reasons could be suggested to explain the lack of performance such as the composition of the migration buffer or the adhesion of the protein to the nitrocellulose membrane. Moreover, during the conception of the RDT in IDRI/InBios, the sera conservation conditions were not optimal; indeed, some of the serum seems to be dried out in the wells, even if the reactivity of the samples was validated and confirmed by another ELISA during the secondment at IDRI. Therefore, further work is required to evaluate the reasons for this discrepancy.

In conclusion, the first prototype rapid diagnostic test for the detection of super-spreaders has been developed based on a new protein, for its adaption into a field-friendly tool. Further improvements could be made to the sensitivity of the RDT. On full development of the RDT, the impact of such a tool on transmission, when applied to the mixed canine population, will require quantification by mathematical modelling dynamic scenarios. This is the subject of the next chapter, with the aim of modelling the most efficient screening method in the field, and generating recommendations to improve the Visceral Leishmaniasis Control and Surveillance Programme (VLCSP).

CHAPTER 7 Modelling canine VL transmission: impact of novel diagnostic tool for super-spreaders on canine VL transmission rates

7.1 Introduction

In most mathematical models of infectious diseases, the heterogeneity of transmission is not not sufficiently taken into consideration, whenever it concerns hosts or vectors. Furthermore, models including super-spreaders in intervention settings are even fewer. However, targeting them for preventive measures is assumed to increase the efficacy of interventions (Woolhouse et al., 1997; Lloyd-Smith et al., 2005). Moreover, the failure of identifying these transmission events reduces the efficiency of control measures as discussed thoroughly in the introduction (section 1.5.3). In the case of ZVL, Costa et al. (2013) has offered the most complex mode; in addition to the basic SEI model, it includes infectiousness and imperfect diagnosis of dogs. The imperfect diagnosis was represented by new boxes being "correctly diagnosed" and "incorrectly diagnosed" (Costa et al., 2013). However, there was no specification for differences in intensity of infectious dogs including super-spreaders, which can have large impacts on model predictions: the existence of such heterogeneities is likely to lead to higher transmission rates than homogeneous mixing, and a clustering of infection (Courtenay et al., 2002b). Models on infection in dogs has shown that sensitivity and specificity of diagnostic tools have a direct impact on the success of the intervention (Rock et al., 2016). To date, xenodiagnosis is the only substantiated method to provide conclusive data to discern infectious from non-infectious dogs. Due to the need for identifying highly infectious dogs, a newly proposed diagnostic test was developed for the specific detection of super-spreaders in a mixed canine population (Chapters 5-6). The present study aimed to develop mathematical models based on this novel diagnostic and its impact on transmission rates. Using a deterministic model, VL transmission rates were calculated for the different categories of infectiousness known as never, mildly and highly infectious. The impact of the targeted group was evaluated by the reduction in incidence. Two diagnostic tools designed for the identification of super-spreaders were used in this chapter: both based on the novel antigen KL914 but in different formats (RDT and ELISA) as described in Chapters 5 and 6. The impact of these newly proposed tools was quantified under different implementation scenarios, including important parameters such as screening and intervention rate, frequency and coverage

of the screening. Due to the continuous turnover of dog populations, the screening frequency is one limiting factor and is essential to understand towards development of efficient campaigns. In addition, the impact of a culling interventions was analysed. The aim of this preliminary mathematical study was to develop a working model incorporating test diagnostic components and heterogeneities in canine infectiousness, to identify the most efficient way to apply the novel diagnostic tool in the field, and to use the model to provide initial recommendations to the Visceral Leishmaniasis Control and Surveillance Programme, as currently established by the Ministry of Health (MOH) in Brazil.

7.2 Material and Methods

7.2.1 Initial model of VL transmission

The compartmental model was schematically represented in Figure 7.1. Transmission dynamics of VL within the dog population were based on the following deterministic equations (*1−5*). The basic model SEI (susceptible, *S*; exposed, *E*; infectious, *I*) was modified to divide the infectious group (*I*) in three subgroups according to their infectiousness. The never-infectious group (I_N) included infected dogs that never transmit the parasite to the sandflies vector; the low infectious group (I_L) is made of mildly infectious dogs, whereas high infectious group (I_H) are the super-spreaders, being the target of the diagnostic tool. Note that the exposed box of this model included the latent infected dogs, therefore all are infected but not all have seroconverted yet.
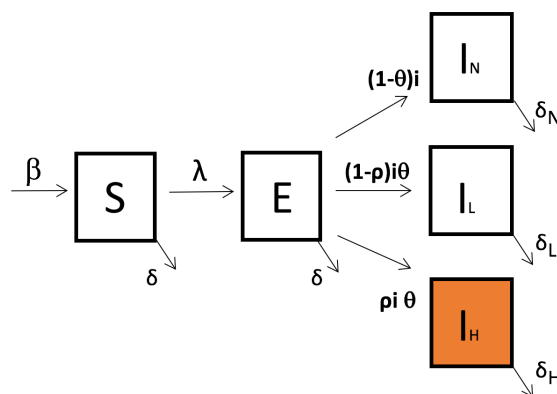
Figure 7.1 – Representation of compartmental models to calculate VL transmission between dogs (S, susceptible; E, exposed; I_N, infected and non-infectious; I_L, infected and mildly infectious; and I_H, infected and high infectious) with parameters β as birth rate, δ as death rate, ρ as proportion of super-spreaders, *i* as latency rate, and θ as proportion of infected dogs that become infectious.

Changes in the number of dogs through time are given by the following deterministic equations:

$$dS/dt = \beta N - \lambda\, S - \delta S \qquad (1)$$
$$dE/dt = \lambda\, S - i\, E - \delta E \qquad (2)$$
$$dI_N/dt = i(1-\theta)\, E - \delta_N I_N \qquad (3)$$
$$dI_L/dt = (1-\rho)i\theta\, E - \delta_L I_L \qquad (4)$$
$$dI_H/dt = \rho i\, \theta\, E - \delta_H I_H \qquad (5)$$

where β is the birth rate of dogs, δ is the death rate of dogs (δ_N, δ_L, δ_H are the death rates of the different types of infectious dogs), λ is the transmission rate (determined below), ρ is the proportion of infectious dogs that are highly infectious, i is the rate of latency of dog, and θ is the proportion of infected dogs that become infectious.

Transmission rate (λ) and vectorial capacity (C) are respectively defined as:

$$\lambda = C\, p_D \left(\frac{p_{VL} I_L}{N} + \frac{p_{VH} I_H}{N} \right) \qquad (6)$$

$$C = \frac{F}{N}\, \frac{a^2\, e^{-\mu\tau}}{\mu} \qquad (7)$$

where p_D is the probability of infected fly transmitting to dog; p_{VL} is the probability of low-infectious dog transmitting to fly; p_{VH} is the probability of highly infectious dog transmitting to fly; F is the total number of sandflies; a is the biting rate of sandflies on dogs, $e^{-\mu\tau}$ is the probability of sandfly surviving the latency period with τ as the latent period of the parasite inside sandflies and μ as the mortality rate as sandflies. The contribution in transmission and prevalence for highly, mildly and never infectious dog was computed separately. Probabilities of high and low infectious dogs transmitting the parasite to the sandflies were defined by Courtenay et al. (2002b). Moreover, the birth rate of dogs (β) is assumed to be equal to the total death rate, so that the total dog population remains constant:

$$\beta = \delta(S+E) + \delta_N I_N + \delta_L I_L + \delta_H I_H \qquad (8)$$

where the natural death rate of dogs (δ) and the death rate of never-infectious dogs (δ_N) are similar; and where the death rate of low-infectious dogs (δ_L) and highly infectious dogs (δ_H) are higher.

All estimates of parameters were taken from published sources (Courtenay et al., 2002b; Dye, 1996; Reithinger et al., 2014; Costa et al., 2013, Nunes et al., 2008 and Quinnell et al., 1997). Annotations are summarised in Table 7.1. All analyses were realised in MATLAB R2018.

7.2.2   *Inclusion of the diagnostic tool*

Two diagnostic tools were used in this chapter: the RDT and the ELISA, both based on the same antigen (KL914) as described in previous chapters. When applying them, the number of diagnosed dogs in each group was based on their performance with sensitivity (d) and specificity (d_z). These variables were determined in previous chapters, sensitivities for ELISA and RDT were 99.9% and 91% respectively, whereas their specificities were 87.5% and 96.4%. Note that the loss of performance for the RDT compared to the ELISA was recurrent in several other studies and discussed in Chapter 6. Although, the selected threshold allows high sensitivity and specificity leading to a specific detection of super-spreaders, the model allows correctly and incorrectly diagnosed individuals to enter the "diagnosed" group. For example, a lack of specificity (large *1-d_z*) of the diagnostic will lead to a large number of false positive dogs. The screening rate (σ), being essential to the success of the diagnostic, is the proportion of dogs tested a day. A large range of screening rates were modelled including 20 logarithmically equally spaced values between 0.001 and 0.1 per day. However, in a tool implementation scenario, the daily screening rate is difficult to set up. Therefore, a pulsed intervention was also modelled where the pulsed screening function takes a time vector, the number of screenings per year, the screening coverage (proportion of dogs tested per day during the screening period), and the duration of the screening (in days). While modelling diagnostic interventions, the first query to know is how the tool needs to be used in the field to have the most efficient impact; this implied to vary the screening rate (constant or pulsed) and the intervention rate (culling or treatment) to evaluated their combined impact on transmission rate and incidence. The breakpoint in transmission was the reference to determine the screening rate at which the diagnostic tool should be applied (instantaneously followed by the intervention). Below that point, VL infection is unable to be maintained in the canine population and will progressively decline to eventually reach zero. The most efficient screening rate was calculated and compared to current field applications.

Practically, the detection tool will be positive for correctly diagnosed super-spreaders (I_H) and misdiagnosed dogs from all the other groups (S, E, I_N and I_L); whereas the test will be negative for misdiagnosed super-spreaders (I_H) and correctly diagnosed other dogs (S, E, I_N and I_L). As result of the detection, the total rate of positive and negative diagnosis was determined as follows:

$$\text{Total rate of positive detection (DP)}$$
$$= [I_H.d + (S+E+I_N+I_L)(1-d_z)] \, \sigma \qquad (9)$$

$$\text{Total rate of negative detection}$$
$$= [I_{H.}(1-d) + (S+E+I_N+I_L) \, d_z \,] \, \sigma \qquad (10)$$

where d is the true positive rate of detecting super-spreaders (the sensitivity of the diagnostic); (1-d) is the false negative rate of diagnosing super-spreaders; d_z is the true negative rate (the specificity of the diagnostic); (1-d_z) is the false positive rate.
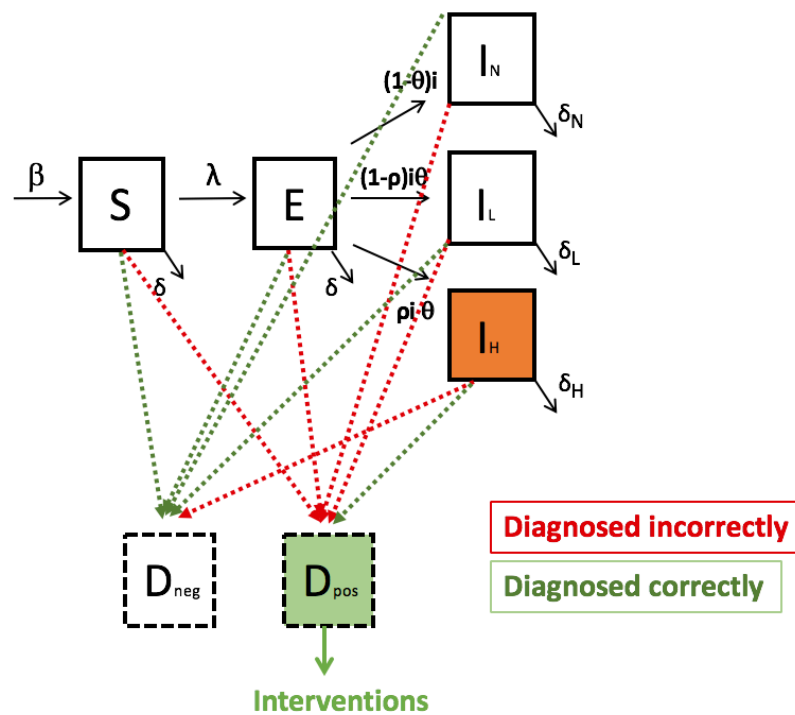
Figure 7.2 – Schematic representation of the detection of S, E, I_N, I_L and I_H at different rates using the novel diagnostic tool (D_{pos}, positive result at diagnosis; and D_{neg}, negative results for diagnosis).

7.2.3   Interventions modelled based on diagnostic tool

The current intervention modelled in this study was a culling scenario. Exclusively applied on the positively detected dogs by the diagnostic tool (at rate DP = $[I_H d +(S+E+I_N+I_L)(1-d_z)]$ σ), correctly or not, the model evaluates the impact by the proportional reduction in VL transmission rate. <u>These interventions were assumed to be instantaneous after the testing</u>. Culling intervention was applied with various degrees of success. The proportion of positively detected dogs that are removed by the culling program was defined as the parameter κ ranging from 10 to 100%. This leads to the following modifications in the equations:

$$dS/dt = \beta - \lambda\,S - \delta S - \kappa\sigma(1\text{-}d_z)\,S \qquad (1')$$
$$dE/dt = \lambda\,S - i\,E - \delta E - \kappa\sigma(1\text{-}d_z)\,E \qquad (2')$$
$$dI_N/dt = i\,(1\text{-}\theta)\,E - \delta_N I_N - \kappa\sigma(1\text{-}d_z)\,I_N \qquad (3')$$
$$dI_L/dt = (1\text{-}\rho)i\theta\,E - \delta_L I_L - \kappa\sigma(1\text{-}d_z)\,I_L \qquad (4')$$
$$dI_H/dt = \rho i\,\theta\,E - \delta_H I_H - \kappa\sigma d\,I_H \qquad (5')$$
$$\beta = \delta(S+E) + \delta_N I_N + \delta_L I_L + \delta_H I_H + \kappa(DP) \qquad (8')$$

Moreover, the course for the intervention was varied in order to determine the time (and the conditions) needed to reach a certain percentage in reduction of transmission to dogs. Here, the basic reproduction number (R0) was not calculated, but the proportional reduction in transmission rates; for which, it was assumed that a minimum 80% reduction would have a significant impact on VL incidence. Before the screening, the population dynamics were run for 20 years without intervention to allow them to reach equilibrium, and allows a better visualisation of the impact of the intervention in the plots. Each intervention was modelled with *(1)* a constant screening rate applied daily over the time course of the intervention and *(2)* a pulse intervention where the number of screenings per year, the screening coverage, and the duration of the screening are variables (as described above). Therefore, the variables in this scenario are sensitivity and specificity of tool, time of intervention, culling rate, coverage and frequency combined in the screening rate (either constant or pulse). Note that these rates are proportions of the total dog population. The reduction in transmission is always measured as the percentage difference in the transmission after the intervention versus before the transmission at equilibrium. The impact of super-spreaders removal was compared to current culling intervention where all infected dogs were removed independently of their infectiousness.

Table 7.1 – Definitions and estimates of parameters used in this model			
Variable	**Definition**	**Estimates**	**References**
F	Total number of sandflies	50000	Assumed value
N	Total number of dogs	1000	Assumed value
S	Number of susceptible dogs	*Variable*	–
E	Number of exposed dogs (latency infected)	*Variable*	–
I_H	Number of highly infectious dogs (super-spreaders)	*Variable*	–
I_L	Number of low infectious dogs *(labelled as mildly previously)*	*Variable*	–
I_N	Number of never infectious dogs	*Variable*	–
F	Number of sand flies	*Variable*	–
β	Birth rate	*Defined above*	–
λ	Transmission rate	*Defined above*	–
C	Vectoral capacity	*Defined above*	–
δ	Natural death rate	0.0011/day	Courtenay et al., 2002b
$δ_N$	Death rate of never-infectious dogs	0.0011/day	Courtenay et al., 2002b
$δ_L$	Death rate of low-infectious dogs	0.003006/day	Courtenay et al., 2002b
$δ_H$	Death rate of high-infectious dogs	0.003006/day	Courtenay et al., 2002b
θ	Proportion of infected dogs that become infectious	0.43	Courtenay et al., 2002b
ρ	Proportion of infectious dogs that are highly infectious	0.39	Courtenay et al., 2002b
p_D	Probability of infected fly transmitting to dog	0.321	Reithinger et al. 2004
p_{VL}	Probability of low-infectious dog transmitting to fly	0.017	Courtenay et al., 2002b
p_{VH}	Probability of highly infectious dog transmitting to fly	0.39	Courtenay et al., 2002b
i	Rate of latency (incubation period of dogs)	0.005/day	Quinnell et al., 1997
a	Biting rate of sandflies on dogs	0.333/day	Dye et al., 1991
τ	Latent period of *L. infantum* in sand flies	7 days	Dye, 1996
μ	Sand fly mortality rate (1/ μ = life expectancy)	0.42/day	Dye, 1996
d	Sensitivity of diagnostic tool	ELISA: 0.99 RDT: 0.61	Chapters 6 and 7
d_z	Specificity of diagnostic tool	ELISA: 0.875 RDT: 0.964	Chapters 6 and 7
σ	Screening rate (including frequency, coverage and duration)	*Variable*	–
κ	Rate of immediate culling	*Variable*	–

7.3 Results

7.3.1 *Transmission model and conditions for diagnostic tool*

The dynamics of the dog population including the three groups of infectiousness (highly, mildly and never infectious) was initially run without any interventions as basic model for VL transmission. The dynamics of the groups are represented in Figure 7.3.
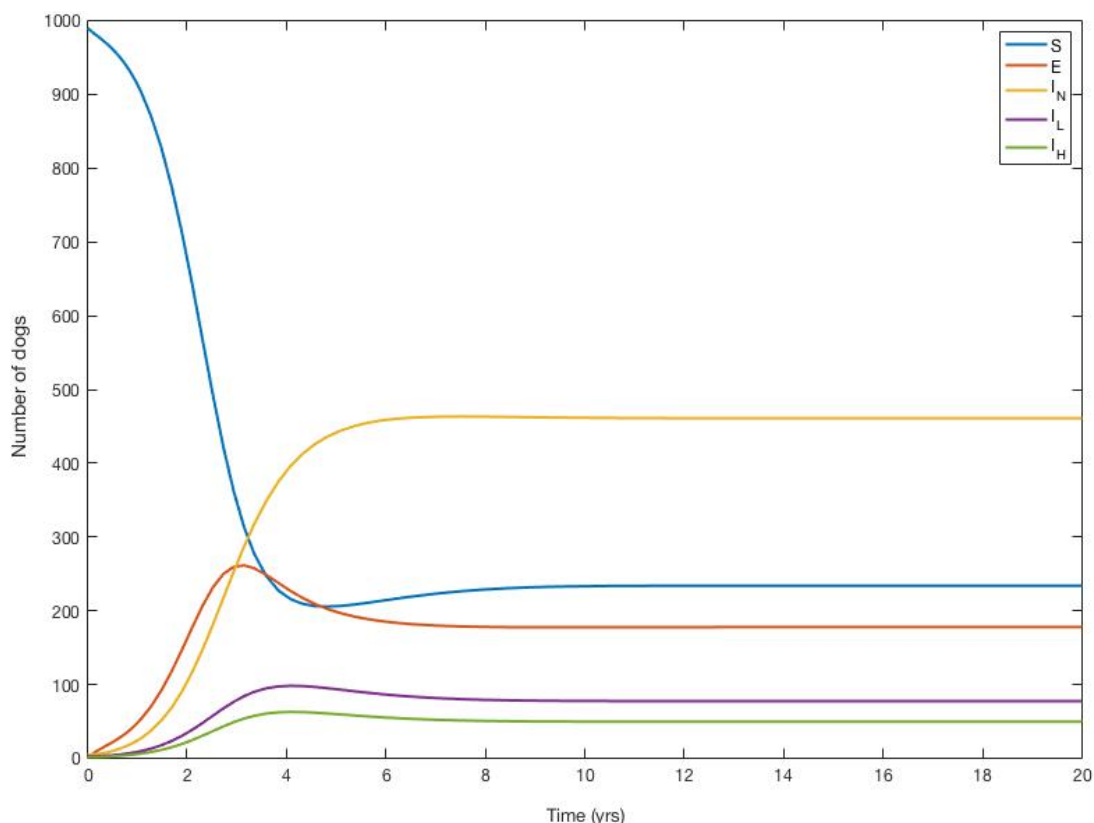
Figure 7.3 – Population dynamics and number of dogs in each category (S, susceptible; E, exposed; I_N, never infectious; I_L, mildly infectious and I_H, highly infectious) over a period of 20 years to reach the equilibrium in the initial population scenario.

In this model, transmission rate was based on estimates for parameters taken from published sources (Table 7.1) based on which, the equilibrium numbers of infected dogs in the model with a sandfly-to-dog ratio of F/N=50 are roughly: E=178, I_N=461, I_L=78, I_H=50, which is a prevalence of (178+461+78+50)/1000=76.7% if all latent dogs are included, or (461+78+50)/1000=58.9% if you exclude all latent dogs. This result is similar to previous prevalence estimated in endemic areas of Brazil (Quinnell et al., 2001; Felipe et al., 2011; Fraga et al., 2012; Quinnell et al., 2013).

7.3.2 Current field screening

The current culling measures applied by the Visceral Leishmaniasis Control and Surveillance Programme, as currently established by the Ministry of Health (MOH) in Brazil, involves all infected dogs independent of their infectiousness. The model was thus adjusted so that the diagnostic tool would include all infected dogs (not only super-spreaders), which modifies the equation (*9*):

$$\text{Total rate of positive detection (DP)}$$
$$DP = [(E+I_N+I_L+I_H).d + S(1-d_z)]\,\sigma \qquad (9')$$

Current testing in Brazil comprises two diagnostic tests (as described in Chapters 1 and 4), first by DPP-RDT, followed by a confirmatory ELISA. Used in sequence, the sensitivity drops to 70% (0.823*0.85) while the specificity of both tests increases to 99.5% (0.928 + [1-0.928]*0.928). Current testing conditions were estimated as follow: (*1*) a pulsed intervention with 1 screening per year, 50% coverage, duration of 14 days for each screening and 50% culling; and (*2*) a continuous intervention with a daily screening rate is 0.0014, representing 50% of the dog population screened over 1-year, and 50% culling of the positively detected dogs. These parameters seem more feasible in the field. Indeed, a 100% coverage in the field is not really achievable as there will always be some people and dogs absent from the village during the screenings and some owners will refuse to take their dog to be screened, or won't be able to make it to the screening location.

Under these conditions, the current MOH recommended test showed little impact on transmission outcomes, with a slight reduction in transmission and a new equilibrium in the dog dynamics (Figure 7.4). Overall, the continuous and pulsed interventions look very similar. The results over time are reported in table 7.2, reaching a plateau phase after 5 years. The reduction in transmission for a pulsed and continuous interventions are 8.36% and 15.95%, respectively. The continuous model shows better performance as the pulsed intervention allows a recovery in transmission between pulses. This suggests that the MOH testing regime are unlikely to be effective to control *Leishmania* infection under current conditions. Note that all figures start with a period of 20 years of equilibrium, without any intervention, and that the percentage of reduction are compared to the non-intervention levels.

**Dynamics of population after 20 years intervention
using current field tool and conditions**

(A) Pulsed intervention
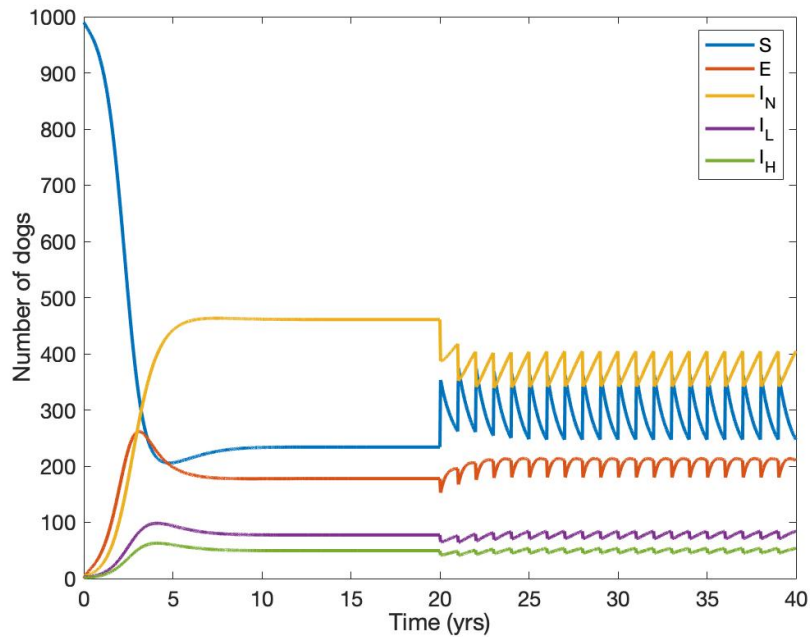
(B) Continuous intervention
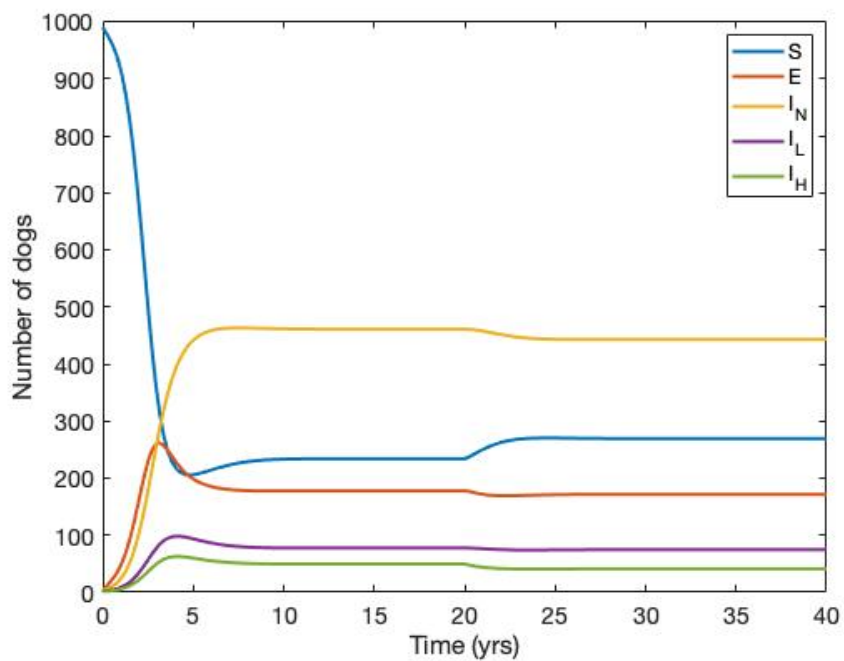
Figure 7.4 – Plot of the population dynamics with 20 years intervention (A) with a pulsed intervention with 1 screening per year, 80% coverage, duration of 2 weeks for each screening and 50% culling (B) with a continuous intervention where the daily screening rate is 0.0014. Screening was based on the current field method : DPP-RDT + ELISA used in this sequence with sensitivity and specificity of 70% and 99.5%. The intervention starts after 20 years of equilibrium.

Table 7.2 – Reduction of transmission rate (%) over time (A) with a pulsed intervention with 3 screenings per year, 50% coverage, duration of 2 weeks for each screening and 50% culling (B) with a continuous intervention where the daily screening rate is 0.0014. Screening was based on the current field method : DPP-RDT+ELISA in sequence. Note that the intervention starts after 20 years of equilibrium.

Time from intervention	Reduction of transmission rate (%)	
	Pulsed intervention	Continuous intervention
1 year	1.80	10.57
2 years	2.19	15.19
5 years	8.18	16.39
10 years	8.36	15.95
15 years	8.36	15.95
20 years	8.36	15.95

7.3.3 Culling scenario with continuous intervention

Going back to the application of the newly-proposed tools, the culling scenario was first modelled with continuous screening regime. The continuous intervention is based on a daily screening rate, a proportion of dogs that need to be screened every day over a certain time course to reach a minimal reduction of 80% in VL transmission rate, which is expected to have a significant impact on VL incidence. Only the dogs diagnosed as positive by the tool (at rate DP) undergo the intervention, which <u>is assumed to be instantaneous</u>. A range of screening rates and the culling proportions were modelled to evaluate their combined impact on the transmission reduction, for interventions over 1, 2 and 20 years (Figure 7.5). From these plots, the effects of RDT and ELISA screening effort and positive dog removal efficiency post testing are explored. For example, to reach a transmission reduction between 80% and 90% with the culling intervention lasting one year and using the RDT as diagnostic tool, several implementation scenarios are possible as summarised in Table 7.3.

Figure 7.5 – Contour plots showing the impact of screening rate and culling rate variations on the transmission rate (colour bar), calculated for 1, 2 and 20 years of intervention, using both the ELISA and the RDT (with their sensitivity and specificity, d and d_z, constant).

Table 7.3 – Interpretation of Figure 7.5 (A) for which, to reach a reduction in transmission of 80 to 90% after 1 year of intervention, optimal conditions of screening and culling rates were calculated. The performance of RDT (d and d_z) was constant.

Example of interpretation of Figure 8.5 (A)			
Reduction in VL transmission	Time of intervention	Screening rate (daily proportion dogs)	Culling rate (% of DP)
80 – 90%	1 year	0.078	20 – 30
		0.061	30 – 40
		0.048	30 – 50
		0.030	50 – 85
		0.023	70 – 100

To determine the optimal screening rate, the reduction in transmission (%) is shown when culling of detected dogs is undertaken at 100% (Figure 7.6). An important result here is that the minimal screening rate to have an impact on transmission is significantly higher than the estimated field screening rate. Indeed, the breakpoint in the transmission for RDT is observed around 0.02 (proportion of dogs screened per day) and for ELISA, it is around 0.01 (Figure 7.6). For both tools, theses screening values are 7 to 14 times higher than the currently estimated values for Brazil. Indeed, in the literature, Costa et al. (2013) reports screening rates of 0.0028 and 0.0021 corresponding, respectively, to 8.3% dogs screened per month (100% of the population per year), and to 6% per month (Costa et al., 2013); whereas Courtenay et al. (2002b) simulated screening rates of 0.0014/day which is 4% per month (50% of the population per year) in their model.

Impact of the screening rate variation on reduction in transmission (%)	
(A) for RDT	(B) for ELISA
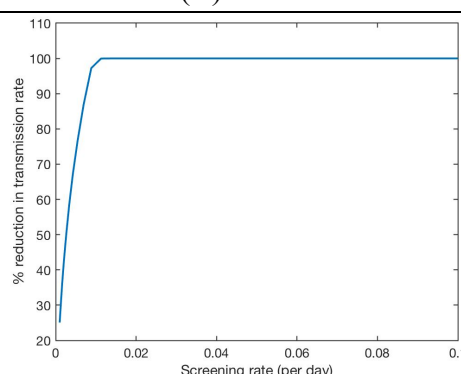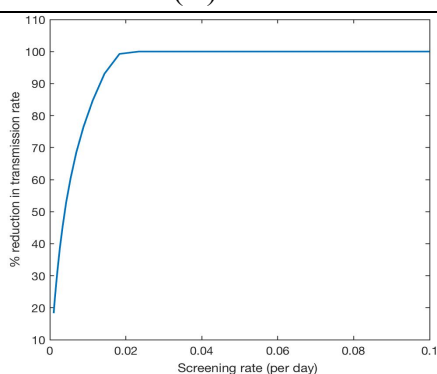
Breakpoint in transmission around a screening rate = 0.02/day

Breakpoint in transmission around a screening rate = 0.01/day

Figure 7.6 – Plot of the screening rate versus the transmission rate, in the optimal culling scenario (100% of DP) while using the RDT (A) or the ELISA (B) as diagnostic method.

For these screening conditions, the population dynamics were modelled over time leading to a considerable decline in the number of the exposed/infectious dogs within 5 years of intervention, while only the healthy dogs (*S*) are remaining (Figure 7.7). The intervention based on ELISA has a faster impact than when using the RDT, though eventually reach a similar reduction in transmission. After 1 month of intervention, the VL transmission rate in dogs decreased by 41.1% using the ELISA and 27.67% using the RDT (Table 7.4). In the short term, the more efficient tool is the ELISA than the RDT. The RDT reached a reduction in transmission of 99% after 10 years of intervention; whereas the ELISA offers a 99% reduction within 3 years of intervention and a complete elimination of VL transmission within 12 years of intervention (Table 7.4). Note that all figures start with a period of 20 years of equilibrium, without any intervention, and that the percentage of reduction are compared to the non-intervention levels.

Table 7.4 – Impact of the time spent in intervention on the reduction in transmission, calculated for both the ELISA and the RDT at a culling rate of 100% and the screening rate set at their breakpoint of transmission. The performance of the RDT and ELISA (*d* and *d_z*) were constant. The percentage of reduction are compared to the non-intervention levels.

Time since start of intervention	Reduction of transmission rate (%)	
	RDT	ELISA
1 month	27.67	41.40
3 months	57.88	74.38
6 months	75.51	87.75
9 months	82.71	92.24
1 year	86.60	94.75
2 years	93.24	98.49
3 years	95.68	99.51
4 years	96.89	99.83
5 years	97.64	99.94
10 years	99.12	99.99
15 years	99.60	100
20 years	99.80	100

Dynamics of population for culling rate at 100% after 20 years intervention

RDT
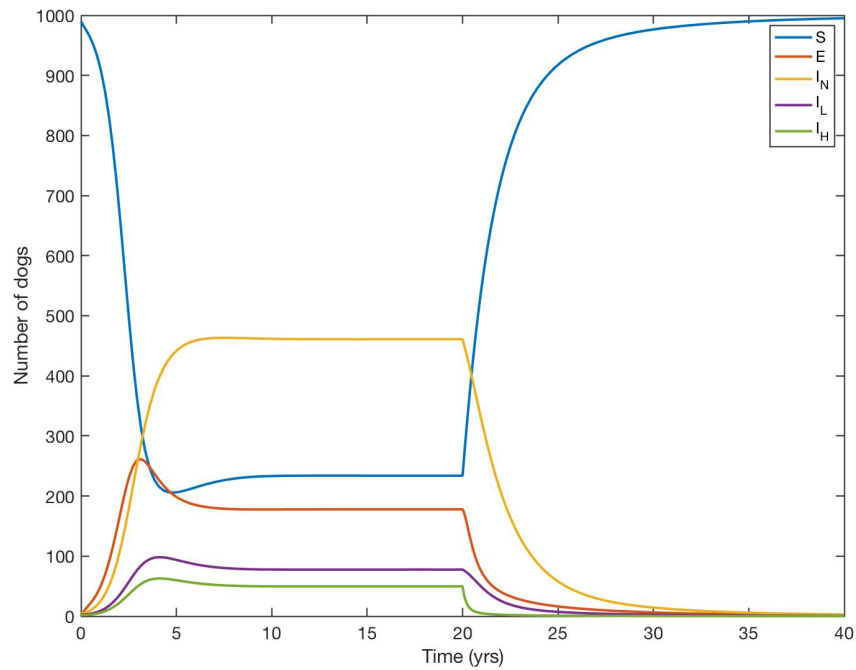
ELISA
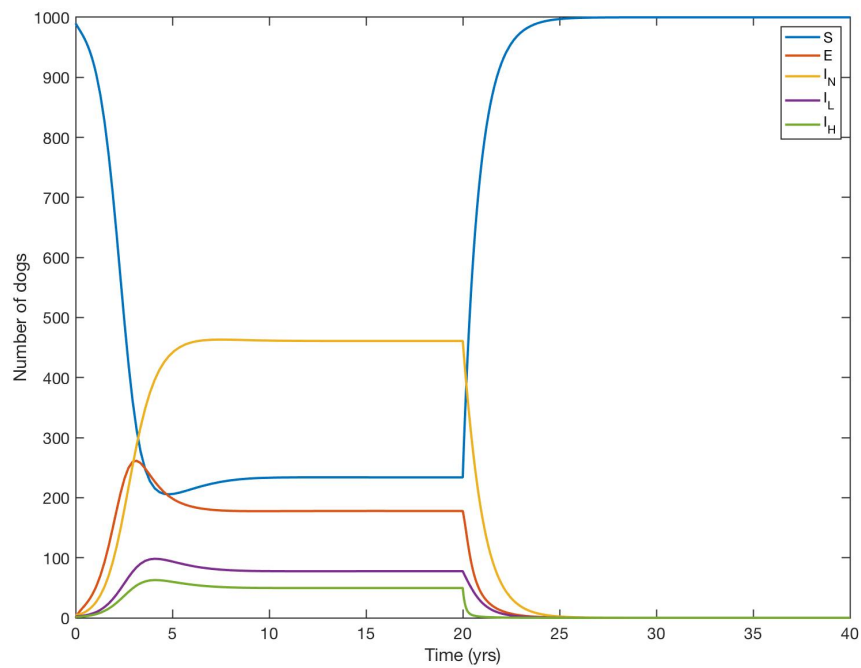
Figure 7.7 – Plot of the population dynamics over time when applying the culling on 100% of the positively detected dogs, with a screening rate is set at the breakpoint of transmission. Screening performed with RDT (A) and ELISA (B) using their fixed sensitivity and specificity. Note that the intervention starts after 20 years of equilibrium.

7.3.4 *Pulsed intervention for the culling scenario*

For implementation, continual diagnostic screening of the dog population is an unlikely scenario. Therefore, a pulsed intervention was modelled rather than a continuous intervention. For a fixed screening duration (14 days), the screening rate is the product of the coverage and the frequency, where coverage is the proportion of dogs within the population screened over the screening period, and the frequency is interpreted as the number of pulse screenings per year. Thus, the screening rate will increase either by increasing the number of screenings or the coverage in each screening, or both. A first simulation was performed for one pulsed intervention per year for 20 years with coverage of 100% and culling of 100%, offering a reduction in transmission of 29.88% for ELISA and 24.55% for RDT. In Figures 7.8, the pulse effect can be observed. Between each pulse, the population recovers meaning that the transmission of VL increases in the interim period. To determine the frequency (number of pulses) and the coverage needed to reach a significant reduction in transmission, these parameters were modelled at a range of values to identify the most efficient combination (Figure 7.9). Note that coverage was varied between 40% to 90% of the dog population over the course of each screening; for example, a coverage of 50% corresponds to a proportion of 0.0357 of the total dog population to be screened daily over the 14 days pulse screening period. The impacts on transmission for a pulsed intervention applied over 5 years, assuming the culling intervention occurs to 100% of the positively detected dogs, is shown in Figure 7.9. Results show that even one screening per year is sufficient to have a little impact on the transmission, even if not significant (less than 20% reduction). To be significant, more screenings need to be performed annually. Under the defined conditions, 5 screenings per year with the ELISA diagnostic would result in at least 70% reduction in transmission independently of the coverage; whereas, a minimum of 7 or 8 pulse screenings are needed for the RDT for the same results. This is due to the higher sensitivity and specificity for the ELISA. Based on these figures, different possibility of intervention with a significant impact on transmission can be created (assuming 100% of detected dogs undergo intervention). In the pulsed interventions, there is recovery in the interim period between pulses. Thus, it takes longer than continuous interventions to reach the same impact. For example, an intervention applied for 5 years needs a minimum of 5 screenings per year to have any impact on transmission; whereas in the same pulsed intervention over 20 years, the

coverage and the frequency of screening required to reduce transmission by the same amount decrease substantially.

Dynamics of population with 1 annual pulsed intervention for 20 years

RDT
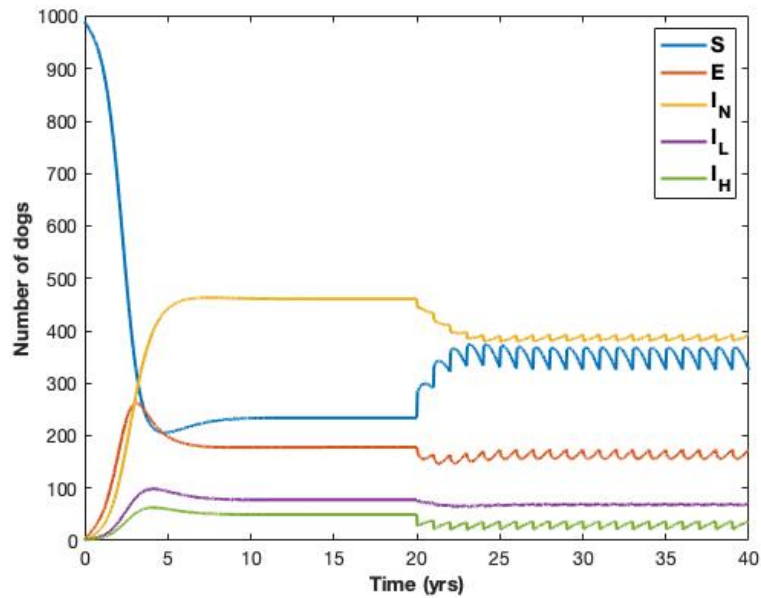
ELISA
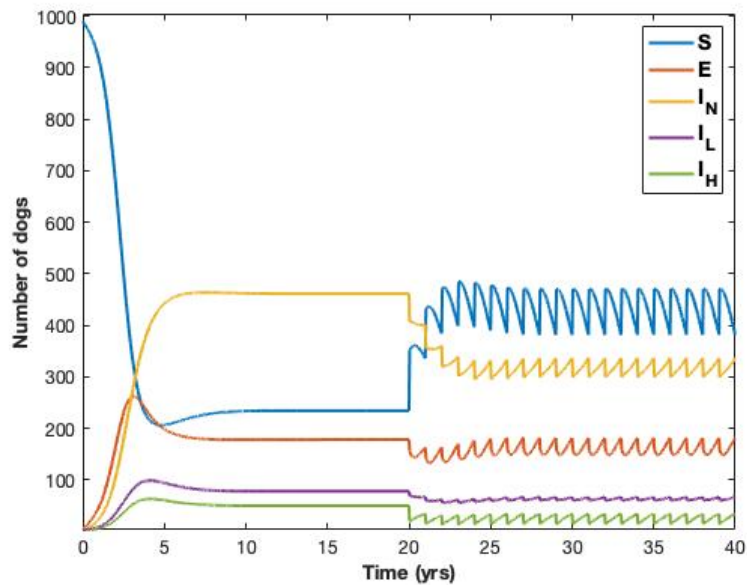
Figure 7.8 – Plot of the population dynamics over time when applying one pulse intervention, a coverage of 100% and a culling of 100% of the positively detected dogs. Screening performed with RDT (A) and ELISA (B) using their fixed sensitivity and specificity. Note that the intervention starts after 20 years of equilibrium.
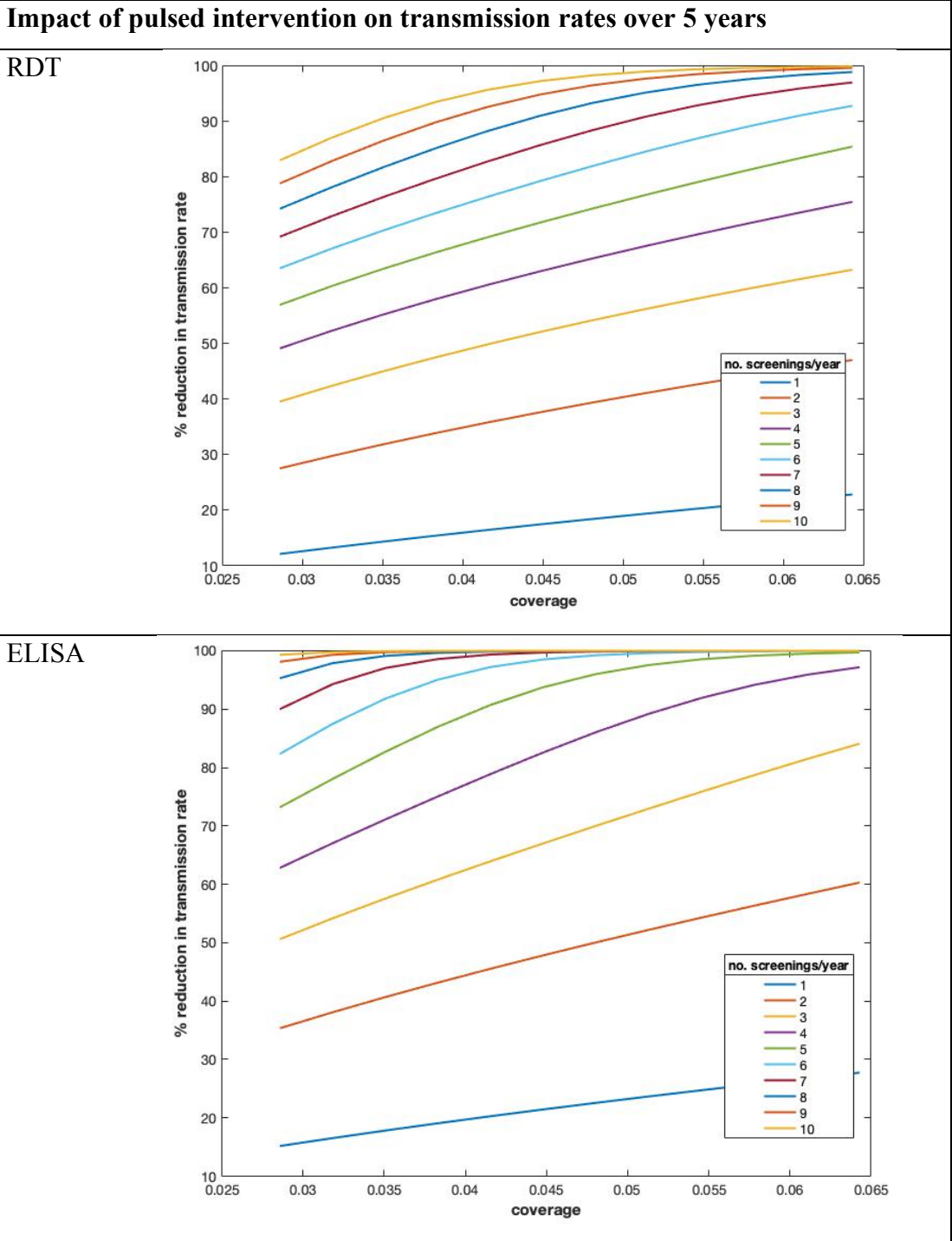
Figure 7.9 – Plots of the impact of the pulsed intervention on the reduction in transmission while varying both the coverage and the number of screenings, for (A) RDT and (B) ELISA, when the pulsed intervention is applied for 5 years, assuming that 100% of the detected dogs undergo the intervention.

The impact of dog removal post identification also influences transmission. This effect was also measured for pulse interventions of different screening frequencies. The heatmaps are represented for ELISA and RDT over 5 years of intervention (Figure 7.9). As 4-dimentional plots are difficult to interpret visually, heatmaps were present for fixed coverages of 50% and 100%, varying the number of screenings per year between 1 to 10, and the proportion of diagnosed dogs culled between 0 to 1. Plots indicate the culling proportion needed to reach a reduction in the transmission rate. Overall, results show that the culling proportion can be low as long as the number of screenings per year and the coverage are high. This is due to the instantaneous intervention; results would be different if a delay is included (discussed in section 7.4).

Impact of culling on the transmission rate after 5 years

(A) For RDT
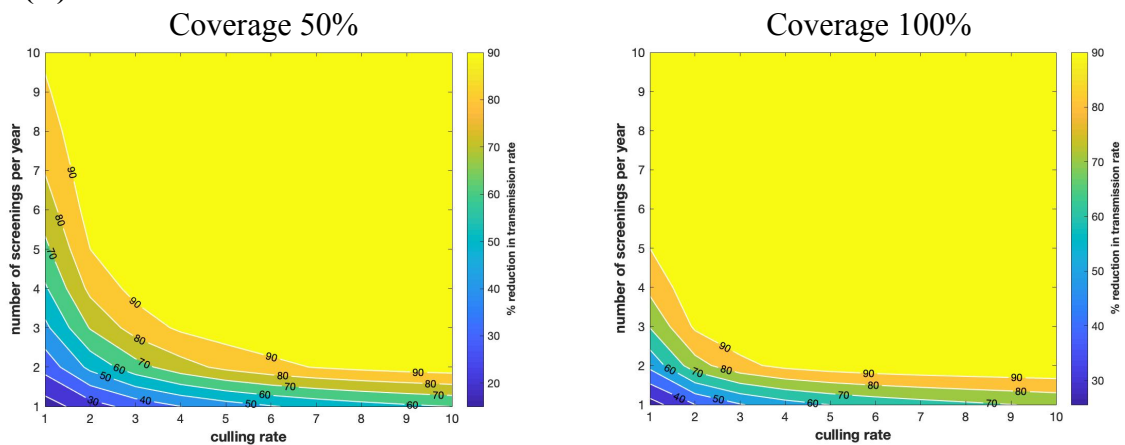
(B) For ELISA
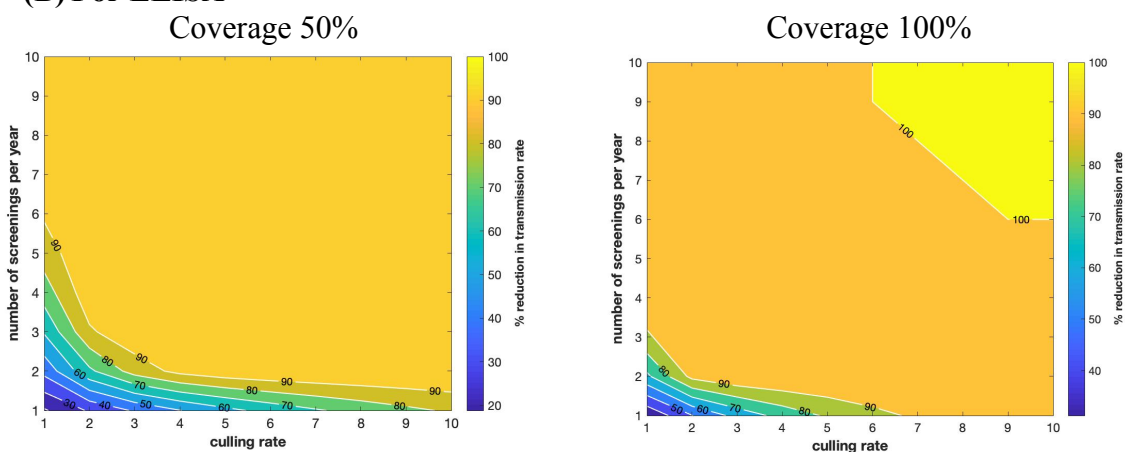
Figure 7.10 – Impact of culling proportion on the transmission rate after 5 years of pulse intervention with variable range of culling, number of screenings per year and coverages of 50 and 100%. Screening tool were (A) RDT and (B) ELISA, with constant performances.

Finally, a last simulation was performed assuming a coverage of 100%, a culling of 100% and 5 pulse screenings per year to observe the impact on the intervention over time. Results were reported below in Table 7.5 for a period of 20 years. The associated plots of the population dynamics over 10 years with 5 screenings per year are shown in Figure 7.11.

Table 7.5 – Impact of the time spent in intervention on the reduction of transmission, calculated for both the ELISA and the RDT at a culling rate of 100%, a coverage at 100% and 5 screenings per year. The performance of RDT and ELISA (d and d_z) were constant. Note that the percentage of reduction here are compared to the non-intervention levels.

Time since intervention	Reduction of transmission rate (%)	
	RDT	**ELISA**
1 year	75.66	87.39
2 years	85.19	94.47
3 years	88.32	97.05
4 years	89.56	98.31
5 years	90.03	99.00
6 years	90.11	99.39
7 years	90.01	99.63
8 years	89.83	99.77
9 years	89.63	99.86
10 years	89.43	99.33
15 years	88.86	99.99
20 years	88.75	99.99

Dynamics of population after 10 years of pulse intervention
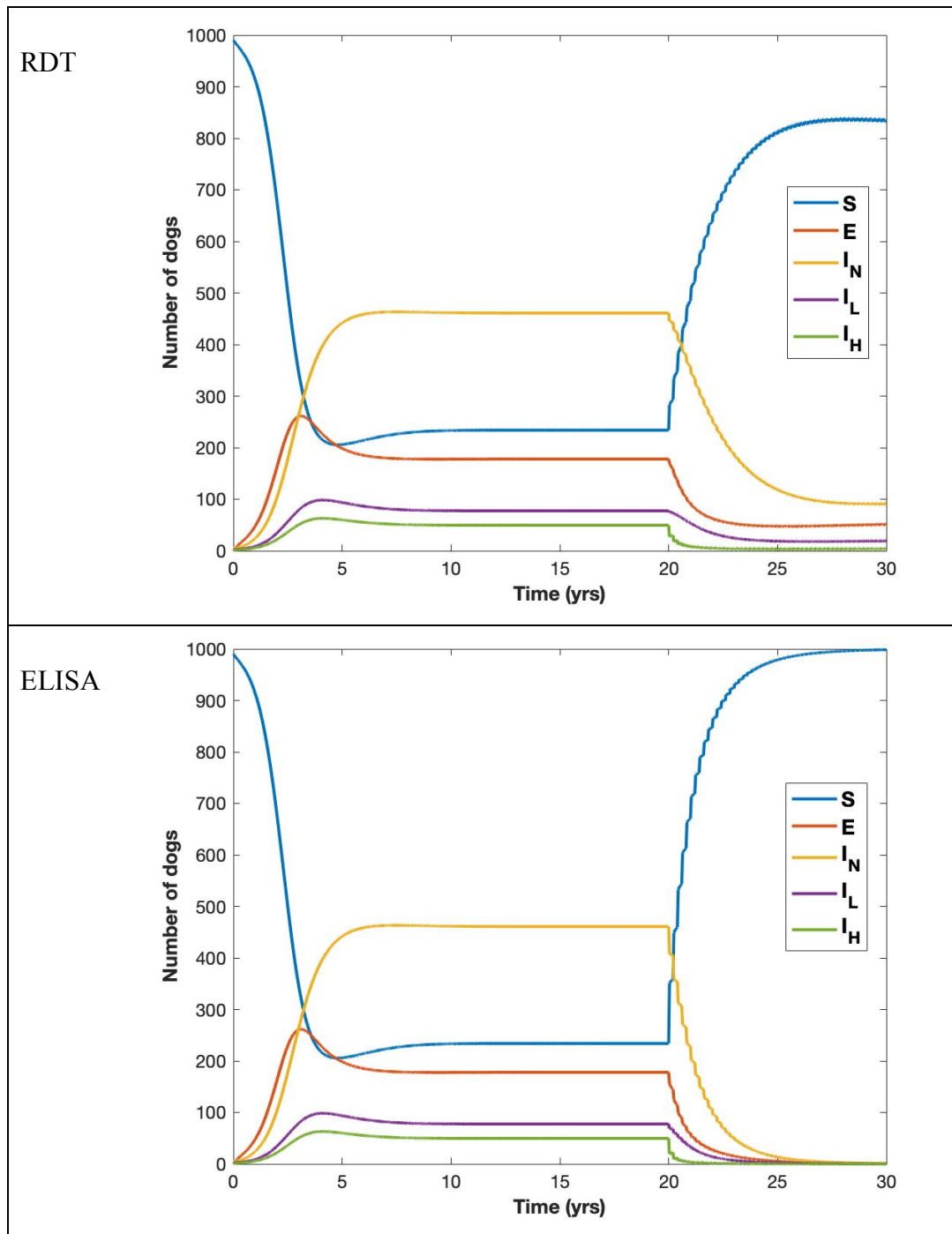
Figure 7.11 – Plot of the population dynamics over time when applied a pulse intervention is applied for 10 years with a culling rate of 100%, a coverage at 100% and 5 screenings per year. The performance of the RDT and ELISA (*d* and *d*$_z$) were constant. Screening performed with RDT (A) and ELISA (B) using their fixed sensitivity and specificity.

7.3.5 Comparative analysis : field versus novel screening

The current MOH tool (DPP+ELISA) under a regime described in section 1.3.2 was compared to the threshold-based tests (KL914-RDT and KL914-ELISA) in their optimal conditions developed above. As a reminder, conditions were (*1*) a pulsed intervention with 5 screenings per year, 100% coverage, duration of 2 weeks for each screening campaign and 100% culling; and (*2*) a continuous testing scenario at a culling rate of 100% and screening rate of 0.02. Note that, once again, the continuous intervention is based on a daily screening rate which correspond to a proportion of dogs that need to be screened daily over a certain time course to reach a minimal reduction in VL transmission rate. Indeed, a screening rate of 0.0014 is equivalent to 50% of the whole population screened over 1year; and a screening rate of 0.0028 equivalent to 100% of the whole population screened over 1 year. Results for the comparative study suggest that there was a significant difference between the performance of the current tools (which targets all infected dogs) and the KL914-RDT and KL914-ELISA targeting only super-spreaders (Table 7.7).

Table 7.6 – Comparative table of the newly-proposed ELISA and RDT, versus the current field screening in their respective conditions; and their impact of the time spent in intervention on the reduction of transmission. For the ELISA and the RDT, the pulse intervention involves 100% culling rate with a coverage at 100% and 5 screenings per year whereas the continuous testing had a screening rate at 0.02 and the culling rate of 100%. For the current tests, the coverage is 50% to represent the reality of screening in the field.

Comparative study of the reduction of transmission rate over time						
	Pulse intervention			Continuous intervention		
Time	RDT	ELISA	Current[1]	RDT	ELISA	Current[1]
1 year	75.66	87.39	1.80	86.60	94.75	10.57
2 years	85.19	94.47	2.19	93.24	98.49	15.19
5 years	90.03	99.00	8.18	97.64	99.94	16.39
10 years	89.43	99.33	8.36	99.12	99.99	15.95
15 years	88.86	99.99	8.36	99.60	100	15.95
20 years	88.75	99.99	8.36	99.80	100	15.95

[1] Current field screening tools : DPP-RDT + ELISA used in this sequence.

However, if the screening conditions are improved drastically for the current field tools (DPP-RDT and ELISA in sequence) to 5 screenings per year, 100% coverage, duration of 2 weeks for the pulse intervention; and, to a screening rate of 0.02 for the continuous scenario. Results suggest that there was no significant difference between the performances. Although the newly-proposed methods (ELISA and RDT) would apparently be the same as the current strategy (if occurring that they were applied properly in the field), in practice the newly-proposed tools are better as fewer dogs are killed to reach the same reduction in transmission (as discussed below).

7.4   Discussion

The detection of super-spreaders is aimed to lead to a reduction in transmission and the proportion of new canine infections within the reservoir population, as modelled for most infectious diseases (Woolhouse et al., 1997; Lloyd-Smith et al., 2005; Baggaley et al., 2006). In ZVL, given the performance of the newly-proposed tools to identify super spreaders in a cohort of dogs (described in Chapters 5 and 6), the requirement for mathematical modelling arises due to the nature of endemic canine populations that at any one time will comprise dogs in all stages of the infection development. For example, the average window of opportunity to detect super spreaders before their onset of infectiousness is relatively short, thus suggesting that both the screening frequency and coverage to identify super spreaders need to be carefully quantified. The other important reason for modelling is to simulate logistically feasible and theoretical implementation scenarios, including parameters such as testing frequency, testing regime (pulsed versus continuous), coverage, and positive dog culling efficiency. The above-described mathematical model was developed as a deterministic compartmental model incorporating the infectiousness groups with focus on super-spreaders, the novel diagnostic tools (KL194 in RDT format and ELISA), and various culling interventions.

First of all, the optimal conditions were defined for the newly-proposed detection tools, and their impact evaluated on the proportional reduction in transmission. In the continuous screening scenario over one year, screening rates (proportion of dogs screened) required to reach 80% reduction in transmission were between 0.02 and 0.01 per day, which is 7 to 15 times higher than the values estimated in Brazil. In the literature, Costa et al. (2013) reports screening rates of 0.0028 and 0.0021

corresponding, respectively, to 8.3% dogs screened per month (100% of the population per year), and to 6% per month (as tested here); whereas Courtenay et al. (2002b) simulated screening rates of 4% per month (50% of the population per year) in their model. Under these screening conditions, the impact on transmission was considerable. Indeed, a 90% decline was observed in the number of the exposed/infectious dogs within 5 years when applying the ELISA and RDT to detect super-spreaders. After only 1 month of intervention, canine transmission decreased by 41.1% using ELISA and 27.67% using RDT. Similarly, for a pulse intervention applied of 5 years, a minimum of 5 screenings per year proved at least 80% reduction in transmission (which was assumed to be significant). Based on these results, an increased rate of testing e.g. quarterly testing each year and high coverage, are required to generate a downward trend in transmission. However, in reality, community continual screening is not conducted, nor will it ever be for logistic reasons. Brazilian campaigns to detect infected dogs are conducted as a pulse (cross-sectional) surveys, and at non-systematic frequencies, particularly as the control program is decentralised and each municipality is autonomous in their control activities. Thus, simulations of pulse screening are the more realistic, though the optimum frequency and coverage requires precise identification; by nature, pulse screening is not expected to be as efficient as continuous screening since the infectious population can recover in the inter-pulse screening intervals. As the modelling shows the length of time for which the pulse interventions need to be sustained to reach similar benefits as continuous culling is prolonged.

Contrastingly, simulations of the current MOH screening tool (DDP+ELISA) within field testing regime, targeting canine infection rather than infectiousness, showed that the protocol was perform poorly: neither pulse nor continuous testing regimes resulted in significant reductions in transmission. However, assuming an improved testing efficiency in comparative simulations applying the KL914-RDT and KL914-ELISA designed to detect super-spreaders, versus the Brazilian DPP+ELISA test for infection, resulted in significant (>80%) reductions in transmission, sooner by continuous testing than by pulse testing as expected, with surprisingly no substantial differences among the three tests when used in optimal conditions (compared to the current field regimes). Although the newly-proposed methods (ELISA and RDT) would apparently be the same as the current strategy; practically, the newly-proposed tools offer the advantage to reduce drastically the number of dogs killed to reach the same reduction in

transmission as for the current tools. Indeed, over 176,000 dogs were previously eliminated from 1990 to 1997 and more than 160,000 dogs from 2003 to 2005 using the field tools to detect seropositivity (Ministerio da Saude, Brazil, 2006; Lemos et al., 2008; Romero et al., 2010; Harhay et al., 2011); whereas the novel tools are focusing on a much small proportion of the infected dog population, and only 20% of the actual number of dogs killed would be removed in these conditions.

The model, as developed in this Chapter, represents the initial steps in capturing dog dynamics, making simplifying assumptions for modelling convenience. The structure of the model, itself, needs discussion. The exposed box (E) included latent infected dogs, therefore all are infected dogs, but not all of them will have seroconverted at this stage. This will influence the performance to detect infection, as tests are based on serology. It would also be possible to improve the model by including additional "exposed" compartments to represent three classes of infectious dogs (EI_N, EI_L, EI_H) as a convenience to apply the diagnostic. Similarly, the model performance would be improved by including a separate detection parameter for the low and mildly infectious dogs in the model, as the onset of infectiousness is likely to be different compared to highly infectious dogs. However, the choice of model structure, as currently designed, was because super-spreaders were the absolute target of the diagnostic tool. The values of detection of low and mildly infectious were not determined in this project. Consequently, sensitivity and specificity were specifically determined towards the highly infectious group (I_H). In previous chapters, the method to determine performance of detection was measured based on ROC using the super-spreaders as the positive group and the never-infectious dogs as the control group. However, these never-infectious dogs are now included I_N box. Anything else was assumed to be equally mis-diagnosable, at rate 1-d_z, including S, susceptible; E, exposed; I_N, infected and non-infectious; and I_L, infected and mildly infectious.

Moreover, a major assumption of the modelling exercise was that the intervention was applied instantaneously on detection of positive dogs. This simplifying assumption was made for modelling convenience; including a delay between diagnosis and culling would be more realistic by defining delay differential equations. Time between detection and culling is estimated between 80 and 180 days (Braga et el., 1998; Vieira et al., 1998). A previous mathematical model demonstrated that such delays lead to the

failure of the current culling intervention independently of the diagnostic tools used (Courtenay et al., 2002b). A study from Braga et al. (1998) showed that dog seroprevalence was reduced by 27% when the culling intervention was applied 7 days after diagnosis, whereas it was only reduced by 9% when the delay reached 80 days. Similarly, while modelling different scenarios, Courtenay et al. (2002b) demonstrated that only a diagnostic tool with high sensitivity and no time delay between diagnosis and culling theoretically would result in significant reductions of transmission. When the interval was increased to 120 days, simulations showed that the reduction in transmission coefficient was minimal. Even if the use of a highly performant diagnostic test could slightly compensate the intervention delay, the proportional reduction of infectious dogs was higher than when there is no delay, with results of 0.04–0.16 compared to near zero. The optimal outcomes were achieved where there was no delay between testing and infected dog removal (Courtenay et al., 2002b). This makes the case for using a specific RDT.

The current model structure also assumes a stable dog population where death equals birth with immediate replacement; dogs lost through natural mortality or culling are replaced into the susceptible class. The model does not account for delays in canine replacement, estimated to range from 0 to 19 months (mean: 120 days) (Nunes et al., 2008). Furthermore, it does not account for a proportion of replacement dogs being potentially infected on arrival (Moreira et al., 2004; Andrade et al., 2007). Most often these replacements are young dogs or puppies (Nunes et al., 2008), and therefore more likely to be uninfected on arrival. However, not exclusively so, as one study estimated 30.6% of replacement dogs tested *Leishmania* seropositive at first follow-up post their arrival (unspecified interval) (Nunes et al., 2008), suggesting that at least a proportion are likely to have imported infection. Thus, new recruits in the model need to be appropriately distributed across infection classes. An improvement of the model would be to add "delay differential equations" for the loss and the replacement of dogs, and also to account for delays between screening and removal (as mentioned above). More generally, a sensitivity analysis of all these parameters should be conducted to identify which parameters in the model are likely to lead disproportionately to model uncertainty. For example, a recent sensitivity analysis in a model of canine VL identified that the infection status of replacement dogs as a key parameter in addition to sand fly related parameters (Buckingham-Jeffery et al., 2019). The last assumption

made in the model was relative to sandflies for which all parameters were set as constant. However, a recent model of canine VL have identified components of the vector biology and vectorial capacity as key parameter in modelling transmission (Buckingham-Jeffery et al., 2018).

The model outputs in this chapter represent parameter estimates taken from Brazilian studies (Courtenay et al., 2002b; Dye, 1996; Reithinger et al., 2014; Costa et al., 2013, Nunes et al., 2008 and Quinnell et al., 1997). The simulated heterogeneities in canine population infectiousness was parameterised from longitudinal studies in Brazil where 80% of transmission events from dogs to sandflies was due to relatively few dogs (Courtenay et al., 2002b). Although the concept of super-spreaders is not new, future models should vary the proportions used here. For example, meta-analysis of the available canine infectiousness data for the New and Old Worlds, indicated that the proportion of infectious dogs in the infected dog population may be higher in European than in Brazilian studies, 0.86 and 0.45 respectively (Quinnell and Courtenay, 2009), perhaps related to a higher susceptibility of the vector in Europe than in Brazil (Quinnell and Courtenay, 2009). Thus, model predictions for European scenarios could be contrasted to the Brazilian settings.

Currently, there are no other transmission and intervention models of ZVL including heterogeneities to identify specifically the class of super-spreaders. Examples of other infectious disease models where control of super-spreaders was modelled showed that targeting super-spreaders can be highly beneficial, and helps validate the rationale for the current study (as discussed in the general introduction). One example is a retrospective analysis of control measures for Middle East Respiratory Syndrome (MERS) (Lee et al., 2016). Results suggested that when control measures were focused on containing infections from super-spreader class, the outbreak duration and size were remarkably reduced. Other studies on various infectious diseases from Anderson and Medley, (1985), Woolhouse et al. (1997), Lloyd-Smith et al. (2005), Baggaley et al. (2006), Galvani (2003) and more recently, Skene et al. (2014) showed that targeting super-spreaders for therapeutic or preventive measures is assumed to increase the efficacy of interventions while failure to target these weakens efforts to achieve herd immunity by vaccination and also severely limits the ability to reduce disease at the population level. Targeting super-spreaders seems thus to be practically effective.

Finally, this model focused on a culling scenario of dogs; however, post-identification, further scenarios are possible. In Brazil, treatment with human-designed drugs has recently been permitted as an option to culling for infected dogs (Ministerio de Saude, 2016). Treatment of positively detected dogs rather than their removal will reduce turnover of the population including potential infected entering the population. In reality, infected dogs do not spontaneously recover (Quinnell et al., 1997), and treatment does not lead usually to complete cure (Noli and Auxilia., 2005; Saridomichelakis et al., 2005b; Pineda et al., 2017). Thus, models need to be developed to incorporate possible scenarios. Parasite clearance after treatment ranges from 10% to 80% with an average clearance of around 30% (reviewed by Noli and Auxilia., 2005). For example, treatment with marbofloxacin decreased the parasite load in 72% of dogs, from 6.2±3.4 to 4.7±3.41 units (Pineda et al., 2017), being a reduction of 25% of the total parasite loads. As treatments reduce the parasite levels without clearing it completely, the assumption was that there could be an impact on the intensity of the infectiousness due to strong positive correlation between parasites load and the super-spreaders (described in previous chapters and elsewhere). Therefore, for modelling purposes, identified highly infectious dogs might be optionally moved into a lower infectious class, under the assumption that some will be treated. In the model of Dye (1996), treated dogs were moved to an additional box labelled "resistant dogs" which is not supported by the data on the effectiveness of treatment. Gradoni et al. (1987) was the first to report significant reductions in infectiousness to sand fly vectors resulted from treating dogs with antimonials. Afterwards, several other studies also demonstrated a considerable reduction of the infectiousness of infected dogs within a few months, and up to 150 days post-treatments (Alvar et al., 1994; Guarga et al., 2002; Ribeiro et al., 2008; Miro et al., 2011 and da Silva et al., 2012). A recent trial published in Brazil (De Mari et al., 2017) concluded similarly the non-infectivity of dogs to sandflies for three months after miltefosine treatment, corresponding to significant reductions in parasite loads in bone marrow (De Mari et al., 2017). However, all reported impacts of treatment on infectiousness were significant for limited periods of time only, treatment being short-lived and not effective for a complete cure of parasite infection (as reviewed by Travi et al., 2018). The frequent relapses due to the incomplete elimination of *Leishmania* parasites in treated dogs (Saridomichelakis et al.,

2005b; Noli and Auxilia, 2005) is also not taken into consideration in mathematical models of canine VL. To start addressing this question, the model was adjusted for therapeutic treatments with movements of identified dogs into the lower infectiousness class. Simulations of treatment was a continuous scenario, the breakpoint in transmission was determined for a treatment of 100% efficacy for a screening rate of 0.03/day for the RDT and 0.01/day for the ELISA, which are similar to the culling data. Using these screening rates, the population dynamic was modelled with 100% efficacy (data not shown). The super-spreaders were eliminated within 1 or 2 years, which was a faster impact than the culling. However, while using ineffective treatment (i.e. 30%), the transmission dropped for the first couple of years before rising again to reach a new lower equilibrium in the transmission dynamic.

To conclude, the results from this chapter are considered as outcomes of preliminary model development and thus should be treated with caution. We must not forget that some interventions may be far easier to deploy in the field than others. Further work is needed to address model structure and to correctly incorporate dog dynamics. Nonetheless, the modelling has provided initial insights into super-spreader detection requirements and the important gaps in the knowledge of some of these epidemiological processes. Although the newly-proposed method appears to be similar as the current strategy; practically, it offers the advantage to reduce drastically the number of dogs killed to reach the same reduction in transmission as for the current tools. Indeed, over 176,000 dogs were previously eliminated from 1990 to 1997 and more than 160,000 dogs from 2003 to 2005 using the field tools to detect seropositivity (Ministerio da Saude, Brazil, 2006; Lemos et al., 2008; Romero et al., 2010; Harhay et al., 2011); whereas the novel tool is focusing on a much small proportion of the infected dog population, and only 20% of the actual number of dogs killed would be removed in these conditions.

CHAPTER 8   General discussion

8.1   Summary of findings

Despite the large range of strategies to reduce the transmission of *Leishmania infantum*, the incidence of the infection has remained high and unchanged since 2001 with an average of 3,300 new human cases each year in Brazil (WHO Report, February 2018). As described in Chapter 7, a mathematical model has been developed to evaluate the effectiveness of current dog culling strategy for limiting transmission of *Leishmania infantum* infection. The model predicts that the current strategy of testing-and-slaughtering dogs, which are assumed to be infected based upon available serological tests for infection, is not sufficient to eliminate VL transmission in the canine population. This is corroborated by Ashford et al. (1998); Costa and Vieira (2001); and Courtenay et al. (2002b). As discussed throughout this thesis, current antigen-based serological tests for *Leishmania* are deployed to identify infected dogs, reflecting the presence of antibodies in blood. The IgG detection can be variably interpreted as exposure, current infection, latent infection, or after-cure (as discussed in Chapter 3). The existence of super-spreaders hinders the control interventions. Targeting infectious dogs would be more effective against VL transmission than the current mass slaughter of the infected canine population (Courtenay et al. 2002b). To date, xenodiagnosis is the only method that provides conclusive method to determine infectiousness, but is difficult to set up in the field as requiring specific material and is time-consuming. To contribute to solving the issue, this PhD project aimed to develop a high-performance diagnostic tool to identify super-spreaders of *Leishmania* in the canine population, which are expected to be responsible for most of the transmission of infection from dogs to sand flies, and thus to humans. By immunological test, current and novel antigens were tested on a sera collection from a naturally infected dog cohort in Brazil for which samples were collected longitudinally.

One of the major findings in the project is the potential of serodetection of infectiousness, which was demonstrated for the first time in Chapter 4 and is in contrast with the study of de Mendonca et al. (2017) which failed to detect dogs that transmit *Leishmania* to the sandfly vector using serological tests. Highly infectious dogs present

higher levels of antibodies which were detected by the *Leishmania* antigen-based ELISA. Results indicated that the super-spreaders could be detected within the mixed population. The carefully selected-threshold allowed a specific detection of target. Furthermore, the test allowed a detection of the dogs before a large fraction became infectious. Among the existing antigen, rK28 out-performed all the others. Assuming that threshold-based antigens can detect super-spreaders (as shown in Chapter 4) and that infectiousness correlates with parasite loads, the threshold-based antigen would also detect infectious dogs with high and low parasite loads. In spite of this, a major concern was the current multiple purposes of this antigen in the field. Indeed, the rK28 antigen is currently used in the Dual-Path Platform to screen for canine infection (Almeida et al., 2017), and as confirmatory test of disease in human as well as in dogs, resulting into confusion for field workers and case management (Ministerio de Saude, 2016; Chapter 4). Therefore, an alternative addressed in this project was to develop a novel protein designed specifically for the detection of super-spreaders, named KL914 (Chapter 5). The conception of the first rapid diagnostic tool for the detection of super-spreaders is a challenge that was partially resolved in this study through the design of a brand-new protein, and its adaption into a field-friendly tool. The current RDT development represents the initial prototype with potential for improvement, as discussed in Chapter 6.

Using the newly-proposed diagnostic tool, implementation scenarios simulated by modelling suggested that even given a high-performance diagnostic tool to identify super-spreaders, the effort required in the field needs to be drastically increased to reach a significant decrease (>80%) in transmission within the dog population. As discussed in Chapter 7, elimination of transmission from dogs using current tests recommended by MOH (Dual-Path Platform for screening, followed by ELISA as confirmatory test) requires continued testing and infected dog removal (treatment or culling) to maintain low incidence. The RDT offers an alternative strategy: concentrating intervention measures targeting only dogs responsible for maintaining $R_0 > 1$, thus reducing the number of seropositive dogs sacrificed (Moreno and Alvar, 2002). Implementation is nonetheless complex and identifying a small select group of dogs in the mixed population would probably require high coverage screening.

8.2 Global discussion and context of this project

One of the major general gaps in the scientific knowledge concerns the causes of infectiousness. Canine infectiousness is associated with more severe clinical signs of the disease, high IgG antibody titers and high parasite loads (Courtenay et al., 2002b; Courtenay et al., 2014; da Costa-Val et al., 2007; Guarga et al., 2000; Magalhães-Junior et al., 2016; Borja et al., 2016; de Sousa Gonçalves et al., 2016). Even if the reasons why certain dogs become super-spreaders and infect disproportionately large numbers of contacts still remains unclear (discussed in Chapters 1 and 4), one hypothesis is that highly infectious dogs have larger parasite loads compared to mildly and non-infectious dogs, making them more attractive to sandflies. There is, indeed, a positive relationship between the level of parasite loads and the intensity of the infectiousness with time since infection, where highly infectious dogs have higher parasite loads in skin, in hair and in bone marrow (Courtenay et al., 2014; de Sousa Gonçalves et al,. 2016).

The current approach to controlling transmission in Brazil focuses on dogs as the reservoir, assuming that humans are a dead-end host. However, a recent paper from Ferreira et al. (2018) demonstrates that humans can transmit *L. infantum* to the vector, including asymptomatic VL cases, and HIV-VL co-infected patients, as noted in previous studies in Europe by Molina et al. (1999), Alvar et al. (1997) and Alvar et al. (2008). Xenodiagnosis was positive in 5/20 patients negative for HIV and 9/20 positive for HIV (Ferreira et al., 2018). Among asymptomatic patients, 4/19 of patients without HIV infected sand flies, and one asymptomatic patient with HIV had a positive xenodiagnosis (Ferreira et al., 2018). Despite the small sample, this study supports previous a report on human infectiousness with *L. infantum* in Brazil (Costa et al., 2000). The epidemiological significance of human versus dog infectiousness in maintaining the transmission cycle has to be confirmed, through mathematical modelling.

Similar questions can then be asked about the role of asymptomatic versus symptomatic canine infection. Several studies indicated that infectiousness is higher in symptomatic than asymptomatic dogs (Quinnell and Courtenay 2009; da Costa-Val et al., 2007; Guarga et al., 2000; Magalhães-Junior et al., 2016), with a single study showing that asymptomatic dogs may be equally infectious as symptomatic dogs (Laurenti et al.,

2013). Other studies report that infectiousness is independent of clinical symptomology (Molina et al., 1994; Guarga et al., 2000). However, longitudinal studies in Brazil demonstrated that dogs classified as asymptomatic at a single time point, as in cross-sectional studies, usually go on to develop progressive disease, and so should be more appropriately described as pre-symptomatic (Courtenay et al., 2002b). Another interesting observation is that the proportion of infectious dogs is higher in European than in South America studies with 0.86 and 0.45, associated with different sand fly species (*Phlebotomus perniciosus* versus *Lu. longipalpis*, respectively), which may be due to a higher susceptibility of the vector in Europe than in Brazil (Quinnell and Courtenay, 2009; Rock et al., 2016).

In the Indian subcontinent (Bangladesh, India, Nepal), the transmission of VL due to *L. donovani* is anthroponotic. A key question is relative to the roles of kala azar cases, post kala azar dermal leishmaniasis (PKDL) cases, and asymptomatic infections in transmission. This is important for case management and the distribution of limited resources. Part of the VL elimination program in the Indian Subcontinent, the identification of this cryptic carriers leads to a diagnostic tool issue, despite being essential as they are reservoirs of *Leishmania* infections (Sundar et al., 2008). Early diagnosis allows fast treatment of patients; however, the RDT based on antigen rK39 only detects the antibodies without differencing current from past exposure (Sundar et al., 2006a; Sundar et al., 2006b). Moreover, WHO recommend the use the diagnostic tool only for VL suspects, and people who present clinical symptoms such as fever for at least 2 weeks and have an enlarged spleen (WHO report, 2008).

The current test development to identify canine super-spreaders was performed on a cohort population of naïve dogs in Amazon Brazil. It is possible that inter-population variation in dog infection rates and infectious dog population dynamics might occur (e.g. owner care regulated governing the general health status of dogs). However, it is unlikely that the fundamental immunological relationships between infection, parasite burden and anti-*Leishmania* antibodies differs markedly between canine populations. Exceptions might include differences in parasite virulence or co-infections. VL co-infection with HIV generally leads to the failure of serological diagnostic test in humans, mainly due to reducing cellular and humoral immune responses. The lower number of T-cells leads to the inability to recognize *Leishmania* antigens and to

stimulate the appropriate B-cells humoral responses (Gradoni et al., 1993; Desjeux et Alvar, 2003). Moreover, co-infected patients can become a human reservoir and maintain the transmission cycle, as already mentioned by Ferreira et al. (2018). The incidence of co-infections is decreasing in Europe due to the better control for HIV and the access to treatments, whereas the co-infection is still problematic in Africa or Latin America (Desjeux et Alvar, 2003).

The development of serodetection tools should also consider the region-specific diversity of antigens, as well as the variation in leishmanial polymorphisms. Indeed, strains of *Leishmania* vary geographically: *L. infantum* in Europe and Latin America, and *L. donovani* in South Asia and East Africa. For example, antigen rK39, derived from a gene of *L. infantum* originated from Europe, and is successfully used in Brazil and in Asia, but shows lower sensitivities in East Africa due to the significant diversity between the rK39 homologues among the African *L. donovani*, with limited binding of diagnostic antibodies (Bhattacharyya et al., 2013). Similar results were found for antigen K9 and K26 (Bhattacharyya et al., 2013).

The vector-related parameters were not considered in the thesis, whereas sandflies are essential to maintain the transmission cycle of parasite *Leishmania*. A number of studies suggest the importance of exposure to sandfly bites and saliva (Gomes et al., 2007; Barral et al., 2006; Gomes and Oliveria, 2012). In a recent study, Quinnell et al. (2018) demonstrated the importance of saliva in the establishment of infection, but not the infectiousness or the severity of the disease (Quinnell et al., 2018). Moreover, the variation of seasonality impacts the abundance of sandflies and thus the exposure of dogs to bites and saliva, which explains the inter-individual variation (Quinnell et al., 2018)

The highly controversial dog test-and-slaughter strategy under the Visceral Leishmaniasis Control and Surveillance Program in Brazil (fully described in Chapter 1) leading to the slaughter of million dogs has had very little impact on human infection rates. As discussed in Chapter 7, a recent alternative to culling is treatment of infected dogs with human drugs. The Ministério de Saudé in Brazil legalised the treatment of *Leishmania*-infected dogs in 2018. Despite some studies concluding that canine treatment results in significant reductions in infectiousness, the effects are short-lived

and not effective for a complete cure of the parasite loads. Thus, treated dogs may still be an important reservoir for infection (discussed in Chapter 8). Reductions in tissue parasite loads post-therapy could render a test designed for super-spreaders less effective. Incomplete cure may also lead to drug resistance by *Leishmania* (Noli and Auxilia, 2005; Pineda et al., 2017). In European endemic countries, private canine treatment is more common and human cases are rare. However, in many developing regions where human case burdens are substantial, the likelihood that dogs will not be treated or will not complete the drug course is high, and drugs will be too expensive for most dog-owners.

Other preventive measures include insecticide-impregnated collars, spot-on products on dogs, vaccination and immunomodulation (as reviewed by Travi et al., 2018). Even if most of these measures have shown evidence of reduction of infection on individual intervention, their efficacy at community-level is not clearly established and their impact on infectiousness was not been assessed. As described in Reithinger et al. (2014), the collars efficacy depends on the coverage of dogs within the population including the addition of newly introduced dogs, and the replacement of lost collars (Reithinger et al., 2014). This high coverage has the same logistic limitation as the implementation of the diagnosis of super-spreaders (Chapter 8). Moreover, being a high cost intervention, the application of collars would require financial support by the government in Brazil. All the above-mentioned preventive measures may only impact on infection and transmission if no other reservoirs occur (e.g. wild reservoirs, such as opossums) and if the management of VL includes street dogs. To date, only domestic dogs are proven to be reservoirs of *Leishmania infantum*.

8.3   Study limitations and further work

The general gaps in the scientific knowledge about VL include: **(1)** the dynamics of VL transmission; **(2)** a gold-standard for diagnosis; **(3)** detection of asymptomatic infections and symptomatic infections; **(4)** the distinction in positive serology results between natural infection and vaccine; **(5)** determinants of dog susceptibility or resistance to infection; and finally, **(6)** determinants of dog infectiousness (as discussed throughout the thesis). In addition to the study limitations discussed in each chapter,

several other points are worth making for further improvement in the research on detecting infectiousness.

While this project focused on total antibody responses, it could be interesting to analyse the different subclasses of antibody. Previous trials on the dog cohort (Quinnell et al., 2003; Carson et al., 2010a) highlighted that all IgG subclasses of the crude *Leishmania* antigen (CLA) positively correlated with the intensity of the infectiousness of naturally infected dogs. The highest level of isotype was for IgG1 followed by lower levels of IgG4, IgG3 and IgG2 in that order. Moreover, there was a significant antibody titre difference between highly and mildly infectious dogs for IgG 1-3-4, though not significant for IgG 2. It would be interesting to perform further assays on subclasses of the novel antigens, used in this study, to evaluate their ability to detect infectiousness and super-spreaders.

One other important challenge, particularly in European setting where the canine vaccines are more widely used, is development of a DEVA assay to differentiate antibody responses to natural infection versus vaccination. Otherwise, it potentially interferes with sero-surveillance programs. As described in Chapter 1, the available vaccines in Brazil, such as Leishmune® and Leish-Tec®, have led to discussion about their efficacy. For example, several cases were reported of vaccinated dogs that develop the disease and became infectious to sandfly (pers. com.). CaniLeish® based on excreted-secreted proteins from *Leishmania* was approved in 2011, offering prophylactic action by reducing the transmission of *Leishmania* from vaccinated dogs to sandflies (Oliva et al., 2014). However, there is a lack of studies to confirm or refute that the appropriate community-wide coverage with a canine vaccine would impact on human infection incidence.

Finally, the present study describes the design, the development and the initial assays of a novel diagnostic tool (KL914-RDT) for the identification of highly infectious dogs, which are primarily responsible for the transmission of zoonotic visceral leishmaniasis. The application of bench research into the field was the major objective of this project (discussed in Chapter 6). Further work is required to improve the point-of-care sensitivity of the RDT before the diagnostic tool can be applied in field trials. The

reason why antigens perform consistently weaker in rapid diagnostic tests than in the ELISA remains unclear (Pattabhi et al., 2010; de Silva Solcà et al., 2014). Several reasons could be suggested to explain the lack of performance such as the composition of the migration buffer, the adhesion of the protein to the nitrocellulose membrane, or the sera conservation conditions (discussed in Chapter 6). Further work is required to evaluate the reasons for this discrepancy.

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Sera samples came from dogs (n=26) in a prospective study in Brazil (Quinnell et al., 1997). Dogs were well-characterised for infection and infectiousness, as listed below. The number (N◦) and the identity (ID) of the dog are reported. For every sample collection, the number of days spent in the field was reported with the results of the experiments. Infection was determined based on PCR, serology and parasite culture. Xenodiagnosis was performed to determine the infectiousness of the dog to the sandflies. The percentage of infected flies was used to classify dogs in infectiousness (highly, mildly, or never infectious).

N◦	ID	Days in the field	Infection	Xenodiagnostic	Infected flies (%)	Classification
1	A29	0	0	-	-	
	A29	66	0	0	0	
	A29	120	0	0	0	
	A29	195	0	0	0	
	A29	262	0	0	0	
	A29	330	1	1	2.3	Mildly
	A29	398	1	1	18.75	Mildly
	A29	467	1	1	0	Mildly
2	A31	0	0	-	-	
	A31	63	0	-	-	
	A31	132	0	0	0	
	A31	182	1	0	0	
	A31	255	1	0	0	
	A31	321	1	0	0	
	A31	391	1	1	10	Mildly
	A31	456	1	1	14.2	Mildly
	A31	526	1	1	46.8	Mildly
	A31	586	1	1	-	Mildly
3	A32	0	0	-	-	
	A32	63	0	-	-	
	A32	132	0	0	0	
	A32	182	0	0	0	
	A32	256	1	0	0	Never
	A32	335	1	0	-	Never
	A32	390	1	0	0	Never
	A32	456	1	0	0	Never
	A32	527	1	0	0	Never
	A32	586	1	0	0	Never
	A32	659	1	0	0	Never
	A32	722	1	0	-	Never
	A32	796	1	0	-	Never
4	A37	0	0	0	0	
	A37	83	0	0	0	
	A37	153	1	0	0	
	A37	209	1	1	67.93	Highly

	A37	278	1	1	16.13	Highly
	A37	353	1	1	0	Highly
	A37	418	1	1	27.62	Highly
	A37	488	1	1	83.33	Highly
	A37	556	1	1	40	Highly
	A37	614	1	1	23.81	Highly
	A37	688	1	1	0	Highly
	A37	749	1	1	28.2	Highly
5	A38	0	0	-	-	
	A38	83	0	0	0	
	A38	153	1	0	0	Never
	A38	211	1	0	0	Never
	A38	284	1	0	0	Never
	A38	353	1	0	0	Never
6	A40	70	0	-	-	
	A40	127	1	0	-	
	A40	185	1	1	0	Mildly
	A40	258	1	1	1.9	Mildly
	A40	327	1	1	0	Mildly
	A40	392	1	1	0	Mildly
	A40	462	1	1	0	Mildly
	A40	531	1	1	0	Mildly
	A40	589	1	1	0	Mildly
	A40	662	1	1	-	Mildly
	A40	723	1	1	0	Mildly
7	A41	0	0	-	-	
	A41	66	0	0	0	
	A41	125	0	0	0	
	A41	183	1	0	0	Never
	A41	259	1	0	-	Never
	A41	323	1	0	0	Never
	A41	397	1	0	-	Never
	A41	473	1	0	-	Never
	A41	537	1	0	0	Never
	A41	589	1	0	0	Never
	A41	663	1	0	-	Never
	A41	724	1	0	-	Never
8	A43	0	0	-	-	
	A43	70	0	0	0	
	A43	127	0	0	0	
	A43	192	0	0	0	
	A43	262	1	-	-	
	A43	330	1	0	0	
	A43	398	1	-	-	
	A43	467	1	0	0	
	A43	535	1	1	33.3	Mildly
	A43	591	1	1	0	Mildly
	A43	663	1	1	15.4	Mildly
	A43	726	1	1	-	Mildly

189

		798	1	1	-	Mildly
	A43	798	1	1	-	Mildly
9	A78	0	0	0	-	
	A78	73	0	-	-	
	A78	129	0	0	-	
	A78	198	1	0	0	
	A78	272	1	1	6.25	Highly
	A78	341	1	1	4.22	Highly
	A78	408	1	1	36.73	Highly
	A78	477	1	1	45.45	Highly
10	A79	0	0	-	-	
	A79	68	0	-	-	
	A79	131	1	0	-	
	A79	189	1	1	0	Highly
	A79	265	1	1	84.1	Highly
	A79	331	1	1	10	Highly
11	A83	0	0	-	-	
	A83	68	0	-	-	
	A83	123	0	-	-	
	A83	192	1	0	0	
	A83	264	1	1	42.5	Highly
	A83	333	1	1	3.27	Highly
12	A84	0	0	-	-	
	A84	131	0	-	-	
	A84	192	0	-	-	
	A84	192	0	-	-	
	A84	265	0	-	-	
	A84	333	1	-	-	
	A84	398	1	-	-	
	A84	459	1	0	0	Never
	A84	534	1	0	0	Never
	A84	583	1	0	0	Never
	A84	662	1	0	-	Never
	A84	723	1	0	0	Never
	A84	798	1	0	-	Never
13	B12	0	0	-	-	
	B12	77	0	0	0	
	B12	135	1	0	-	Never
	B12	207	1	0	-	Never
	B12	277	1	0	-	Never
	B12	347	1	0	-	Never
	B12	413	1	0	-	Never
	B12	487	1	0	-	Never
	B12	550	1	0	0	Never
	B12	606	1	0	0	Never
	B12	679	1	0	-	Never
	B12	742	1	0	-	Never
	B12	813	1	0	-	Never

14	B24	0	0	0	0	
	B24	71	0	-	-	
	B24	122	0	0	0	
	B24	190	1	0	0	
	B24	262	1	1	3.7	Mildly
	B24	336	1	1	0	Mildly
	B24	400	1	1	0	Mildly
	B24	465	1	1	0	Mildly
	B24	538	1	1	0	Mildly
	B24	595	1	1	-	Mildly
	B24	666	1	1	-	Mildly
	B24	731	1	1	0	Mildly
	B24	805	1	1	-	Mildly
15	B25	0	0	-	-	
	B25	71	1	0	0	Never
	B25	122	1	0	0	Never
	B25	190	1	0	0	Never
	B25	264	1	0	0	Never
	B25	334	1	0	-	Never
	B25	400	1	0	-	Never
16	B26	71	0	0	0	
	B26	122	1	0	0	Never
	B26	190	1	0	0	Never
	B26	264	1	0	0	Never
	B26	334	1	0	-	Never
	B26	400	1	0	-	Never
	B26	468	1	0	0	Never
	B26	536	1	0	0	Never
	B26	595	1	0	0	Never
	B26	667	1	0	-	Never
	B26	737	1	0	0	Never
	B26	805	1	0	-	Never
17	B40	0	0	0	0	
	B40	71	0	-	-	
	B40	140	0	0	0	
	B40	202	0	0	0	
	B40	274	0	0	0	
	B40	343	1	0	0	Never
	B40	408	1	0	-	Never
	B40	480	1	0	0	Never
	B40	544	1	0	0	Never
	B40	593	1	0	0	Never
	B40	672	1	0	-	Never
	B40	733	1	0	0	Never
	B40	808	1	0	-	Never
18	C10	0	0	0	-	
	C10	72	0	0	0	
	C10	125	0	0	0	
	C10	195	1	1	1.3	Mildly

	C10	261	1	1	0	Mildly
	C10	330	1	1	0	Mildly
	C10	400	1	1	14.8	Mildly
19	C14	0	0	-	-	
	C14	60	0	-	-	
	C14	124	0	-	-	
	C14	197	0	-	-	
	C14	261	1	-	-	
	C14	330	1	1	5	Highly
	C14	401	1	1	5	Highly
	C14	466	1	1	55.6	Highly
20	C2	0	0	-	-	
	C2	77	1	-	-	
	C2	127	1	-	-	
	C2	202	1	-	-	
	C2	267	1	0	0	
	C2	335	1	0	0	
	C2	401	1	0	0	
	C2	471	1	-	-	
	C2	530	1	-	-	
	C2	604	1	-	-	
	C2	667	1	1	2.6	Mildly
	C2	743	1	1	1	Mildly
21	C21	0	0	-	-	
	C21	43	0	-	-	
	C21	106	1	-	-	
	C21	181	1	1	2.5	Mildly
	C21	246	1	1	0	Mildly
22	C8	0	0	-	-	
	C8	73	0	0	0	
	C8	121	0	-	-	
	C8	193	0	-	-	
	C8	265	0	0	0	
	C8	333	0	0	0	
	C8	400	0	0	0	
	C8	467	0	0	0	
	C8	527	0	0	0	
	C8	598	1	-	-	
	C8	667	1	1	40	Mildly
	C8	736	1	1	40	Mildly
23	C9	0	0	-	-	
	C9	73	0	-	-	
	C9	122	0	-	-	
	C9	196	0	-	-	
	C9	262	0	-	-	
	C9	331	1	1	-	Mildly
	C9	399	1	1	-999	Mildly
	C9	467	1	1	-999	Mildly

	C9	526	1	1	-999	Mildly
	C9	597	1	1	-999	Mildly
24	E20	0	0	-	-	
	E20	67	1	-	-	Never
	E20	140	1	-	-	Never
	E20	206	1	-	-	Never
	E20	275	1	0	0	Never
	E20	348	1	0	0	Never
	E20	409	1	0	0	Never
	E20	467	1	0	0	Never
	E20	541	1	0	0	Never
	E20	602	1	0	0	Never
	E20	677	1	0	0	Never
25	E4	0	0	-	-	
	E4	65	1	-	-	
	E4	139	1	-	-	
	E4	209	1	-	-	
	E4	284	1	1	50	Highly
	E4	344	1	1	50	Highly
	E4	413	1	1	50	Highly
26	E40	59	0	-	-	
	E40	135	0	-	-	
	E40	202	1	-	-	
	E40	269	1	-	-	
	E40	341	1	1	25	Highly
	E40	405	1	1	25	Highly
	E40	456	1	1	25	Highly
	E40	534	1	1	25	Highly
	E40	594	1	1	25	Highly

Appendix II - Possible thresholds values for each candidate and the proportion of sample detection among groups of dogs (never, mildly, highly infectious)

Proportion and the related percentage of samples detected in each group (never, mildly, and highly) for each possible value of cut-offs, indicating the performance of the diagnostic tool. Cut-offs were determined based on point xenodiagnosis and applied to samples selected for longitudinally classified dogs within the xenodiagnoses study as described in the methods section. The cut-off options include the conventional (mean+2/3SD), values from the ROC-curve analysis such as the Youden Index and other values selected to maximise sensitivity and specificity of the test as well as values labelled "max never" and "min highly" as values corresponding to 75% and 95% of the never samples detection. From the complete table of threshold values, the "max never"-threshold and Mean+3SD are usually too high as there is a 0% detection of never infectious, but also limited detection of super-spreaders, as opposed to threshold values based on "min highly," which offer complete coverage of super-spreader detection, and also a high detection of never infectious dogs (up to 50% for some candidates). As a result, the optimal cut-off must be a value in between. Among the remaining options, the threshold with the highest detection of super-spreaders and the lowest detection of never infectious dogs was selected as the default.

rK39	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	0.604	0%	(0/72)	29.55%	(13/44)	65.52%	(19/29)
Mean+3SD	0.663	0%	(0/72)	29.55%	(13/44)	65.52%	(19/29)
Mean+2SD	0.515	2.78%	(2/72)	31.82%	(14/44)	65.52%	(19/29)
2Mean	0.439	9.72%	(7/72)	34.09%	(15/44)	68.97%	(20/29)
Min highly	0.234	38.89%	(28/72)	45.45%	(20/44)	100%	(29/29)
Youden Index	0.272	26.39%	(19/72)	45.45%	(20/44)	96.55%	(28/29)
75% CI Never	0.305	25.00%	(18/72)	43.18%	(19/44)	86.21%	(25/29)
95% CI never	0.254	34.72%	(27/72)	45.45%	(20/44)	96.55%	(28/29)

K28	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	0.877	0%	(0/72)	27.27%	(12/44)	89.66%	(26/29)
Mean+3SD	1.085	0%	(0/72)	18.18%	(8/44)	34.48%	(10/29)
Mean+2SD	0.858	2.78%	(2/72)	27.27%	(12/44)	89.66%	(26/29)
2Mean	0.808	4.17%	(3/72)	29.55%	(13/44)	93.10%	(27/29)
Min highly	0.733	6.94%	(5/72)	31.82%	(14/44)	100%	(29/29)
Youden Index	0.720	6.94%	(5/72)	31.82%	(14/44)	100%	(29/29)
75% CI Never	0.581	25.00%	(18/72)	38.64%	(17/44)	100%	(29/29)
95% CI never	0.457	36.11%	(26/72)	56.82%	(25/44)	100%	(29/29)

K26	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	1.084	0%	(0/72)	4.55%	(2/44)	20.69%	(6/29)
Mean+3SD	0.975	1.39%	(1/72)	13.64%	(6/44)	41.38%	(12/29)
Mean+2SD	1.882	0%	(0/72)	0%	(0/44)	0%	(0/29)
2Mean	0.641	8.33%	(6/72)	29.55%	(13/44)	79.31%	(23/29)
Min highly	0.248	51.39%	(37/72)	63.64%	(28/44)	100%	(29/29)

		Detection of never		Detection of mildly		Detection of highly	
Youden Index	0.531	15.28%	(11/72)	34.09%	(15/44)	90%	(26/29)
75% CI Never	0.402	25.00%	(18/72)	45.45%	(20/44)	90%	(26/29)
95% CI never	0.371	27.78%	(20/72)	45.45%	(20/44)	93%	(27/29)

K34	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	0.907	0%	(0/72)	31.82%	(14/44)	37.93%	(11/29)
Mean+3SD	1.036	0%	(0/72)	22.73%	(10/44)	20.69%	(6/29)
Mean+2SD	0.813	1.39%	(1/72)	38.64%	(17/44)	51.72%	(15/29)
2Mean	0.735	5.56%	(4/72)	40.91%	(18/44)	55.17%	(16/29)
Min highly	0.362	50.00%	(36/72)	47.73%	(21/44)	100%	(29/29)
Youden Index	0.522	25.00%	(18/72)	40.91%	(18/44)	93%	(27/29)
75% CI Never	0.528	25.00%	(18/72)	40.91%	(18/44)	93%	(27/29)
95% CI never	0.420	40.28%	(29/72)	45.45%	(20/44)	93%	(27/29)

K9	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	0.822	0%	(0/72)	11.36%	(5/44)	17.24%	(5/29)
Mean+3SD	0.686	2.78%	(2/72)	13.64%	(6/44)	20.69%	(6/29)
Mean+2SD	0.516	5.56%	(4/72)	20.45%	(9/44)	37.93%	(11/29)
2Mean	0.350	9.72%	(7/72)	38.64%	(17/44)	58.62%	(17/29)
Min highly	0.099	63.89%	(46/72)	93.18%	(41/44)	100%	(29/29)
Youden Index	0.235	19.44%	(14/72)	59.09%	(26/44)	83%	(24/29)
75% CI Never	0.189	25.00%	(18/72)	65.91%	(29/44)	86%	(25/29)
95% CI never	0.215	23.61%	(17/72)	59.09%	(26/44)	83%	(24/29)

6H	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	1.190	0%	(0/72)	4.55%	(2/44)	13.79%	(4/29)
Mean+3SD	0.874	2.78%	(2/72)	9.09%	(4/44)	27.59%	(8/29)
Mean+2SD	0.655	5.56%	(4/72)	15.91%	(7/44)	37.93%	(11/29)
2Mean	0.434	9.72%	(7/72)	29.55%	(13/44)	58.62%	(17/29)
Min highly	0.093	68.06%	(49/72)	84.09%	(37/44)	100%	(29/29)
Youden Index	0.223	27.78%	(20/72)	50.00%	(22/44)	82.76%	(24/29)
75% CI Never	0.283	25.00%	(18/72)	43.18%	(19/44)	68.97%	(20/29)
95% CI never	0.268	26.39%	(19/72)	45.45%	(20/44)	72.41%	(21/29)

8e	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	0.935	0%	(0/72)	4.55%	(2/44)	6.90%	(2/29)
Mean+3SD	0.510	1.39%	(1/72)	20.45%	(9/44)	37.93%	(11/29)
Mean+2SD	0.380	2.78%	(2/72)	27.27%	(12/44)	51.72%	(15/29)
2Mean	0.240	9.72%	(7/72)	31.82%	(14/44)	65.52%	(19/29)
Min highly	0.072	54.17%	(39/72)	84.09%	(37/44)	100%	(29/29)
Youden Index	0.156	23.61%	(17/72)	45.45%	(20/44)	86%	(25/29)
75% CI Never	0.150	25.00%	(18/72)	45.45%	(20/44)	86%	(25/29)
95% CI never	0.150	25.00%	(18/72)	45.45%	(20/44)	86%	(25/29)

Lin14/4	Cut-offs	Detection of never		Detection of mildly		Detection of highly	
Max never	2.466	0%	(0/72)	11.36%	(5/44)	27.59%	(8/29)
Mean+3SD	2.343	1.39%	(1/72)	13.64%	(6/44)	27.59%	(8/29)
Mean+2SD	1.733	5.56%	(4/72)	27.27%	(12/44)	51.72%	(15/29)
2Mean	1.029	19.44%	(14/72)	40.91%	(18/44)	72.41%	(22/29)
Min highly	0.277	45.83%	(33/72)	70.45%	(31/44)	100%	(29/29)
Youden Index	0.802	23.61%	(17/72)	43.18%	(19/44)	90%	(26/29)
75% CI Never	0.791	25.00%	(18/72)	43.18%	(19/44)	90%	(26/29)
95% CI never	0.658	29.17%	(21/72)	45.45%	(20/44)	90%	(26/29)

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