Development of a new sand fly exposure test to evaluate vector control tools

Vývoj nových metod pro studium expozice hostitelů vůči flebotomům

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Ph.D. thesis/Dízertační práce

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AUTHOR’S DECLARATION / PROHLÁŠENÍ AUTORKY

I declare that this Ph.D. thesis was written by myself and the results presented within this thesis were accomplished by myself or in the collaboration with the co-authors of the presented papers. I proclaim that the literary sources were cited properly and neither this work nor the particular data have been used as a final work towards any other university degree.

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Prague, December 19th, 2018

Praha, 19. prosince 2018

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I declare that Laura Adrienne André Willen substantially contributed to the experimental work in the projects presented in her Ph.D. thesis and that she had a principal role in the writing of one publication and one manuscript presented.

Prohlašuji, že Laura Adrienne André Willen se významně podílela na experimentální práci projektů shrnutých v této dizertační práci a je autorkou textu dvou uvedených publikací.

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# TABLE OF CONTENT

- **AUTHOR’S DECLARATION / PROHLÁŠENÍ AUTORKY** ................................................................. I
- **SUPERVISOR’S DECLARATION / PROHLÁŠENÍ ŠKOLITELE** ................................................... II
- **ACKNOWLEDGEMENTS / PODĚKOVÁNÍ** ................................................................................ III
- **TABLE OF CONTENT** ................................................................................................................ IV
- **LIST OF ABBREVIATIONS** .......................................................................................................... V
- **ABSTRACT** ................................................................................................................................ VI
- **ABSTRAKT** ............................................................................................................................... VII
- **INTRODUCTION** .......................................................................................................................... VII
- **1** CANINE LEISHMANIASIS AND ITS CONTROL ............................................................. 1
  - 1.1 **EPIDEMIOLOGY OF CANINE LEISHMANIASIS ACROSS THE MEDITERRANEAN** ................. 2
  - 1.2 **CONTROL OF CANINE LEISHMANIASIS USING SYNTHETIC PYRETHROIDS** ..................... 4
  - 1.3 **VACCINATION OF DOGS** .................................................................................................. 6
  - 1.4 **CULLING OF SEROPOSITIVE DOGS COMBINED WITH INSECTICIDE SPRAYING** .......... 8
- **2** EVALUATION OF VECTOR CONTROL METHODS USING ANTI-SALIVA ANTIBODIES AS MARKERS OF EXPOSURE ........................................................................ 11
  - 2.1 **SALIVA OF HAEMATOPHAGOUS ARTHROPODS AND ITS EFFECT ON THE HOST** ............. 11
  - 2.1.1 Salivary exposure markers in various vectors ................................................................. 13
  - 2.1.2 Salivary exposure markers in sand flies ............................................................................ 16
  - 2.1.3 B-cell epitopes as exposure marker ............................................................................... 18
  - 2.2 **SPREADING UP THE PROCES: LATERAL FLOW IMMUNOCHEMOTOGRAPHIC ASSAYS** ....... 19
  - 2.2.1 The use of rapid tests in vector-transmitted diseases ..................................................... 21
- **3** OBJECTIVES ......................................................................................................................... 26
- **4** PUBLICATIONS ....................................................................................................................... 27
- **5** SUMMARY AND CONCLUSIONS ....................................................................................... 28
- **6** REFERENCES ............................................................................................................................ 32
LIST OF ABBREVIATIONS

Ab  Antibody
ADP  Adenosine diphosphate
Ags  Antigens
CanL  Canine Leishmaniasis
CATT  Card agglutination test for trypanosomiasis
CDC  Centers for Disease Control and Prevention
CL  Cutaneous Leishmaniasis
CS  Circumsporozoite
DAT  Direct Agglutination Test
ELISA  Enzyme-linked immunosorbent assay
HAT  Human African trypanosomiasis
HIV  Human immunodeficiency virus
ITN  Insecticide treated nets
ICT  Immunochromatographic test
IgG/M  Immunoglobulin G/M
LFA  Lateral flow assay
LFIA  Lateral flow immunoassay
MCL  Mucocutaneous Leishmaniasis
NC  Nitrocellulose membrane
OC  Oligochromatography
OD  Optical density
PCR  Polymerase chain reaction
RDT  Rapid diagnostic test
SGE  Salivary gland extract
SGH  Salivary gland homogenate
VL  Visceral leishmaniasis
VLSCP  Visceral Leishmaniasis Surveillance and Control Programme
WHO  World Health Organization
WNV  West-Nile virus
ZVL  Zoonotic Visceral Leishmaniasis
**ABSTRACT**

In the Mediterranean basin, human visceral leishmaniasis caused by the protozoan parasite *Leishmania infantum* is a zoonotic disease that gives rise to 1,200 to 2,000 new cases annually. The domestic dog constitutes its main reservoir, of which some may suffer from a severe chronic disease, canine leishmaniasis (CanL). The sand fly *Phlebotomus perniciosus* is considered to be the principle vector. Saliva of bloodfeeding vectors of diseases has been used in the past to assess host exposure to vector bites and to evaluate vector control tools. This Ph.D. focused on saliva of *P. perniciosus* to identify exposure markers that could be used in the preparation of a new vector exposure tool.

The first part of this Ph.D. aimed at validating the use of a recombinant salivary protein of *P. perniciosus* – rSP03B – in endemic settings of CanL. During a cross-sectional study, no significant differences between the antibody (Ab) response against whole saliva or the rSP03B were observed between different regions across the Mediterranean basin. Furthermore, the rSP03B was shown to resemble the native protein. During a subsequent study this protein was used to assess the seasonal dynamics of the canine Ab response to *P. perniciosus* in an endemic area of *L. infantum*. This study elucidated that also in a heterogeneous dog population both salivary gland lysate as well as the rSP03B ELISA followed expected trends of *P. perniciosus* activity, with significantly lower anti-*P. perniciosus* IgG levels in non-transmission season. These results validated the Ab response against the rSP03B protein as a universal exposure marker for *P. perniciosus* and encouraged its further use.

The second part of this thesis was aimed at accelerating the use of this salivary protein to allow a rapid evaluation of vector control programs in the future. Therefore, a rapid vector exposure test based on the rSP03B protein was prepared which detects with a high sensitivity (100%) and specificity (87%) canine exposure to *P. perniciosus*. This rSP03B sero-strip was verified to substitute the conventional ELISA by showing an almost perfect degree of agreement with the latter.

Finally, during the third part of this Ph.D. thesis the rSP03B sero-strip was successfully optimized, which resulted in a higher specificity (95%) of the test and ensured test suitability with whole canine blood. The main focus was to ascertain field applicability of the test. In order to do so, 186 dogs from different CanL endemic areas and 154 longitudinally sampled dogs were screened with the SGH-ELISA and the rSP03B sero-strip. Moreover, cross-reactions between non-vector species were excluded and favorable cross-reactions with other *L. infantum* vectors belonging to the subgenus *Larroussius* were confirmed. The results supported the use of the test in a field population of naturally exposed dogs and showed its ability to distinguish recent from past exposure. The presence of favorable cross-reactions between members of the *Larroussius* subgenus expands its use in the field.
ABSTRAKT

V oblasti Středozemního moře je ročně zaznamenáno 1200-2000 nových případů lidské viscerální leishmaniózy. Toto onemocnění je zoonózou působenou parazitem *Leishmania infantum* a přenášenou flebotomy. Hlavním rezervoárem jsou psi, kteří mohou též onemocnět závažným a chronickým onemocněním, psí leishmaniózou; jedním z hlavních přenašečů je *Phlebotomus perniciosus*. Sliny krevsajících přenašečů byly opakovaně využity v studiu pobodání hostitelů a sledování účinnosti insekticidních zásahů. Tato disertační práce byla proto zaměřena na identifikaci slnních antigenů, které mohou sloužit jako markery pobodání psů druhem *P. perniciosus* a mohou být použity k vývoji nových diagnostických metod.

Prvá část práce byla věnována využití rekombinantního slinného proteinu *P. perniciosus* – rSP03B – v různých endemických oblastech psí leishmaniózy. Během průřezové studie byla zjištěna podobná protištítková odpověď u psů pocházejících z různých oblastí okolo Středozemního moře a bylo potvrzeno, že rekombinantní protein je antigenně shodný s nativním proteinem ze slin. V následující longitudinální studii byla sledována sezónní dynamika protivítěžkové odpovědi proti *P. perniciosus*. Bylo prokázáno, že odpověď proti slinám i rSP03B koreluje se sezónní aktivitou *P. perniciosus* a je signifikantně nižší během zimních měsíců. Výsledky potvrdily, že protištítková odpověď proti rSP03B může být využita jako univerzální marker pobodání flebotomem *P. perniciosus*.

Druhá část práce se zabývala využitím tohoto rekombinantního proteinu při vývoji rychlého testu, použitelného při hodnocení výsledků boje s flebotomy. Byl připraven chromatografický test (séro-strip) založený na proteinu rSP03B. Tento test měl vysokou sensitivitu (100%), velmi dobrou specifitu (87%) a bylo ověřeno, že může plně nahradit ELISA testy využívající slinné proteiny.

Během třetí, závěrečné fáze práce byl tento rSP03B séro-strip optimalizován pro využití v terénu. Použitím jiného expresního systému pro rSP03B byla zvýšena specifita testu na 95%, díky další drobné úpravě je vhodný i při použití celé psí krve. Test byl ověřen na vzorku 186 psů z různých endemických oblastí a 154 psů z longitudinalní studie. Bylo též zjištěno, že nereaguje zkrůženě se séry hostitelů pobodaných jinými krevsajícími členovci, ale pouze s jinými flebotomy podrodu *Larroussius*, kteří jsou též přenašeči *L. infantum*. Výsledky prokázaly, že test je vhodný pro psy přirozeně vystavené pobodání *P. perniciosus* a že je schopen rozlišit expozici nedávnou od časově vzdálené. Existence zkrůžených reakcí mezi zástupci podrodu *Larroussius* je pozitivní a rozšiřuje využitelnost testu v různých ohniscích.
INTRODUCTION

1 CANINE LEISHMANIASIS AND ITS CONTROL

*Leishmania* (Kinetoplastida: Trypanosomatidae) is a protozoan parasite and causative agent of cutaneous (CL), mucocutaneous (MCL) and visceral (VL, Kala-azar) leishmaniasis in humans in both the Old and the New World. Leishmaniases have an annual global incidence of about 0.6 – 1 million CL cases and 50,000 – 90,000 VL cases [1]. The course of infection is defined by the virulence of the parasitic strain and the genetic background and immune status of its host. In the vertebrate host, *Leishmania* parasites live as amastigotes inside a parasitophorous vacuole in phagocytic cells (mainly macrophages) (reviewed in [2]). Human VL has shown an association with the human immunodeficiency virus (HIV) and constitutes an important opportunistic disease in immunocompromised patients (reviewed in [3]).

*Leishmania* parasites are transmitted as promastigotes, by the bite of female sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (reviewed in [4]). This transmission can either be zoonotic – coming from a wide range of infected animals – or anthroponotic, from man-to-man. *Leishmania infantum* is the main causative agent of zoonotic VL (ZVL) across the Mediterranean region, including northern Africa and parts of Asia [5]. Both wild and domestic dogs may be infected with *L. infantum*, although the main reservoir host fueling and amplifying the leishmanial cycle in the region is the domestic dog. When dogs are infected with *Leishmania* parasites most of them develop a protective cellular immune response and remain asymptomatic [6,7], whereas in a minority of dogs a severe progressive systemic disease may appear, referred to as canine leishmaniasis (CanL). The type of the immune response, and hence the course of infection leading either to resistance or overt disease, is most likely genetically controlled (reviewed in [8]). CanL develops slowly and comprises a wide variety of symptoms; including skin lesions, loss of weight or poor appetite, local or generalized lymphadenopathy, ocular lesions, renal failure, and diarrhoea among others (reviewed in [8]). The wide variety of the symptoms is most likely a result of the heterogeneity of immune responses in the individual hosts. Transmission from dogs to humans can take place when suitable conditions are present, resulting in cases of human ZVL in southern European countries with an estimated annual incidence of 1,200 to 2,000 cases [9]. Important to note is that although dogs with clinical symptoms transmit *Leishmania* more readily than asymptomatic dogs, both of them transmit *Leishmania* to sand flies and hence pose a risk for human disease [10].
Compared to Europe, in Latin America dogs are also the main reservoir but the principle vector of ZVL is *Lutzomyia longipalpis*. More than 90% of human ZVL cases across a dozen Latin American countries are reported from Brazil, where 2,000 – 3,000 cases of ZVL are recorded every year [11,12].

The main focus of this thesis was to develop a vector exposure tool that allows rapid evaluation of vector control interventions. The work performed during this Ph.D. thesis was focused on the control of CanL in the Western Mediterranean basin, where *P. perniciosus* is the main vector of *L. infantum*. During the remaining part of this chapter I will briefly discuss the epidemiology of CanL in the Mediterranean basin together with its control, either by vector or reservoir control. The second chapter focuses on ways to evaluate vector control interventions, with a special emphasis on sand fly saliva and lateral flow assays.

### 1.1 Epidemiology of Canine Leishmaniasis Across the Mediterranean

The distribution of CanL is related to the presence of phlebotomine sand flies – constituting the unique vector of this parasite. Transmission of CanL is mainly rural and periurban, usually in houses that keep dogs in their garden together with rubbish, accumulated garbage or organic material [13] – conditions that favor the presence of sand flies [14]. Eight species of closely related phlebotomine sand flies (Diptera, Psychodidae) are proven vectors of CanL in the Mediterranean basin. They occupy mainly humid, sub-humid and semi-arid niches in different parts of the region, leading to development of foci of CanL, and they show a clearly typical seasonal activity [15]. The proven vectors across the region are the following: *P. perniciosus*, *P. ariasi*, *P. perfiliewi*, *P. neglectus*, *P. langeroni*, *P. tobbi*, *P. balcanicus*, and *P. kandelakii* [14,15]. However, not all of them have the same distribution: e.g. *P. ariasi* occurs only in the western part of the Basin and is generally found in cooler and more humid bioclimatic zones at higher altitudes, whereas *P. perniciosus* ranges from Portugal in the west to Italy in the east, predominating in warmer and drier bioclimatic zones at lower altitudes [16–18]. In some places a co-occurrence of two vector species of the subgenus *Larroussius* is reported, of which the best studied one is in Catalonia, Spain where *P. ariasi* and *P. perniciosus* occur sympatrically [19]. Since both of them have a similar behavior, the presence of two sympatric vectors should not be of great importance in control [14].

CanL endemicity stretches across the Mediterranean Sea and its prevalence varies from region to region – with new foci still being discovered [20,21]. Stable and unstable endemic regions of CanL are scattered across European countries and differ in their transmission cycle; with stable CanL endemic regions encompassing dog-to-dog transmission, hence ensuring the appearance of new cases every season, whereas unstable CanL endemic regions mainly involve sporadic or periodic...
autochthonous cases (reviewed in [22]). Although CanL is prevalent in all countries bordering the Mediterranean Sea, the prevalence rates are not uniform. Dog populations only living a few kilometres apart can already differ tremendously in prevalence rates of CanL, most likely due to the strict ecological requirements in order for *Leishmania* to be transmitted. Even more so, prevalence rates can fluctuate broadly over time [23,24] – even within the same season – and is most probably due to human intervention [25,26], or to changes in the vector populations. When considering the European countries in the Mediterranean basin which are endemic for CanL, cases have been reported in Cyprus, Greece, Albania, Croatia, Italy, Malta, France, Spain and Portugal (reviewed in [27]). An analysis on the distribution of CanL in Western Europe showed that the overall canine seroprevalence in Italy, France, Spain and Portugal equaled 23.3% [28], with Italy, Spain and Portugal reporting point prevalences of higher than 80%. Even more so, seroprevalences seems to be on the rise in many traditional endemic areas [23,29,30]. Important to note is that the seroprevalence most likely underestimates the real number of infected dogs in the area, since prevalence of infection was shown to be significantly higher than seroprevalence [6,31].

Recently, the geographical distribution has expanded to more northern latitudes and altitudes due to ongoing climate changes [13,29,32]. A continuous rise of temperatures in the Mediterranean subregion has been recorded, which is likely to affect the presence of arthropod borne diseases in areas previously devoid of them. Besides the increase in infected dogs in endemic areas [33,34], autochtonous cases of CanL [35] and human ZVL [36] have also been reported in new areas. In Northern Italy for example, autochtonous CanL and human ZVL cases were shown to be related to a geographic and density expansion of two vector species – *P. perniciosus* and *P. neglectus* – over a period of 30 years [4]. Furthermore, relatively recent autochtonous cases of CanL have been reported in Germany [37], the UK [38], Hungary [39], Romania [40], and Finland [41]. However, it is important to note that cases reported from Finland, the UK and Germany included non-vector borne transmissions involving transplacental transmission or direct contact.

Apart from autochthonous cases, imported cases of CanL are probably more common in Northern European countries (reviewed in [42,43]). Dogs that accompany their owners to endemic countries are of great risk to become infected and are the prime source of the growing number of dogs with CanL in non-endemic regions, even in regions free from sand flies. In recent years, around 700 imported CanL were reported from non-endemic Northern European countries [32].

Since currently no vaccines are available to prevent establishment of human ZVL, controlling ZVL in the Mediterranean basin and Latin-American countries is mainly focused on a combined control of the canine reservoir and the vector. Sand flies that feed on infected dogs, whether or not overt clinical symptoms are present, ingest the *Leishmania* parasite and subsequently transmit it to
the host of their next bloodmeal [10]. Different methods and tools have been proposed and
developed in order to break this contact between the vector and the reservoir host and hence inhibit
the parasite’s transmission cycle, including insecticide impregnated dog-collars, spot-on formulas,
CanL vaccines, and culling of infected dogs. In the next chapter I will shortly touch upon these
currently used vector and reservoir control strategies and address their merits and drawbacks.

1.2 CONTROL OF CANINE LEISHMANIASIS USING SYNTHETIC PYRETHROIDS

Synthetic pyrethroids are known to possess insecticidal properties [44] and are frequently
used to treat bed nets in vector-based malaria intervention strategies, following recommendation by
the WHO [45]. Regarding the control of sand flies, the efficiency of deltamethrin-impregnated dog
collars, worn during the transmission season, or spot-on formulas containing permethrin (a synthetic
pyrethroid) or a combination of permethrin with imidacloprid (a neonicotinoid) in reducing the
incidence of *Leishmania* seroconversion has been widely tested in laboratory as well as in field trials
[46,47,56–61,48–55] and has become the most effective tool in order to prevent *L. infantum*
infection in these animals [62]. Synthetic pyrethroids have a toxic and irritating effect on sand flies,
causing them to experience disorientation which leads to a sudden abandonment of the host and
death of the sand fly soon after it landed on the treated animal [62]. Therefore, these compounds are
an excellent tool to prevent the sand fly from taking a bloodmeal and infect its host, consequently
combining individual protection (due to anti-feeding properties) with mass protection caused by
lethal-by-contact activity [62]. Neonicotinoids, on the other hand, are insecticides that act by
affecting the central nervous system of insects which eventually leads to paralysis and death of the
insect.

The use of deltamethrin-impregnated dog collars as a control method to protect dogs from
sand fly bites has been evaluated in a study performed on dogs kept under natural climatic
conditions in kennels and outdoor enclosures in southern France [51]. Deltamethrin is a lipophilic
compound and as such spreads into the dermal secretions of most of the dog’s body [63]. PVC plastic
collars impregnated with this synthetic pyrethroid have been shown to slowly release deltamethrin
for several months [64] and a high degree of protection against bites of sand flies is obtained starting
from the second week of wearing the collar [51]. Another study performed in China bathed dogs in
water containing 2.5mg of deltamethrin [65], and found that only 2.8% of sand flies fed on treated
dogs, whereas 61.5% fed on control dogs. Even more so, the majority of sand flies that fed on treated
dogs died within 4-5 hours of exposure. A recent laboratory study further investigated the anti-
feeding efficacy of these slow release deltamethrin collars against *P. perniciosus* during a period of
one year [53], being the first study investigating the specific duration during which these collars
prevent sand fly feeding as well as the degree of this feeding-prevention. They demonstrated that a strong and sustained anti-feeding effect on sand flies is obtained for up to one year, with a 97% reduction in the mean numbers of engorged P. perniciosus females when deltamethrin collars were applied. This anti-feeding efficacy was sustained for more than 94% throughout the study. Furthermore, between day 7 and day 196 a significantly higher insecticidal effect was obtained for the dogs wearing the deltamethrin collars. A two-year field study that evaluated whether deltamethrin-impregnated collars could render protection against CanL was performed in Italy [48]. They found a cumulative protection rate of 50.8% for the impregnated dog collars, together with a significantly lower number of dogs with signs of CanL (36% vs 90%, for collared and uncollared dogs respectively). Another study performed in Iran reported similar results, stating that the mass use of deltamethrin-impregnated dog collars does not only protect domestic dogs but is also effective in reducing the number of VL cases in children [57].

Regarding the use of a topical solution of 65% permethrin, laboratory studies have demonstrated that it is as capable in reducing the number of bloodfed sand flies as a deltamethrin impregnated collar [54,55]. However, they also noted that using collars prolongs the protective effect compared to spot-on formulas – even though it takes 2 weeks before the lipophilic insecticides in the collar are spread over the dog’s body [54]. Even more so, when they took into account the death rate of sand flies that did feed, the reduction in number of bloodfed flies surviving 24 hours was greater for deltamethrin-impregnated collars than for the 65% permethrin spot-on treatment (91% vs. 37%, respectively) [54]. This encourages the use of collars above spot-on formulas. However, it has to be noted that spot-on lotions are easier to use and wear-and-tear commonly associated with collars is not a concern. Furthermore, a field trial performed in Brazil studied the effect of 65% permethrin spot-on on the prevalence of CanL and the abundance of sand flies [56]. Dogs were given three spot-on treatments during the course of the study, which resulted in a 50% reduction in infection rate one month after the last application. The authors concluded that regular use of such a spot-on formula during months of high risk for CanL, together with other control measures, could reduce the prevalence of CanL in hyperendemic areas. Even more so, a study in a CanL endemic region in Italy showed that only 2.5% of dogs treated with either a 65% spot-on solution of permethrin (Expot) or a deltamethrin impregnated collar (Scalibor) seroconverted compared to an incidence of 15% in the untreated dogs [47]. As such, the risk of infection was reduced with 84%, based on serological results.

Topical solutions containing 10% imidacloprid/ 50% permethrin have also been evaluated in previous studies, of which the first one assessed its repellent and insecticidal efficacy against P. papatasi [59]. This study found a repellent efficacy of 95% at day 1, which decreased to 80% at day 5 and finally to 56% at day 29; while a low insecticidal efficacy was obtained: 60% at day 1 and 29.3% at
day 29. Another study evaluating the same combination of this imidacloprid/permethrin spot-on solution against *P. perniciosus* found similar results, with a high repellent efficacy and a moderate to low insecticidal efficacy [61]. The high repellent effect being the potential cause for the low insecticidal effect observed in both studies. A cross-sectional study performed in a CanL endemic area in Spain evaluated the effect of different insecticides – either deltamethrin-impregnated collars, permethrin or imidacloprid/permethrin spot-on formulations – and found that applying preventive anti-sand fly insecticide products on a regular basis does not have an effect on the CanL seroprevalence [60]. An exception was made for when the deltamethrin-impregnated collar was combined with the imidacloprid/permethrin spot on, which showed a significant reduction in the risk of *Leishmania* seropositivity.

When the efficacy of two dog collars (a polymer matrix collar containing a combination of 10% imidacloprid with 4.5% flumethrin (“Seresto”) and a collar containing 4% deltamethrin (“Scalibor”) protector band) against flea infestations was assessed and compared to the CaniLeish vaccine [58], an efficacy of 88.3% for the Seresto-treated dogs and 61.8% for the Scalibor-treated dogs in preventing *L. infantum* infection was obtained. Contrastingly, dogs treated with the CaniLeish vaccine did not show a significant difference in frequency of active infections compared to control dogs [58]. The outcome of all these studies stresses the importance of using impregnated collars or 65% permethrin spot-on formula as control methods to protect dogs against sand fly bites and *L. infantum* infection, by not only killing sand flies after feeding but also exhibiting a strong anti-feeding effect, hence breaking the contact between the reservoir host and the vector. Spot-on formulas are more suitable for short-term protection of dogs when accompanying their owners to endemic countries; impregnated dog collars, on the other hand, should be recommended especially for hunting dogs or dogs living in domestic gardens in endemic areas, to ascertain they are protected during the whole season. The last study consequently brings us to a second control method for CanL in endemic countries: vaccination, which will be discussed in more detail in the following chapter.

### 1.3 Vaccination of Dogs

It is widely accepted that the protective response against *Leishmania* comprises a mixed Th1/Th2 response in which a dominant Th1 profile correlates with resistance to developing CanL and a Th2 dominance is associated with susceptibility [8,66–68]. Therefore, in order to have an effective vaccine against CanL a strong and long-lasting Th1-dominated response should be induced [69].

One such vaccine, which is also the first commercially licensed vaccine for CanL in Europe, is CaniLeish (Virbac Animal Health, France) and is based on excretory-secretory antigens (Ags) of *L. infantum* with Quillaja saponaria (LiESP-QA-21) as adjuvant [70]. A primary vaccination course of
three injections with three-week-intervals has to be performed, after which a booster vaccination is annually injected. This vaccine has been tested in previous studies focusing on its ability to stimulate an appropriate, specific, Th1-dominated immune response against *L. infantum* in dogs [71,72]. They confirmed that vaccination with the CaniLeish vaccine induces such an immune response rapidly and maintains it for at least one year after the last injection. Although these analyses were performed *in vitro*, authors did support the expectation of an effective immune response *in vivo*. Indeed, when vaccinated dogs were experimentally challenged with *L. infantum* one year after vaccination a 57% efficacy against active infection was obtained [69,73]. A longitudinal field evaluation of the CaniLeish vaccine in two study sites – one in Naples, Italy and one near Barcelona, Spain – revealed that a significant reduction in the number of actively infected animals and the probability of developing symptomatic disease was achieved by vaccination [74]. Vaccinated dogs that did develop CanL generally showed a slower progression and less severe clinical symptoms. Overall, this study obtained a vaccine efficacy of 68.4% and a protection rate of 92.7%. Although the probability of a dog to become PCR positive was similar between the control and the vaccinated group, PCR positive but subclinical vaccinated dogs reverted to the *Leishmania*-free status more frequently [74].

All of these studies show the rapid onset and consequent conservation of an appropriate Th1-mediated immune response together with a high clinical efficacy of the CaniLeish vaccine when dogs were experimentally or naturally challenged with *L. infantum*, hence supporting its role in the control of CanL. However, it has to be noted that the vaccine does not provide complete protection to 100% of dogs [74]. Even more so, a study that evaluated the efficacy of two dog collars together with the CaniLeish vaccine showed a clear inefficacy of the vaccine compared to the use of the impregnated dog collars [58]. This study therefore concluded that the use of the CaniLeish vaccine has to be recommended as part of an integrated control program in combination with repellents or insecticides and cannot replace the use of anti-vectorial measures in CanL endemic areas. Such a combined approach will increase the immune system’s ability to control a possible infection as well as decrease the level of challenge that it must control.

Contrarily to Europe, two different vaccines are commercially available in Brazil - the Leishmune (Zoetis, Brazil) and the LeishTec (Hertape Calier, Brazil) – both having a similar vaccination course as the CaniLeish vaccine: three initial injections followed by annual booster injections. The Leishmune vaccine, the first commercially licensed vaccine for CanL, is based on the fucose-mannose ligand of *L. donovani* together with a saponin adjuvant and has shown to render an 80% vaccine efficacy against CanL during a field study [75]. Furthermore, the vaccine demonstrated a 95% significant and long lasting protection against canine disease, which was hypothesized to be responsible for the observed decline in human disease in the study area [75,76]. The LeishTec vaccine
on the other hand uses the recombinant A2 Ag of *L. donovani* – an amastigote specific Ag – together with a saponin adjuvant and has been shown to mount a Th1 immune response in vaccinated dogs with an eventual vaccine efficacy of 43%, as observed when vaccinated dogs were experimentally challenged with *L. chagasi* four weeks after the last vaccine dose [77]. Although the vaccine efficacy of the LeishTec is clearly less than the efficacy of the Leishmune or the CaniLeish vaccine, the authors concluded that the rA2 was able to render a partial protection against *Leishmania* infection by limiting *Leishmania* replication and preventing severe disease [77]. All in all, none of the currently available vaccines for CanL has optimal efficacy.

### 1.4 Culling of Seropositive Dogs Combined with Insecticide Spraying

Elimination of seropositive dogs (i.e. culling) is, together with residual insecticide spraying and early diagnosis and treatment of human cases, the main focus of the Visceral Leishmaniasis Surveillance and Control Programme (VLSCP) in Brazil [78]. Indeed in China an intensive control program that comprised treatment of cases, use of insecticides, and killing of infected dogs eliminated the disease [79]. However, in Brazil, despite the widespread use of culling of seropositive dogs in the country, national health records were not able to associate it with a decline in the number of human cases [80].

A handful of trials performed in the past on dog culling methods obtained different results regarding its efficiency to reduce canine or human disease in the area. A study performed in Brazil comparing the use of a diagnostic test standardly used during mass-screening programs to the use of a more sensitive diagnostic test did see a difference in canine seroprevalence before and after intervention, with a significantly higher reduction in the area where the more sensitive diagnostic test was used [81]. Another study also observed a decrease in the incidence of CanL when seropositive dogs were removed, together with a significant decrease in the incidence of human cases [82]. Incidence of dog seroconversion in their study significantly decreased from 36% to 14%, however – importantly – this decrease was not significant when compared to the control area. Even more so, these findings indicate that removal of seropositive dogs is an insufficient control method for complete elimination of CanL – despite the fact that they might be a good tool to reduce the force of transmission of infection among dogs. The authors of the study propose two possibilities for the continued transmission; the first one being the efficiency and timing of dog removal, the second one being the presence of other reservoirs in the area. Furthermore, they highlight that the significant decrease observed in the incidence of human cases could be attributed to other factors, e.g. increased precautions in the population against acquiring the disease.
Although the previous studies showed a decrease in incidence of VL, another study performed over a 12-month period in Brazil observed an increase in human seropositivity rates from 15% to 54% in areas where infected dogs were eliminated together with an equal increase in the control area [83]. This study did not find any significant differences between the incidence of human serological conversion when comparing the intervention to the control area at any time point. Authors concluded that the reason for the fact that no decline in human cases was observed after eliminating infected dogs can be attributed to the fact that humans might serve as a significant reservoir for the infection in the area. Another field study performed in Brazil showed that removing seropositive dogs with active disease soon after detection may indeed affect the cumulative incidence of seroconversion in dogs temporarily [84]. However, they detected newly infected dogs every four months throughout the study, indicating that the transmission of *L. infantum* was not interrupted and culling of dogs can not be regarded as an efficient measure to eliminate CanL. A study performed by Nunes et al [85] tested the relationship between the rate of eliminating dogs compared to the incidence of human disease during the period from 1999 to 2008 and stated that dog euthanasia rate caused a decrease in transmission potential and was related to incidence of human disease. Furthermore, a study performed by Werneck et al [86] compared the effectiveness of insecticide spraying to dog culling or a combination of both methods and assessed their effect on the incidence of *L. infantum* infection in humans in Brazil. The outcome of the study is comparable to previously performed studies and reinforces the idea that current control programs in Brazil – involving culling of seropositive dogs and insecticide spraying – are not effective. The use of insecticides as vector control tool could theoretically be highly effective, however it’s failure within the Brazilian control programme is possibly linked to the fact that insecticide spraying mainly reduces biting rates within and around houses and does not necessarily reduce overall sand fly population numbers. This together with its low coverage, short-lived residual activity, and higher densities of vectors outside houses (e.g. animal sheds) leads to its ineffectiveness in controlling ZVL [87–89].

Important to note is that, even though some studies did show a decrease in disease incidence, none of these studies were able to achieve complete elimination of transmission in the studied areas. A systematic review that analyzed the effectiveness of VL control programs concluded that there is a lack of scientific evidence for the effectiveness of these interventions, including animal reservoir control, vector control with insecticide spraying or a combination of both [90]. This has been confirmed by a recent study performed in Brazil evaluating the prevalence and incidence of asymptomatic infections by *L. infantum* in children in areas where the VLSCP had been carried out for four or six years. This study clearly showed an ineffectiveness of the VLSCP at reducing ZVL transmission [91].
Different reasons lay at the basis of why culling fails to control ZVL; variations in disease incidence, the need for a high proportion of dogs to be killed in order for it to be effective, only seropositive dogs are being eliminated without taking into account infectious dogs that are not spotted by current serological tests, long interval between diagnosis and culling of the dog, or eliminating infected dogs that may never become infectious and hence making room for the introduction of new susceptible dogs – a practice that has been shown to occur frequently in Brazil [92]. Furthermore, a main argument against the implementation of the culling programs is the unnecessary sacrifice of healthy dogs that, due to low specificity of currently available diagnostic tests, are erroneously diagnosed. Researching the infectiousness of dogs, e.g. proportion of infected dogs that become infectious, the variation in infectiousness between dogs, the latent period between infection and infectiousness, and the sensitivity of diagnostic tests to identify infectious from non-infectious dogs is essential in order to guide current control programs, such as the culling of dogs, or to develop new tools to identify and control infectious dogs.

A study aimed to address these unresolved questions was performed in Brazil during a longitudinal study by using a mathematical model [93] and showed that only 43% of infected dogs become infectious with a small proportion of dogs becoming highly infectious. However, it has to be noted that these highly infectious dogs could be responsible for 88% of all transmission in case sand flies would bite all dogs equally. Even more so, the probability of a dog to be infectious was shown to be correlated with the presence of clinical symptoms. The authors concluded that a high incidence of infections and infectiousness in endemic areas together with a lack of sensitive diagnostic tests and the time delay between the diagnosis and culling of the dog most likely lies at the basis of the failure of dog culling. Therefore, in order for culling to be a successful control method for ZVL a very high proportion of infectious dogs should be removed each year, pointing out the need for a highly sensitive diagnostic test. A newer study investigated the effectiveness of culling dogs when implementation of the program occurs imperfectly, also by using a mathematical model [94]. The results of their model suggest that strategies in the culling of dogs should differ in areas where there is a high or a low transmission. In areas with a high transmission a more integrated approach should be chosen, by also including asymptomatic but infectious dogs in the program. Contrarily, culling of only symptomatic dogs appeared to be sufficient in low transmission areas to maintain a prevalence of less than 1%. The need to include asymptomatic dogs in high transmission areas raises concerns, due to the increased chance of putting down healthy dogs. Therefore, in these areas combined strategies should be prioritized.
2 EVALUATION OF VECTOR CONTROL METHODS USING ANTI-SALIVA ANTIBODIES AS MARKERS OF EXPOSURE

Evaluation of the effectiveness of anti-vectorial measures and transmission of vector-borne diseases are currently performed by bioassay tests in the lab, by measuring the incidence, morbidity or mortality of vector-borne diseases in controlled clinical field trials or by entomological methods (e.g. human landing catches, capturing with traps). However, for large-scale field applications these methods have their limitations and ethical concerns, especially regarding human landing catches. Therefore, since saliva of bloodfeeding arthropods was shown to elicit an antibody (Ab) response in the host, the use of the host’s Ab response against certain salivary proteins has been proposed as a useful tool to measure host exposure to vectors of diseases and could be used as a method to evaluate vector control interventions. In the following section, different properties of saliva of bloodfeeding arthropods will be briefly discussed. The main topic of this chapter, however, is the use of saliva in estimating host exposure to disease vectors, with a special emphasis to sand flies, and will be discussed in the last part of this chapter.

2.1 SALIVA OF HAEMATOPHAGOUS ARTHROPODS AND ITS EFFECT ON THE HOST

Haematophagous arthropods are known to possess specialized saliva containing a complex spectrum of proteins. This specific cocktail of molecules might, however, be affected by age of the vector and environmental changes [95–97]. Components present in saliva of all haematophagous arthropods are known to cause allergic reactions in humans – making these arthropods a real physical nuisance. Apart from allergic reactions, salivary proteins of disease vectors have been identified that influence the host haemostasis’ mechanisms such as vasoconstriction, formation of a primary platelet plug or vessel strengthening by blood coagulation (reviewed in [98,99]). A strategy employed by the sand fly Phlebotomus longipalpis and the black fly Simulium vittatum to circumvent the vasoconstriction associated with the primary response to blood vessel damage is the secretion of a vasodilator called maxadillan [100,101]. Phlebotomus sand flies do not express maxadillan but have a vasodilator substitute, the adenosine and 5’AMP vasodilators [102]. Overcoming the host’s vasoconstriction response is not the only obstacle in order to obtain a decent bloodmeal, platelet aggregation and consequently coagulation also need to be controlled. The most well-known platelet inhibitor ubiquitously found in saliva of haematophagous arthropods is apyrase – an enzyme that prevents the formation of a platelet plug after injury to the blood vessel by hydrolyzing ADP released by damaged cells and activated platelets (reviewed in [98]). Other examples are aegyptin present in saliva of A. aegypti [103] or triplatin – a salivary protein of T. infestans [104]. The aegyptin protein binds collagen,
leading to inhibition of collagen-induced platelet aggregation and adhesion (reviewed in [99]). Apart from inhibiting platelet aggregation, triplatin was also shown to inhibit vasoconstriction. Activated platelets lead to coagulation which eventually causes – through the formation of thrombin – blood clotting and a complete arrest of haemorrhage. Anti-coagulants found in sand flies comprise Lufaxin [105], which is also an inhibitor of inflammation, and the salivary protein SP15 [106]. Furthermore, other proteins – such as yellow-related proteins from sand flies or lipocalin present in saliva of Rhodnius prolixus – were shown to bind biogenic amines and interfere with the hemostatic system in that way [107,108].

Besides the influence of saliva on the host hemostasis, it has also been shown to influence the host’s immune system – generally promoting a Th2 environment – and suppress pain perception at the bite site (reviewed in [98]). Pain perception at the bite site is suppressed by adenosine deaminase enzymes found in saliva of several bloodfeeding arthropods [109]. The change of immune environment in the host in the direction of a Th2 response is not only beneficial to the act of taking a bloodmeal but might also have an impact on pathogen transmission. Several studies have revealed the enhanced effect of saliva on pathogen transmission, consequently promoting infection. This is observed for salivary components of triatomine bugs, ixodid ticks, and mosquitoes enhancing the transmission of T. cruzi [110]; Thogoto virus [111] or Borrelia burgdorferi [112]; and West Nile [113,114], La Crosse [115] or Cache-valley [116] virus, respectively (reviewed in [98]). Regarding sand flies, transmission of Leishmania major was proven to be enhanced by salivary gland extracts of L. longipalpis and resulted in lesions that were five to ten times larger and contained 5,000 times more parasites than the controls [117]. The vasodilator maxadilan of L. longipalpis was shown to play a role in the enhanced parasite transmission [118].

On the contrary, pre-exposure of hosts to uninfected bites has proven to protect against the establishment of several vector-borne infections. This is an interesting finding since it proposes the use of salivary proteins as vaccine candidates. This has been observed for animals pre-exposed to tick bites, which protected against tularaemia [119] and Lyme borreliosis [120]. Sand fly saliva has received much attention in the past for its potential to be used as a vaccine strategy against leishmaniasis. Bites of uninfected P. papatasii sand flies were shown to protect against L. major infection, implying that exposure history of individuals can have an impact on the incidence of CL [121,122]. Subsequent studies on mice identified a salivary protein of P. papatasii – SP15 – that was capable of rendering the same level of protection as whole saliva and was as such proposed as a vaccine candidate against L. major infection [123]. Another example comprises the sand fly L. longipalpis, of which the salivary proteins LJL143 and LJM17 induced protection in immunized dogs when challenged with L. i. chagasi [124]. The LJM11 salivary protein of L. longipalpis, on the other
hand, has been shown to induce a long-lasting immunity against *L. major* without any additional adjuvant, and resulted in an ulcer-free protection [125].

From these examples, it is clear that saliva of bloodfeeding arthropods is very complex with an enormous functional diversity to facilitate the act of taking a bloodmeal. Although these proteins are very important during the host-vector interaction, they are out of the scope of this thesis and will therefore not be discussed any further. This thesis is merely interested in the Ab responses that are elicited in the host against certain salivary proteins, rendering them useful to estimate host exposure to vectors of diseases and evaluate vector control interventions. In the past, studies have focussed on the use of whole salivary glands to estimate host exposure. Later on, they diverged into the identification of antigenic salivary proteins, able to replace the use of whole saliva and overcome the common drawbacks associated with it (e.g. higher chance of cross-reactivity, labor-intensive, time-consuming). The following paragraphs will focus on these vector exposure markers with special attention to studies on sand fly saliva. The last part is dedicated to the identification of salivary B-cell epitopes – the newest addition to the domain of salivary exposure markers.

### 2.1.1 Salivary Exposure Markers in Various Vectors

Research on mosquito saliva already pointed out that the immune response to salivary proteins correlates well with exposure to mosquito bites [126–129]. Ab responses were shown to be genus-specific, which enforces the idea of using these salivary proteins as an indicator of vector exposure [130].

A study in Senegal focused on the exposure of children to malaria by screening for Abs against whole salivary gland extract (SGE) of *Anopheles gambiae*, and compared the results with entomological data obtained by CDC light traps and the outcome of malaria attacks within 3 months follow-up in these children [127]. They found that high anti-SGE Abs levels were present in case a malaria attack presented within three months and that these Ab levels positively correlated with the density of *Anopheles* mosquitoes. Similar results were obtained in Thailand, where people residing in malaria endemic areas showed significantly higher IgM and IgG titers against *An. dirus* salivary proteins, with the IgG response being present at much higher titers in patients experiencing an acute malaria infection [130]. Even more so, in a study in Brazil on *An. darlingi*, the most widespread malaria vector in the region, an association between anti-salivary Abs and clinical immunity against *Plasmodium vivax* was found [131]. A study performed in Indonesia on *An. sundaicus* supported the findings from previous studies and proposed the 31, 33, 41 and 46kDa salivary proteins as most immunogenic and possible biomarker of exposure to *Anopheles* bites [132].
Regarding *Aedes* mosquitoes, vectors of several arboviruses, studies showed that the IgG Ab response against *Aedes albopictus* saliva positively correlates with data from classical entomological methods and cross-reacted with salivary proteins of *Ae. aegypti* indicating that also salivary proteins of this genus could be used to evaluate control measures [128,129]. Furthermore, the IgG and IgM response against salivary Ags of *An. gambiae* or *Ae. aegypti* were shown to be genus specific and were even present in individuals that were merely transiently exposed to either of these two, indicating high sensitivity of these assays [133]. In subsequent studies, these Ab responses have been used to assess the efficacy of vector control methods; one study performed in Angola evaluated the efficacy of insecticide-treated nets used in malaria vector control, another study performed later compared the efficacy of three malaria control methods (Long Lasting Insecticide Treated Nets, Insecticide Treated Plastic Sheeting (ITPS), and Indoor Residual Spraying [134,135]. Both studies confirm the use of the anti-saliva Ab response as an efficient and reliable indicator to evaluate the effectiveness of malaria vector control methods. Furthermore, the study comparing three control methods showed that the use of deltamethrin-impregnated nets together with ITPS was more effective in reducing the density of *Anopheles*, the contact between humans and *Anopheles* mosquitoes, and the prevalence of *Plasmodium*, than any other method used alone [134].

In order to improve the specificity of mosquito exposure markers, attention has been paid to the identification and more importantly validation of immunogenic salivary proteins that can replace the use of whole saliva. The most well-known one up-to-date for mosquitoes is the gSG6 *Anopheles*-specific salivary recombinant protein [136–138]. This protein has been used to test sera from individuals living in a hyperendemic malaria area in Burkina Faso and showed to possess high sensitivity in measuring exposure to *An. gambiae* able to follow seasonal fluctuation of human exposure, with a significant decline after reduced exposure [139]. However, the study found a difference between the Ab response in children and adults, being lower in the latter group, indicating that desensitization to salivary proteins occurs after intense and prolonged exposure [139]. Similar findings were obtained during a study in Tanzania, supporting the use of this protein as an exposure marker to *Anopheles* bites and providing the first evidence for an association between malaria incidence and anti-gSG6 Ab response [140]. In subsequent studies, the Ab response against a newer *Anopheles*-specific salivary protein, the cE5, was compared to the gSG6 and also showed to be highly immunogenic [141]. However, the long-lasting Ab response against the cE5 protein observed in this study renders it unable to show a seasonal fluctuation of *Anopheles* mosquitoes – one of the requirements for it to be used as a proper exposure marker.

In *Glossina* flies – vectors of human African trypanosomiasis (HAT), saliva was shown to predominantly induce Th2 responses [142] and immunogenic proteins in *Glossina* saliva were shown
to be species specific and rely on the infection status of the tested individual [143]. When the IgG response against *Glossina fuscipes fuscipes* saliva was measured according to vector exposure and infection status of the individual, it was proposed to be a marker of exposure for *Glossina* bites whether the individual was infected or not [144]. The Ab response against saliva from *G. p. gambiensis* was also shown to be associated with human exposure to Tsetse flies in West-Africa [145]. However, no association was found between anti-saliva Ab response and the HAT status of the individual [145]. Further studies focused on the identification of an immunogenic protein and highlighted that the rTsal1 protein from *G. m. morsitans* was able to cross-detect Abs directed against *G. f. fuscipes*, while not cross-reacting with Abs against saliva from Anopheline mosquitoes [142]. Hence, indicating that screening for rTsal1-specific IgG can replace saliva-specific IgG in order to determine the risk of human exposure to pan-Tsetse species – also demonstrated in future studies [142,146]. During a recent study, a nanobody-based immunoassay was developed able to detect the presence of anti-Tsal Abs with a high sensitivity without the need for adaptation to different host species [147].

Ab responses against salivary proteins of triatomine bugs, vectors of *T. cruzi* (causative agent of Chagas’ disease), have also been characterized and were shown to be useful in measuring exposure against this vector [148,149]. Measuring the Ab responses to salivary Ags of *Triatoma infestans* in domestic animals was identified to be a useful biomarker for low-level infestation of triatomines [150]. Hence, in a study in Peru on triatomine bugs, the hosts’ Ab response against salivary proteins of *T. infestans* was used as a measure to evaluate the use of long-lasting deltamethrin-impregnated nets and the effect on *T. cruzi* infection. This study showed that nets did indeed protect sentinel animals from bites of *T. infestans* and pointed out the importance of using the Ab response against *Triatoma* saliva to assess animal exposure and efficacy of the nets, instead of merely counting vectors that infested bricks placed in new sentinel protected enclosures as was performed in a previous study [151,152]. A consequent study that focused on the identification of a single immunogenic protein in saliva of *T. infestans* proposed the rTiSP14.6 to detect low level infestation of triatomines [153]. The Ab response against this recombinant protein was shown to increase with exposure to *T. infestans*. Furthermore, this protein assay was sensitive enough to detect sera from chickens in low-infested households and highly specific since no cross-reaction with non-triatomine vectors was observed. The rTiSP14.6 protein did, however, react with sera from chickens exposed to *T. brasiliensis, T. sordida, R. prolixus* and *P. megistus*, supporting the use of this protein as a marker of exposure to five different triatomine species [153]. However, due to its inability to give a sufficiently high reaction with sera from guinea pigs, a later study focused on the IgG and IgM response against triatomine saliva in guinea pigs and demonstrated a strong variability in
the salivary protein profile and immunogenicity of salivary proteins between different strains of *T. infestans* and between different developmental stages of *T. infestans* [148]. This finding also led to the identification of a 35 kDa salivary Ag which had consistent immunogenicity across all strains and developmental stages of *T. infestans* and was proposed for further evaluation as an exposure marker to *T. infestans* [148].

### 2.1.2 Salivary Exposure Markers in Sand Flies

Experimentally and naturally bitten hosts were shown to produce species-specific Abs to sand fly saliva [154,155], which were proven to correlate with the intensity of sand fly exposure [156–158]. Even more so, species variability in sand fly salivary Ags enables the possible identification of species-specific Ags, as it was shown before that only species of the same subgenus likely cross-react with each other [155]. Exposure markers of several sand fly species have already been identified for *P. perniciosus*, *P. papatasi*, *P. argentipes*, *P. orientalis*, and *L. longipalpis*.

Studies on *P. papatasi*, vector of zoonotic CL caused by *L. major*, led to the identification of the salivary protein PpSP32 as being the most antigenic among all since it strongly recognized sera from people naturally exposed to *P. papatasi* bites [159,160]. Furthermore, the recombinant form of the PpSP32 protein was shown to resemble the native protein, supporting its role as a marker of exposure [160]. This protein is so far unique to sand flies and shares significant homology with five different sand fly species (*P. papatasi*, *P. argentipes*, *P. ariasi*, *P. perniciosus* and *L. longipalpis*) [161]. During subsequent studies, the PpSP32 protein was used to screen a large cohort of individuals in Tunisia in order to validate the use of the Ab response against this protein as a biomarker of exposure to *P. papatasi* [162]. Indeed a significant correlation was obtained between the OD values obtained by the PpSP32 and the OD values obtained when whole saliva was used. Furthermore, good sensitivity and specificity values of the assay using PpSP32 were obtained, equaling 80.38% and 71.57% respectively. Even more so, the Ab response against the PpSP32 was shown to not cross-react with Abs against saliva from *P. perniciosus*, also prevalent in Tunisia. All these results together validated the use of the anti-PpSP3 Ab response as a suitable biomarker of exposure to *P. papatasi* [162].

When moving further down to East Africa, the most important sand fly species — *P. orientalis* is the main vector of *L. donovani*, and feeds on different mammals, depending on the host availability [163–165]. The main salivary antigens of this sand fly were identified and comprise a D7-related protein (PorSP67), an Ag 5-related protein (PorSP76), an apyrase (PorSP15), a yellow-related protein (PorSP24), and a ParSP25-like protein (PorSP65) [166]. Of these five proteins the rPorSP24 was shown to be the most reliable protein to screen for dog, sheep or goat exposure to *P. orientalis*, due to its
high correlation with whole saliva, its sensitivity and specificity values, and its high specificity to *P. orientalis* compared to *P. papatasi* and *S. schwetzi* [166].

In the Indian subcontinent the main vector of *L. donovani* is *P. argentipes*. IgG responses against *P. argentipes* saliva were shown to correlate with the number of female *P. argentipes* trapped per household. However, the salivary proteins from the two local species in India, *P. papatasi* – vector of *L. major* – and *P. argentipes*, were shown to cross-react with each other, which could be overcome by implementing a pre-adsorption step in the *P. argentipes* ELISA which neutralizes *P. papatasi*-specific Abs [157]. Furthermore, when VL patients were monitored before, during and after their hospitalization, a significant decrease in Ab titers was observed during their stay in the hospital where they were protected from sand fly bites. However, after returning home Ab levels increased significantly to titers higher than before [157]. The use of sand fly saliva to evaluate vector control interventions such as insecticidal bed nets was further supported by a study in which the impact of deltamethrin-impregnated bed nets was defined by measuring the anti-*P. argentipes* Ab response [167]. The study showed that exposure to *P. argentipes* was reduced by 9-12% in the intervention group while maintaining, however, a significant number of individuals with a high level of anti-*P. argentipes* Abs 24 months after implementation [167]. This suggests that such impregnated bed nets only have a limited effect on *P. argentipes* exposure, as was also shown in a previous study in the same region using sand fly abundancy as reference [168]. All together, this does support the role of salivary Abs in evaluating vector control tools [167].

Concerning *L. longipalpis*, vector of *L. infantum* in the Americas, the most abundant secretory proteins and transcripts have been characterized in the past [169], and two yellow-related salivary proteins – LJM17 and LJM11 – were identified and were shown to positively react with sera from humans living in an endemic area for VL [170]. No cross-reactivity with *L. intermedia* – a sympatric sand fly species – was observed, implying that the Ab response against these two proteins can be used as a universal marker of exposure to *L. longipalpis* [170]. Furthermore, both of these proteins were also shown to positively react with sera from dogs from CanL endemic regions [170]. During a subsequent study in which the reactivity of these proteins was compared to whole saliva, the combination of both proteins was shown to considerably increase the detection of anti-saliva seroconversion and hence was proposed as the best tool to assess exposure to *L. longipalpis* [171].

*P. perniciosus* is the principle vector of *L. infantum* in Western Mediterranean countries, and consequently the main focus of this thesis. Multiple studies in the past have focused on the Ab response against whole saliva and salivary proteins from *P. perniciosus* and have shown that anti-*P. perniciosus* Abs in dogs positively correlate with exposure to bites, as measured by the number of bloodfed females [158]. When different vertebrate hosts were screened for anti-*P. perniciosus* Abs,
high levels were obtained in domestic dogs as well as in wild animals, such as hares and rabbits [172]. During an outbreak of VL in Fuenlabrada (southwestern Madrid) Iberian hares (*Lepus granatensis*) were identified as being active reservoirs [173]. This suggests that using anti-sand fly Ab response could even aid in identifying potential reservoirs of transmission. During subsequent studies, yellow-related proteins and apyrases were highlighted as being highly antigenic *P. perniciosus*’ salivary proteins in dogs [158,174] and were studied in depth in following studies [175,176]. During a longitudinal study performed in Italy, the *P. perniciosus*’ rSP03B (43kDa yellow-related protein) and the rSP01 (35.5kDa apyrase) were evaluated and compared to the use of whole saliva [175]. The rSP03B protein was revealed as the closest resemblance to whole saliva, both showing seasonal dynamics of the Ab response caused by changes in sand fly abundance [175]. This study also showed a significant positive association between anti-*P. perniciosus* Abs and *Leishmania* infection status, possibly due to a higher sand fly challenge resulting in a higher chance of becoming infected [175].

### 2.1.3 B-CELL EPITOPE AS EXPOSURE MARKER

Due to the high number of salivary proteins present in saliva of bloodsucking arthropods, the presence of cross-reactive proteins is very likely. This cross-reactivity can be overcome by selecting only these proteins that are specific and highly immunogenic. An upgrade to an even more species-specific salivary Ag can be obtained by focusing on the identification of B-cell epitopes.

Salivary B-cell epitopes have been identified for several mosquito species. For *Ae. aegypti*, for example, an N-terminal extremity peptide of a 34 kDa salivary protein (N-term 34kDa peptide) has proven to be valuable to assess human exposure [177]. When the peptide was tested in field conditions in Benin, the IgG response against this peptide was associated with the intensity of rainfall in the region and followed the same heterogeneity between individuals and villages as reported in previous studies with whole saliva; encouraging the use of this peptide as an individual exposure marker to *Ae. aegypti* [177]. Recently, this peptide has been shown to cross-react with *Ae. albopictus*, expanding its use as a marker of human exposure to *Aedes* species [178]. Furthermore, when vector control implementation measures against *Aedes* mosquitoes in La Reunion island were evaluated by measuring the IgG response against the N-term 34kDa peptide, a significant decrease in the immune response and the proportion of immune responders was observed one week after implementation [178]. This decrease was faster than when whole saliva was used [128], reinforcing the idea that the IgG response to the peptide is more sensitive to detect early variations in human exposure to *Aedes* bites [178]. Currently, the N-term 34kDa peptide is under development in the form of a rapid vector exposure test [179].
Regarding *Anopheles* mosquitoes, an immunogenic peptide marker corresponding to the salivary protein gSG6 has been identified [180]. The Ab response against this peptide – the gSG6-P1 – correlates with *Anopheles* exposure (as measured by classical entomological methods), was shown to increase significantly during rainy season, and varied across individuals [180]. Furthermore, since the gSG6 protein was shown to be *Anopheles*-specific, these results support the use of this peptide as a marker of exposure to *Anopheles* bites. Indeed, this gSG6-P1 peptide has consequently been validated to evaluate exposure against *An. funestus* in Senegal [181]. Consequent studies revealed that the IgG response against gSG6-P1 was in line with parasite prevalence on population level and was able to distinguish between different malaria transmission settings (hypo, meso, hyper) [182]. Therefore, the gSG6-P1 can not only be used as a marker of exposure to *Anopheles* mosquitoes but can also be exploited as an epidemiological marker for risk of parasite transmission. This has clearly been the most successful peptide in the field of vector exposure, and has therefore also been proposed as a novel tool to evaluate the efficacy of ITNs in malaria vector control, for which it showed to be more sensitive and specific than the use of whole saliva [183].

### 2.2 Spreading up the ProcE: Lateral Flow Immunochromatographic Assays

The most widely used serological method to assess host exposure to bloodfeeding arthropods is the enzyme-linked immunosorbsent assay (ELISA). However, certain drawbacks are commonly associated with the use of such conventional techniques in field settings and can be overcome by using lateral flow assays (LFA) (reviewed in [184,185]). LFAs are fast assays that only require one step in order to achieve a read out. They have a low operational cost, require simple instrumentation, are prepared in a user-friendly format, have a long-term stability under a different set of environmental conditions, and their portability makes them especially unique point of care applications. Since the use of LFAs is brand new in the field of vector exposure, their principle and application in other fields will be discussed in the following paragraphs.

Different LFA formats exist and the one that is chosen depends on what needs to be detected. However, all formats have four main components in common: (1) sample application pad, (2) conjugate pad, (3) nitrocellulose membrane (NC), and (4) absorbent pad. Choosing the optimal material to prepare an LFA is crucial in order to obtain a test with high sensitivity, specificity, and reproducibility (reviewed in [184]). For example, the sample application pad (also called the lower absorbent pad) should be capable to push the fluids in a smooth, homogenous and continuous manner. On the other hand, the conjugate pad – the place where labeled conjugate is impregnated – should be made of material that enables immediate release of the conjugate when in contact with
the moving liquid. Results of the assay can change significantly if the amount of conjugate impregnated, the time it was left to dry properly or the pad’s ability to release the conjugate varies. The choice of the NC membrane is probably the most important factor that could potentially influence the sensitivity of the assay. Being the place where the test and control lines are coated, the NC membrane should provide excellent binding to these probes. However, it should also exhibit low non-specific binding, which might influence the results of the assay significantly. Apart from that, it should also have an optimal wicking rate which ensures proper migration of all fluids (conjugate, sample, buffers) yet also enabling decent binding of these components to the probes present on the test and control line.

Looking at the different formats a bit more into detail, it can be concluded that three major formats exist: (1) the sandwich format, (2) the competitive format, and (3) the multiplex format. The choice of the format depends on the analyte to be tested (reviewed in [185]). For larger analytes or analytes with multiple antigenic sites a direct or sandwich format is used. On the other hand, for smaller analytes that cannot bind two Abs simultaneously the competitive assays are employed. The multiplex option allows the detection of multiple analytes simultaneously, with the number of test lines corresponding to the number of target analytes being tested.

Considering the conjugate labels, the most frequently used one is gold nanoparticles. Others are magnetic particles, fluorescent and luminescent materials, enzymes, and colloidal carbon; all having their own strengths and drawbacks (reviewed in [184]). Using colloidal carbon, for example, is a good option for LFAs due to its low cost, high sensitivity, and very low detection limits. However, some major problems are also associated with it, such as non-specific adsorption of proteins and biomolecules, which might interfere with the specificity of the test. Overall, when color producing labels are used LFAs give a reliable qualitative read-out that provides the user with a clear idea if the desired response is present or not. However, in certain cases a quantitative determination of the result is preferred. In this case, mechanical readers can be used: the strips are inserted into a strip reader and the intensities on the test and control line are simultaneously recorded by imaging software. The selection of the type of detector to be used depends on the label which was employed during analysis (reviewed in [184]).

Finally, different biorecognition molecules can be used during the preparation of LFAs, such as Abs, aptamers, and molecular beacons. When Abs are used as the biorecognition molecule in the LFA the assay is referred to as a lateral flow immunochromatographic assay (LFIA). As broadly described in the previous chapter, the Ab response against specific salivary proteins of disease vectors has been widely accepted as being a marker of exposure for certain haematophagous
arthropods and hence could be used to evaluate vector control methods. Therefore, LFA using Abs as biorecognition molecules will be the main focus during the following paragraphs.

2.2.1 THE USE OF RAPID TESTS IN VECTOR-TRANSMITTED DISEASES

Immunochromatographic LFAs, commonly referred to as rapid diagnostic tests (RDTs) have been widely employed in a broad range of fields. In the next paragraph I will briefly discuss some immunochromatography-based rapid tests used in the control of vector-transmitted diseases, of which the most well-known one – malaria – has more than 200 RDTs on the market today. All of them enabling a quick and easy diagnosis [186]. The first malaria RDTs were developed in the mid-1990s [187,188] and the ones used today are largely based on three Plasmodium Ags, being: P. falciparum histidine-rich protein 2 (PfHRP2), plasmodial aldolase and plasmodial lactate dehydrogenase (pLDH). All of these RDTs use immunochromatography, in which the presence of the parasite Ag is demonstrated when it is captured by the Ab coated on the test line. Currently, more than 90% of RDTs used in the diagnosis of malaria screen for the HRP2 Ag (reviewed in [189,190]).

These RDTs were shown to possess acceptable sensitivity and specificity values and have been employed in both low- and high-endemic areas in the diagnosis of malaria. They were deemed particularly useful as a replacement for microscopy in the diagnosis of malaria in pregnant women [191–193]. However, due to the persistence of the HRP2 Ag in the peripheral blood, these RDTs are not suitable to estimate the response to therapy [194,195]. Even more so, specificity and sensitivity values were shown to vary across batches resulting in false diagnosis [196]. Apart from the variation in the manufacturing process of the kits, false-negative results can also be caused by either a genetic variation in the PfHRP2 amino acid sequence or by deletion of the Pfhrp2 gene in certain isolates (reviewed in [189,190]). Furthermore, very high parasitaemias can cause a prozone effect; flooding of the RDT capture site resulting in a false-negative result [197]. All of these factors considerably affect the specificity of these RDTs. Conversely, false-positive results were shown to be associated with the Ab used for Ag capture, being either IgG or IgM, or with the persistence of the Ag after clearance of the parasite. The sensitivity and specificity of the tests also seemed to be associated with the age of the patient, transmission intensity in the region and the absence of symptoms, resulting in under- or overdiagnosis (reviewed in [189,190]). Part of these difficulties can be overcome by screening for a different Ag, e.g. LDH – an essential energy-producing enzyme present in all four human Plasmodium species. RDTs based on the detection of LDH can be either specific for P. vivax or P. falciparum or are combined with the detection of PfHRP2. The benefit of using LDH as an Ag in an RDT lies in its ability to monitor the parasite’s response to treatment – since the protein does not persist in the bloodstream after clearance of the parasite – and the evasion of the prozone effect commonly
associated with the PfHRP2-based RDTs. However, these RDTs were shown to have very low sensitivity at low parasitaemia (reviewed in [189,190]). Thus, it is clear that although there are plenty of malaria RDTs on the market and currently used in endemic settings, they are also accompanied by substantial difficulties and challenges which need to be addressed and overcome in the future.

Apart from RDTs used for the diagnosis of malaria in humans, a test has been developed which enables the identification of potential malaria vectors and measures sporozoite infection rates [198,199]. This test is based on the detection of the circumsporozoite (CS) protein of *P. falciparum*, *P. vivax* variant 210, and *P. vivax* variant 247, usually detected by performing a CS-ELISA. The rapid VecTest™ Malaria showed a complete correlation with the standard ELISA, with high sensitivity and specificity values, and was made commercially available in the format of a malaria rapid panel dipstick test able to detect *Pf*, *Pv*210, and *Pv*247 malaria sporozoites in field caught *Anopheles* of several species [198,199]. A similar test – the VecTest – was developed to detect West Nile Virus (WNV) in mosquito pools and was later shown to be also useful in the diagnosis of WNV in birds [200].

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a disease caused mainly by the protozoan parasite *T. brucei gambiense* in West and Central Africa. In order to allow rapid diagnosis of the infection, two RDTs were developed in order to screen individuals in primary health centers: the HAT Sero-Strip and the HAT Sero-K-Set [201]. Both RDTs enable the detection of specific Abs against the variant surface glycoproteins of the *T. brucei gambiense* Ag types LiTat 1.3 and LiTat 1.5. Case-control studies attributed very high sensitivity (98.5%) and specificity (98.6%) values to both tests, which did not differ significantly from the standard immune trypanolysis test or the Card Agglutination Trypanosomiasis Test (CATT). Recently, the HAT Sero-Strip has been evaluated in field conditions and proven to be highly sensitive (100%) and specific (97%) with a diagnostic accuracy comparable to the CATT [202]. However, currently the cost of these tests is the limiting factor for their large-scale deployment in central Africa and ways to reduce the unit costs – which are now higher than 1EUR – need to be undertaken [202].

Apart from these Ab-detection RDTs, another HAT rapid test – the HAT-PCR-OC test – has been described and detects in a rapid and sensitive manner *T. b. gambiense* parasites in blood samples by means of oligochromatography (OC), which allows a rapid detection of PCR products in a dipstick form [203]. Although this RDT is not based on immunochromatography and hence is out of the scope of this chapter, it is worth mentioning that the HAT-PCR-OC is the first nucleic acid-based diagnostic dipstick test for vector-borne diseases of which the principle might be extrapolated in the future to the diagnosis of other important infectious diseases. It has to be noted however that drawbacks associated with such OC dipsticks include the need for midlevel-equipped laboratory
facilities, such as clean laboratories with electricity and cold storage. Therefore, for individual rapid diagnosis requiring no specialized equipment, immunochromatographic devices remain the number one.

Another protozoan trypanosomatid – *T. cruzi* – is the causative agent of Chagas’ disease in the Americas. Recently, several immunochromatographic RDTs for its diagnosis have been brought to the market [204–209]. For example, the Trypanosoma Detect MRA rapid test. This RDT has been used to detect *T. cruzi* infection in reservoir animals, for which it showed to possess both a high specificity (94%) and a high sensitivity (96%) [204]. A subsequent study evaluated the same RDT during a multicenter study on human samples, which resulted in a lower (but still acceptable) sensitivity (85%) of the test [205]. Although the results from these and other studies strengthen the use of these RDTs as a diagnostic tool, their retail price is rather expensive compared to standard methods [205]. This is a common problem for immunochromatographic dipsticks and can be overcome by producing RDTs which are more cost-effective than currently available diagnostic test (i.e. higher sensitivity and specificity values).

Finally, going back to the main theme of this dissertation – controlling the spread of ZVL – I will briefly discuss the immunochromatographic tests that have been used so far in diagnosing the disease in the following paragraph. The first RDT produced for the diagnosis of VL was based on a 39-amino-acid-repeat recombinant *Leishmania* Ag (rK39) which is a highly conserved epitope in *L. infantum* and *L. donovani* [210,211]. Currently, RDTs based on two Leishmanial Ags are on the market, the rK39 and the rKE16.

A report published by the WHO describes the performance of five commercially available RDTs that are all based on Ab detection against either the rK39 or the rKE16 protein [212]. They studied the performance of these RDTs in the three global regions of VL endemcity (Indian subcontinent, East Africa, Brazil) and showed that all RDTs possessed a high specificity (>95%) in all areas. However, a varying sensitivity was observed between tests and between regions, ranging from 36.8% to 100%, with the highest sensitivity observed for all RDTs in the Indian subcontinent. They also noted a lower performance of the rKE16 RDT in Brazil and East Africa, which according to the authors is likely due to the fact that the rKE16 Ag used in these RDTs is based on an Indian strain of *L. donovani*, whereas rK39 is based on *L. infantum* [213,214]. Since they observed that the performance of these RDTs was highly comparable in one region, they concluded that differences in test performance is only reflected by the three VL endemic regions tested [214]. According to this study all RDTs possess a high specificity, i.e. almost no false positive cases, and could therefore be used to direct treatment in case of a positive test result in combination with clinical case detection. However, they are not sufficient to rule out diagnosis of VL due to their low sensitivity values and hence the
high probability of being false negative. Negative results, therefore, need to be ruled out by additional serological or parasitological tests. Results from a previous meta-analysis on the performance of the DAT and the rK39 RDTs together with results from a multicentre evaluation on the rK39 RDT led to it being the main tool for VL elimination in the Indian subcontinent when combined with the WHO clinical case definition for VL [214–216]. This meta-analysis found a combined sensitivity of 93.9% and a combined specificity of 90.6% for the rK39 RDT [216]. Another meta-analysis performed years later found a comparable combined sensitivity and specificity of the rK39 RDT of 92% and 95%, respectively [217]. However, a recent study performed in Northern Ethiopia obtained a sensitivity of 84.1% and a specificity of only 27.8% [218]. These results reinforce the finding that the rK39 RDTs have variable functionality in different regions and settings, and future improvement of these RDTs is essential to enhance their accuracy.

Other studies performed in the past evaluated the rK39 RDT for the use with human saliva or urine [219]; compared its functionality when serum or whole blood is used [220,221], evaluated its functionality in an area with a high prevalence of HIV [222], or tested for cross-reactions with *L. braziliensis* to assure its functionality in regions where an overlap of VL and localized CL occurs [223]. Use of the rK39 RDT with saliva resulted in a sensitivity and specificity of 82.5% and 84.06% - 91.57% (depending on the controls used), respectively [224]. Due to its low sensitivity, saliva was deemed incompetent in diagnosing VL [224]. Similar results were observed when urine was used, which resulted in a sensitivity of 96.4% and a specificity ranging from 66.2% - 77.08%, depending on the controls used [219]. Comparably to saliva, the low specificity obtained when the RDT is screened with urine does not allow it to be used in VL diagnosis for now [219]. When the use of serum versus whole blood was compared, an excellent concordance was obtained [220,221]. Furthermore, the study on HIV co-infection concluded that the rK39 RDT has a sensitivity of 64.6% and a specificity of 66.7% in patients co-infected with HIV [222]. Conversely, in HIV-negative patients the sensitivity was 81.0% and the specificity was 81.8% [222], indicating that co-infection with HIV hampers proper diagnosis of VL patients. Whereas the study investigating cross-reactions of the rK39 with *L. braziliensis* concluded that no cross-reaction exists with *L. braziliensis* Abs, allowing the test to be used in areas where a co-occurrence of *L. infantum* and *L. braziliensis* is reported [223].

Apart from diagnosing human VL cases, the rK39 RDT has also been evaluated to diagnose CanL during Brazilian control programs. However, studies pointed towards their inefficiency in identifying asymptomatic but infected dogs [225,226]. Therefore, due to their low sensitivity to detect infection these RDTs are currently only useful in suspected clinical cases and cannot contribute to the control of CanL in these regions [226]. Interestingly, however, since previous studies have suggested that CanL control should be more directed towards infectious dogs rather
than merely seropositive dogs, the rK39 RDT could be of interest since infectious dogs were shown to be more likely RDT positive [225]. The major drawback being its sensitivity (78%) to detect infectious dogs which is probably too low for it to effectively aid in control programs [225].

Besides the rK39, a new generation fusion Ag was designed – the rK28 – and has been translated into an RDT both for humans and dogs [227]. The rK28 protein is a chimeric protein which is designed by fusing multiple tandem repeats of the \textit{L. donovani haspb1}, kinesin and \textit{haspb2} gene [227]. The rK28 RDT is similar to the rK39 in the sense that they are Ab detection tests. Two prototypes previously developed for human use (rK28-LF and rK28-DPP) both showed a higher sensitivity than the rK39 RDT, with a 96% sensitivity for the rK28-DPP and 98% for the rK28-LF [227]. Both of these prototypes were also shown to be highly specific, 100% for the rK28-DPP while a slightly lower specificity was obtained for the rK28-LF (92.5%) [227]. Later on, in 2011, the rK28-DPP for CanL was selected together with an ELISA confirmatory test to replace the previous serodiagnostic protocol for the dog culling program in Brazil. The accuracy of this protocol has been recently evaluated and showed that implementation of this screening protocol in Brazil was more accurate in identifying seropositive dogs compared to the previous protocol, which was based on ELISA and an Indirect Immunofluorescence Test [228]. The higher accuracy was indicated by a higher specificity and positive predictive value of the new protocol, resulting in less healthy dogs being sacrificed aimlessly. However, they also noted that the low sensitivity – reported previously with other VL RDTs – remained, together with its insufficiency to detect infected but asymptomatic dogs [228]. Although these results show an inability of this screening program to reduce the maintainance of (asymptomatic) infected dogs, which still constitute a major public health concern, authors of this study concluded that the rk28-DPP has an excellent accuracy and could contribute to improve CanL diagnosis [228].
3 Objectives

Canine leishmaniasis is a severe progressive systemic zoonotic disease which can be transmitted from dogs to humans, resulting in cases of human zoonotic visceral leishmaniasis in southern European countries with an estimated annual incidence of 1,200 to 2,000 cases. Methods to assess host exposure to the phlebotomine sand fly vector are essential to evaluate vector control tools, currently the prime focus in the control of CanL. Given that the host’s antibody response to sand fly saliva correlates with host exposure, we have validated the use of the antibody response against a salivary protein of *P. perniciosus* – the main vector of CanL in the Mediterranean basin – as marker of exposure. Consequently, we have used this protein to prepare a new vector exposure tool, which is faster and easier to use than conventional serological methods and which proved to be suitable for use in the field where a higher variability of antibodies against sand fly saliva is expected.

The main objectives of this Ph.D. thesis were to:

- Validate the use of the rSP03B yellow-related protein from *P. perniciosus* as a marker of exposure across regions endemic for canine leishmaniasis in the Mediterranean basin and assess the seasonal dynamics of the canine antibody response against *P. perniciosus* saliva in a naturally exposed heterogenous dog population.

- Prepare a test that allows rapid evaluation of exposure to *P. perniciosus* using the rSP03B protein.

- Optimize the rapid test for ideal use in field conditions and evaluate the detectability of the rapid test on serum and blood samples of dogs naturally exposed to *P. perniciosus*. 
4 PUBLICATIONS


The recombinant protein rSP03B is a valid antigen for screening dog exposure to *Phlebotomus perniciosus* across foci of canine leishmaniasis

SHORT COMMUNICATION

The recombinant protein rSP03B is a valid antigen for screening dog exposure to Phlebotomus perniciosus across foci of canine leishmaniasis

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Abstract. The frequency of sandfly–host contacts can be measured by host antibody levels against sandfly salivary proteins. Recombinant salivary proteins are suggested to represent a valid replacement for salivary gland homogenate (SGH); however, it is necessary to prove that such antigens are recognized by antibodies against various populations of the same species. Phlebotomus perniciosus (Diptera: Psychodidae) is the main vector of Leishmania infantum (Trypanosomatida: Trypanosomatidae) in southwest Europe and is widespread from Portugal to Italy. In this study, sera were sampled from naturally exposed dogs from distant regions, including Campania (southern Italy), Umbria (central Italy) and the metropolitan Lisbon region (Portugal), where P. perniciosus is the unique or principal vector species. Sera were screened for anti-P. perniciosus antibodies using SGH and 43-kDa yellow-related recombinant protein (rSP03B). A robust correlation between antibodies recognizing SGH and rSP03B was detected in all regions, suggesting substantial antigenic cross-reactivity among different P. perniciosus populations. No significant differences in this relationship were detected between regions. Moreover, rSP03B and the native yellow-related protein were shown to share similar antigenic epitopes, as canine immunoglobulin G (IgG) binding to the native protein was inhibited by pre-incubation with the recombinant form. These findings suggest that rSP03B should be regarded as a universal marker of sandfly exposure throughout the geographical distribution of P. perniciosus.

Key words. Leishmania infantum, Phlebotomus spp., antibody response, dog, markers of exposure, Mediterranean region, salivary proteins, sandflies.
Leishmaniasis is a widely distributed disease caused by *Leishmania* protozoans and transmitted by phlebotomine sandfly vectors. During blood feeding, sandflies inoculate saliva into the host. Bitten hosts then develop a species-specific antibody response against sandfly antigens that reflects the intensity of sandfly exposure and thus provides a useful marker of exposure to generate epidemiological data (Vilkova et al., 2011; Martín-Martín et al., 2014; Kostalova et al., 2015).

Large-scale serological studies using total sandfly salivary gland homogenate (SGH) are currently impractical because it is difficult to dissect the high numbers of sandflies necessary to obtain sufficient amounts of SGH. Another potential complication refers to variability in the protein composition of sandfly saliva, which has been found to fluctuate depending on physiological factors such as sandfly age and diet (Volf et al., 2000; Prates et al., 2008). Studies in Old World sandfly species also revealed a certain degree of intra- and inter-population variability in protein and mRNA levels (Rohousova et al., 2012; Ramalho-Ortigão et al., 2015). Therefore, salivary recombinant proteins have been suggested to represent valid replacements for the whole salivary gland protein cocktail, and some have already been validated in the field (Drahota et al., 2014; Martín-Martín et al., 2014; Kostalova et al., 2015). The use of specific recombinant salivary antigen circumvents the necessity for the laborious maintenance of sandfly colonies, and potentially provides a more refined way to minimize antigenic cross-reactivity with taxonomically close sandfly relatives. A useful recombinant salivary protein would demonstrate antigenicity comparable with that of SGH, share similar antigenic epitopes with the native proteins, and demonstrate similar antigenic patterns throughout the geographical distribution of a particular sandfly vector.

This study follows the canine longitudinal study conducted in southern Italy by Kostalova et al. (2015), which described the dynamics and diagnostic potential of antibodies recognizing *Phlebotomus perniciosus* (Larroussius subgenus) salivary recombinant proteins in dogs following natural exposure to sandflies over 2 years. Factors such as sandfly antigens, age and expected sandfly dynamics were considered as variables and were therefore carefully evaluated. The most reactive and reproducible antigen was found to be the 43-kDa yellow-related recombinant protein (rSP03B) from *P. perniciosus* saliva. In view of these promising results, the rSP03B antigen was tested in canine sera samples collected cross-sectionally in canine leishmaniasis (CanL) endemic settings in Italy and Portugal. The study evaluated levels of individual canine antigenic responses to *P. perniciosus* rSP03B compared with *P. perniciosus* SGH, and the degree of similarity in these antigenic associations, across endemic canine populations in Portuguese and Italian foci in order to assess the universal use of rSP03B as a marker of natural sandfly exposure. Previous research had confirmed the presence of two native yellow-related proteins in *P. perniciosus* salivary gland transcriptome and proteome (Anderson et al., 2006). Therefore, the antigenic similarity of rSP03B to its native form was studied and the specificity of the anti-rSP03B immunoglobulin G (IgG) antibody response was confirmed by the inclusion of 42-kDa yellow-related recombinant protein (rSP03).

Canine sera originated from three regions: (a) Campania (*n* = 118), a traditional high-risk area for CanL in southern continental Italy (Oliva et al., 2006); (b) Umbria (*n* = 96), an inland area of central Italy recently recorded as a medium- to high-risk area for CanL (Di Muccio et al., 2012), and (c) the metropolitan Lisbon region (*n* = 341), which is well known as a CanL endemic locality on the west coast of Portugal (Cortes et al., 2012). In all three areas, *P. perniciosus* is the only or principal vector of CanL (Bongiorno et al., 2003; Rossi et al., 2007; Alten et al., 2016). *Phlebotomus perfiliewi* (Larroussius subgenus), another vector of *Leishmania infantum*, was found to be abundant in some areas in Umbria (Maresca et al., 2009). However, *P. perfiliewi* is found in association with large animals (cattle and equine species) in rural habitats (Bongiorno et al., 2003). Dogs examined in Umbria included urban pets and animals hosted in kennels, but all lived in populated areas including residential zones surrounding urban centres, which represent typical habitats for *P. perniciosus* (Maroli et al., 1994).

Additionally, sampled dogs may have been exposed to sandflies from other subgenera occurring in study localities (Cortes et al., 2007; Rossi et al., 2007; Maresca et al., 2009). A previous study by Volf & Rohousova (2001) suggested there was no cross-reaction of *Larroussius* species with other sandflies present in these study regions, namely *Phlebotomus papatasi* (Phlebotomus subgenus), *Phlebotomus sergenti* (Paraphlebotomus subgenus) and members of the genus Sergentomyia.

Single sera samples from Campania and Umbria were purposely selected from archived samples collected in 2007–2013 to represent the period from July (i.e. at least 2 months after the beginning of the sandfly season) to October (i.e. the end of the sandfly season). The selected sera were collected from dogs ranging in age from 1.5 to 13 years. The dogs from both Italian regions represented a mixture of hunting breeds and mongrels. Single sera samples from the metropolitan Lisbon region were randomly collected from kennelled dogs (mostly mongrel) at the beginning of the sandfly season in May 2012. These dogs ranged from young (6–12 months) to more senior (> 7 years) dogs.

Samples from Campania consisted of stored sera sent by veterinary clinics to the Istituto Superiore di Sanità for routine serological diagnosis of suspected CanL in owned dogs. Sera from Umbria were collected from healthy dogs that were enrolled on a voluntary basis in the Perugia University CanL surveillance programme. Blood sampling was performed in accordance with the Italian guidelines for animal welfare, following owners’ consent, and did not include additional or unnecessary invasive procedures. The collection of sera in the metropolitan Lisbon region was ethically approved by the board of the Institute of Hygiene and Tropical Medicine, New University of Lisbon (IHMTUNL) (authorization no. 8 2011-PI) in compliance with Portuguese legislation for the protection of animals (Law 113/2013).

Anti-*Leishmania* IgG in canine sera from Campania and Umbria was detected with an in-house indirect fluorescent antibody test (IFAT) using *L. infantum* promastigotes as antigen, as described in Gradoni & Gramiccia (2008). Samples showing an IFAT titre of 1:40 or greater were considered to indicate exposure to *Leishmania*. Immunoglobulin G antibodies against *Leishmania* in canine sera from the metropolitan Lisbon region were detected using an enzyme-linked immunosorbent assay.

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(ELISA) kit (Bordier Affinity Products SA, Crissier, Switzerland) according to the manufacturer’s guidelines (Maia et al., 2010). The result was considered positive when the absorbance of the analysed sample was higher than the absorbance of the weak positive control serum provided with the kit.

A long-term established laboratory colony of P. perniciosus originating from Spain (Murcia) was reared under standard conditions as described in Volf & Volfova (2011). Salivary glands, rSP03B (GenBank accession no. DQ 150622) and rSP03 (GenBank accession no. DQ 150621) from P. perniciosus were obtained for this study as previously described (Kostalova et al., 2015) and used as antigens for testing the canine sera.

Antibodies against P. perniciosus SGH and rSP03B protein were measured by ELISA as described by Kostalova et al. (2015). Each serum was tested in duplicate. Test absorbance values were reported as optical densities (ODs) with subtracted blanks (the ELISA plate background mean absorbance value measured in control wells).

Western blot analysis was used to confirm the similarity of antigenic epitopes between the native yellow-related protein found in P. perniciosus SGH and the corresponding recombinant protein rSP03B. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of SGH (equivalent to 4 μg total salivary proteins per lane) and rSP03B (2 μg per lane) was run on a 12% gel and blotted onto the nitrocellulose membrane using the iBLOT instrument (Invitrogen Corp., Carlsbad, CA, U.S.A.). Membrane with separated proteins was cut into strips and blocked in 5% milk diluted in Tris-buffered saline with 0.05% Tween 20 (Tris-Tw) overnight at 4°C. For the inhibition test, three Italian canine sera possessing high levels of anti-P. perniciosus IgG against SGH and rSP03B were pooled. The positive serum pool was diluted 1 : 50 in Tris-Tw and split into halves. The first half was incubated for 2 h on a shaker with rSP03B (20 μg/mL) and the second half was incubated without rSP03B. Negative control sera (canine sera from a non-endemic locality) were diluted 1 : 50 in Tris-Tw and incubated without rSP03B on a shaker for 2 h. In the next step, part of the positive sera pool, incubated either with or without rSP03B protein, and part of the negative control sera was incubated with strips of separated P. perniciosus SGH. The same procedure was repeated for strips containing rSP03B, except that sera were diluted 1 : 100 in Tris-Tw. After 1 h, all strips were rinsed in Tris-Tw and subsequently incubated for 1 h with peroxidase-conjugated anti-dog IgG (1 : 3000) (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.). The colour reaction was developed by substrate solution containing 3,3′-diaminobenzidine (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). Furthermore, in order to confirm the specificity of the western blot analysis, the same procedure was repeated for rSP03 protein.

Statistical analyses were carried out using R software (http://cran.r-project.org/) and STATA Version 13.1 (Stata Corp., College Station, TX, U.S.A.). Correlations were analysed using the Spearman rank correlation test and medians were compared between groups using a Wilcoxon rank sum test. Optical density values were logarithmized (natural logarithm) for better readability. Statistical analyses of the relationships between SGH and rSP03B OD values among the canine populations were statistically tested by fitting Poisson general linearized models (GLMs) with an In link function as the right-skewed frequency distributions were found not to follow a negative binomial distribution (deviance goodness-of-fit χ² > 56.2; P = 1, d.f. = 549, for each antibody). The full Poisson GLMs included interaction terms to test differences between the regions, both in terms of baseline anti-rSP03B value (intercept where anti-SGH equals 0) and the relationship between antibodies against SGH and rSP03B (slopes). A P-value of < 0.05 was considered to indicate statistical significance.

The use of P. perniciosus rSP03B as an epidemiological tool was tested for investigations of canine exposure to sandfly bites in geographically distinct localities in which P. perniciosus is the prevalent phlebotomine vector. The recombinant protein rSP03B used in this study was obtained from the salivary glands of P. perniciosus in a laboratory-reared colony originating from Murcia in Spain, and was used as an antigen in the serology of dogs living in the Campania and Umbria regions of Italy and in the metropolitan Lisbon region in Portugal.

Levels of canine IgG antibodies reacting with SGH and rSP03B were measured by ELISA. Positive but variable correlations between antibody responses to SGH and rSP03B antigens were observed in sera from all three localities [Campania: r = 0.73, 95% confidence interval (CI) 0.62–0.82 (P < 0.001); Umbria: r = 0.56, 95% CI 0.38–0.71 (P < 0.001); metropolitan Lisbon: r = 0.81, 95% CI 0.76–0.84 (P < 0.001)] (Fig. 1). Table 1 summarizes the OD values for each region and indicates that OD frequency distributions were over-dispersed. To query possible differences in the relationships between SGH and rSP03B antibody responses between geographical regions, the equality of the population-specific regression slopes was tested by fitting a Poisson model. No significant differences were detected (population × antigen interaction terms: Z > −0.85, P > 0.365). Relative to the metropolitan Lisbon region, both the Campania and Umbria populations tended to produce higher baseline antibody responses against rSP03B, although these differences failed to reach significance at the 5% level (Campania: Z = 1.66, P = 0.097; Umbria: Z = 1.95, P = 0.051). One plausible explanation for the putative differences in baseline rSP03B antibody levels among populations is that the populations differ in their condition or past history of infections and that these differences affect general immunological responses to certain antigens, and/or that sandfly biting pressure differs across these populations. The seasonal exposure of dogs to sandflies has been found to lead to antibody response fluctuations related to the period of activity and abundance of vectors (Vilkova et al., 2011; Kostalova et al., 2015). Secondly, as age is a frequent covariate of cumulative exposure used to model cross-sectional age-related prevalence data of Leishmania infection (Courtenay et al., 1994), the average older dog is expected to have experienced more sandfly seasons (Kostalova et al., 2015). Dogs from Campania and Umbria were sampled from July (i.e. during the period of highest sandfly abundance in Italy). All of the animals tested from these two regions had experienced at least two consecutive transmission seasons. Sera from dogs in the metropolitan Lisbon region were sampled in May, which is the beginning of the sandfly season, and were sourced mainly from dogs aged > 1 year. Thus these dogs had experienced at least one transmission season. According to reactivity data shown by Kostalova et al. (2015), dogs will be reactive to saliva at the beginning of the transmission season if they have already been ‘primed’ in the
For salivary recombinant proteins, among which rSP03B proved to be a powerful marker of host exposure to sandflies (Kostalova et al., 2011), as well as for salivary recombinant proteins, among which rSP03B proved to be a powerful marker of host exposure to sandflies (Kostalova et al., 2015). Therefore, the present study analysed the relationship between anti-P. perniciosus antibodies and Leishmania serological status. When using rSP03B antigen, significantly higher levels of specific IgG in Leishmania-seropositive dogs [median = 0.346, interquartile range (IQR) 0.257–0.536] than in Leishmania-seronegative dogs (median = 0.320, IQR 0.229–0.422) were found only in the metropolitan Lisbon region (Wilcoxon rank sum test, W = 5391.5, P = 0.025). In Campania, the differences in antibodies against rSP03B between CanL-seropositive (median = 0.457, IQR 0.357–0.550) and CanL-seronegative (median = 0.379, IQR 0.303–0.499) dogs were marginally significant (Wilcoxon rank sum test, W = 1123.5, P = 0.053). Previous studies on the relationship between anti-P. perniciosus antibodies and seropositivity to L. infantum show variable correlations. In Kostalova et al.
Western blot analysis of salivary gland homogenate (SGH), rSP03B and rSP03 and inhibition test. A mixture of canine sera positive to Phlebotomus perniciosus SGH was pre-incubated with rSP03B or rSP03 and then tested in western blotting against SGH. Arrows indicate the points at which inhibition should take place. The star indicates the position of rSP03. STD, standard; AB, strip stained by Amido black; (+), positive control strip; i43, inhibition strip for rSP03B; i42, inhibition strip for rSP03; (−), negative control strip.

Table 2. Frequencies of Leishmania seropositivity and seronegativity in dogs from different regions.

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Cut-off</th>
<th>Serological status</th>
<th>Anti-L. infantum IgG positive/total animals sampled, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Campania</td>
</tr>
<tr>
<td>IFAT</td>
<td>1:40</td>
<td>Positive</td>
<td>35/118 (30%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>83/118 (70%)</td>
</tr>
<tr>
<td>ELISA</td>
<td>0.26</td>
<td>Positive</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>—</td>
</tr>
</tbody>
</table>

*As determined by the IFAT titre or ELISA cut-off.

IgG, immunoglobulin G; IFAT, indirect fluorescent antibody test; ELISA, enzyme-linked immunosorbent assay.

(2015), a positive association was observed between levels of canine IgG antibodies against sandfly saliva and active CanL infection in dogs sampled longitudinally over 2 years. By contrast, the study by Vlkova et al. (2011) described a negative correlation between levels of specific IgG2 and risk for Leishmania infection. Comparisons between studies are difficult following observations that anti-saliva antibodies wax and wane with sandfly exposure and seasonality (Kostalova et al., 2015). In actively infected dogs, anti-Leishmania antibodies tend to persist after an initial increase, whereas in exposed resistant animals they tend to fluctuate or convert to negative (Oliva et al., 2006). As studies tend to be cross-sectional and use different approaches to determine Leishmania infection status, cross-study comparisons are difficult. Although longitudinal studies have already demonstrated the potential usefulness of the sandfly saliva antigenic response in dogs as a marker for Leishmania infection (Kostalova et al., 2015; R. J. Qinnell, personal communication, 2016), the possibility of using sandfly salivary recombinant proteins in a similar way in cross-sectional surveys still needs to be validated.

In conclusion, this study showed that P. perniciosus rSP03B, the 43-kDa yellow-related recombinant protein, possesses the same antigenic epitopes as its native form in salivary glands, and binds similarly in canine sera from foci in Italy and Portugal. Therefore, it could serve as a universal marker of sandfly exposure across the entire geographical distribution of P. perniciosus, even in dogs of various breeds and ages.

Acknowledgements

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Seasonal dynamics of canine antibody response to *Phlebotomus perniciosus* saliva in an endemic area of *Leishmania infantum*

Seasonal dynamics of canine antibody response to *Phlebotomus perniciosus* saliva in an endemic area of *Leishmania infantum*

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**Abstract**

**Background:** Canine leishmaniosis (CanL) is an important zoonotic parasitic disease, endemic in the Mediterranean basin. In this region, transmission of *Leishmania infantum*, the etiological agent of CanL, is through the bite of phlebotomine sand flies. Therefore, monitoring host-vector contact represents an important epidemiological tool, and could be used to assess the effectiveness of vector-control programmes in endemic areas. Previous studies have shown that canine antibodies against the saliva of phlebotomine sand flies are specific markers of exposure to *Leishmania* vectors. However, this method needs to be further validated in natural heterogeneous dog populations living in CanL endemic areas.

**Methods:** In this study, 176 dogs living in 12 different locations of an *L. infantum* endemic area in north-east Spain were followed for 14 months. Blood samples were taken at 5 pre-determined time points (February, August and October 2016; January and April 2017) to assess the canine humoral immune response to whole salivary gland homogenate (SGH) and to the single salivary 43 kDa yellow-related recombinant protein (rSP03B) of *Phlebotomus perniciosus*, a proven vector of *L. infantum* naturally present in this region. Simultaneously, in all dogs, *L. infantum* infection status was assessed by serology. The relationship between anti-SGH and anti-rSP03B antibodies with the sampling month, *L. infantum* infection and the location was tested by fitting multilevel linear regression models.

**Results:** The dynamics of canine anti-saliva IgG for both SGH and rSP03B followed the expected trends of *P. perniciosus* activity in the region. Statistically significant associations were detected for both salivary antigens between vector exposure and sampling month or dog seropositivity to *L. infantum*. The correlation between canine antibodies against SGH and rSP03B was moderate.

**Conclusions:** Our results confirm the frequent presence of CanL vectors in the study area in Spain and support the applicability of SGH- and rSP03B-based ELISA tests to study canine exposure to *P. perniciosus* in *L. infantum* endemic areas.

**Keywords:** Canine leishmaniosis, *Phlebotomus perniciosus*, Saliva proteins, Markers of exposure, Longitudinal study, North-east Spain

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**Background**

*Leishmania infantum* (Kinetoplastida: Trypanosomatidae) is the causative agent of canine leishmaniosis (CanL), a zoonotic vector-transmitted disease widespread in the Mediterranean region, as well as in other parts of the world [1–3]. Prevalence of *L. infantum* infection in canine populations from endemic areas is highly heterogeneous [4], and not all infected dogs will ever develop clinical signs of the disease [5]. However, infected asymptomatic dogs can act as a reservoir of the parasite and are capable of transmitting *L. infantum* to other dogs, as well as to humans [6, 7].

The transmission of the parasite is mainly vectorial, through the bite of phlebotomine sand flies. In the Mediterranean basin, eight species of the genus *Phlebotomus* have been implicated as vectors of *L. infantum*, according to conventional criteria. From these, all except one belong to the subgenus *Larroussius* [8]. In Spain, CanL transmission is mainly shared by *P. (L.) perniciosus* and *P. (L.)
ariasi [9, 10], with the second species having a narrower distribution but being responsible for maintaining the infection at higher altitudes [11, 12]. Recently, L. infantum DNA was also found in another Larroussius species, P. langeroni, in the south of the country [13].

The detection of anti-sand fly salivary antibodies in the blood of vertebrate hosts has proven to be highly specific [14] and was successfully used as a marker of exposure to L. infantum vectors [15, 16]. In CanL endemic areas, monitoring the canine IgG response to sand fly saliva can be a useful epidemiological tool [15, 17], complementing studies of vector population dynamics and host-vector interactions, as well as enabling the assessment of risk of Leishmania infection [14, 18, 19]. Furthermore, it can be used to measure the effectiveness of vector-control programmes and to assist in the design of better control strategies for the disease [20, 21].

Originally, sand fly whole salivary gland homogenates (SGH) were used to investigate the presence of anti-sand fly saliva antibodies in vertebrate hosts [20–22]. However, its use in large-scale studies is impaired by technical limitations [23]. Additionally, the use of SGH in vector exposure tests may reduce the specificity of detection due to a possible cross-reactivity with saliva of sympatric and closely related sand fly species [24].

An alternative to the use of SGH is the identification of species-specific salivary proteins that can be expressed in recombinant forms and produced in large quantities for use in large-scale epidemiological studies [25, 26]. Recent studies identified P. perniciosus yellow-related protein rSP03B as the most promising candidate to replace SGH in the detection of host markers of exposure to this vector species [16, 17, 26]. This recombinant protein has been tested and validated in dogs and other animals in cross-sectional studies [16, 26], as well as in a canine longitudinal study [17], but no information exists on the seasonal dynamics of either SGH or rSP03B in natural heterogeneous dog populations from endemic areas.

Therefore, the objectives of this study were (i) to investigate the dynamics of P. perniciosus and their relative density in a previously uncharacterized CanL endemic area through the detection of anti-saliva IgG in dogs; and (ii) to evaluate the performance of both SGH and rSP03B antigens as markers of exposure to P. perniciosus in natural canine populations.

Results
Seasonal dynamics of IgG response against salivary proteins from P. perniciosus
Median values of normalized ELISA OD values for SGH ranged from 9.04 (range: 3.94–66.23) in January 2017 to 18.51 (7.93–100.58) in August 2016 (Table 1). For rSP03B, median OD values varied between 12.21 (6.75–53.71) and 19.53 (10.64–124.01) in January 2017 and August 2016, respectively. With both antigens, median OD readings raised from basal values in February 2016 (10.11 and 14.67 for SGH and rSP03B, respectively) to peak in August (18.51 and 19.53 for SGH and rSP03B, respectively), sustained higher readings in October (11.15 and 15.31 for SGH and rSP03B, respectively), and descended again to basal levels in January (9.04 and 12.21 for SGH and rSP03B, respectively) and April 2017 (9.54 and 13.44 for SGH and rSP03B, respectively). Median normalized ELISA OD results obtained per month for both SGH and rSP03B are described in Table 1 and plotted in Fig. 1.

Cut-off values were set at 13 for SGH and 22 for rSP03B. When these were applied to the OD readings obtained in August 2016, 75.76% (25/33) of the dogs were positive to anti-SGH IgG, and 36.36% (12/33) to anti-rSP03B antibodies. In October, these values dropped to 35.98% (59/164) for SGH and 18.9% (31/164) for rSP03B. During the non-transmission season (considered to extend from Table 1 Median values of normalized OD readings for SGH and rSP03B obtained per sampling month in all locations

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>SGH</th>
<th>rSP03B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (Range)</td>
<td>Median (Range)</td>
<td></td>
</tr>
<tr>
<td>February 2016</td>
<td>174</td>
<td>10.11 (5.49–49.62)</td>
<td>14.67 (7.36–41.24)</td>
</tr>
<tr>
<td>August 2016</td>
<td>33</td>
<td>18.51 (7.93–100.58)</td>
<td>19.53 (10.64–124.01)</td>
</tr>
<tr>
<td>October 2016</td>
<td>164</td>
<td>11.15 (5.56–86.44)</td>
<td>15.31 (6.15–112.54)</td>
</tr>
<tr>
<td>January 2017</td>
<td>154</td>
<td>9.04 (3.94–66.23)</td>
<td>12.21 (6.75–53.71)</td>
</tr>
</tbody>
</table>

Abbreviation: N number of dogs sampled per sampling month
November to May), the percentage of seropositive dogs ranged from 14.29% (25/175) in February 2016 to 17.57% (26/148) in April 2017 for SGH and 8.44% (13/154) in January 2017 to 12.16% (18/148) in April 2017 for rSP03B.

Correlation results for IgG response between SGH and rSP03B were $r_S = 0.54$ (95% CI: 0.48–0.60, $P < 0.001$) (Fig. 2).

**Dogs’ exposure to *P. perniciosus* in the study area**
Exposure of dogs to phlebotomine vectors showed some variation according to the location. Median OD readings varied from 9.11 (range: 5.25–20.57) to 14.14 (7.44–55.45) for SGH ELISA and from 12.71 (7.53–64.44) to 17.87 (8.39–112.54) for rSP03B. Minimum median values of response to both SGH and rSP03B corresponded to the same location (Aiguaviva), but maximum median values were registered in different sites for each antigen (Sant Feliu de Guíxols for SGH and Montagut for rSP03B) (Table 2). Figure 3 presents the dynamics of dogs’ IgG response to SGH (Fig. 3a) and rSP03B (Fig. 3b) in each locality.

The percentage of anti-sand fly saliva seropositive dogs per location, defined as the number of dogs that showed a positive IgG titre at least once during the study period, ranged from 13.33% (1/8) in Ordis to 100% in Canet d’Adri (8/8) and Sant Feliu de Guíxols (4/4) for SGH, and from 8.16% (1/12) in Hostalnou de Bianya to 100% (4/4) in Sant Feliu de Guíxols for rSP03B. Total anti-sand fly saliva seropositivity calculated for the study area was 49.43% (87/176) for anti-SGH IgG and 28.98% (51/176) for anti-rSP03B antibodies.

**Dogs’ exposure to *P. perniciosus* and *L. infantum* infection**
Correlation results between antibody response to *P. perniciosus* saliva and *L. infantum* were low both for SGH ($r_S = 0.27$, 95% CI: 0.19–0.35, $P < 0.001$) and rSP03B protein ($r_S = 0.25$, 95% CI: 0.18–0.32, $P < 0.001$).

**Multilevel analysis of the relationship between anti-*P. perniciosus* salivary proteins, month and location and *L. infantum* seropositivity**
The multilevel model results confirmed the annual dynamics of anti-salivary proteins IgG responses. When compared to the first sampling month (February 2016), IgG responses to SGH significantly rose in August ($t = 8.55$, $df = 491$, $P < 0.001$) and October ($t = 6.49$, $df = 491$, $P < 0.001$) and dropped in January ($t = -2.49$, $df = 491$, $P = 0.013$) and April 2017 (no significant difference when compared to February 2016). As expected, the highest log OD estimate was observed in August 2016 and the lowest in January 2017 (Table 3). The same trend was observed in the model run for the rSP03B protein, with comparable levels of significance (Table 4). There were no significant differences in IgG responses for both antigens between each sampling location and the one set as reference, except for Montagut, where significantly higher OD levels were observed for SGH ($t = 2.28$, $df = 166$, $P = 0.024$) and rSP03B ($t = 2.13$, $df = 164$, $P = 0.035$). According to the multilevel model, seropositivity to *L. infantum* proved to be associated with a rise in anti-salivary proteins OD values for both SGH ($t = 2.5$, $df = 491$, $P = 0.013$) and rSP03B ($t = 2.15$, $df = 493$, $P = 0.032$).

**Discussion**
The quantification of anti-sand fly saliva antibodies in vertebrate hosts of *L. infantum* has been previously shown to be an effective way of measuring exposure to the parasite vectors [16]. In the case of dogs, the most frequent host and reservoir of *L. infantum*, this has been proven for *P. perniciosus* [15, 26], as well as for other sand fly species [27–29]. These markers of exposure can then be applied in host-vector epidemiological studies, in *L. infantum* infection risk assessment, and to assist in the design of control strategies for the disease. Therefore, it is important to validate these techniques in natural, heterogeneous populations from endemic areas, in which a higher individual variability is expected.

*Phlebotomus perniciosus* activity period in Spain shows two main peaks, the first in June-July and the second in September-October. These peaks also correspond to the periods of highest *L. infantum* transmission [30–32]. This trend was identified in our study and corresponds to the rise in anti-salivary antibody levels observed between
August and October. Humoral immune response to *P. perniciosus* saliva elicited in experimentally bitten dogs showed that antibody levels significantly rose after 2–4 weeks of continued exposure, peaking in week 5 \[15\]. In our field study, the highest IgG levels were in August, which clearly corresponded to the June-July *P. perniciosus* expected activity peak. Similarly, the high IgG readings obtained in October are likely to correspond to *P. perniciosus* second peak of activity. The lower rise in antibody levels observed at this time point can be explained by an earlier sampling at the beginning of October, which may have hindered the display of a complete seroconversion. The high overall levels of seropositivity to anti-sand fly saliva antigens, especially for SGH (49.43%), strongly support the CanL endemicity status for the region \[33\]. These results also validate both SGH and rSP03B as suitable antigens to assess exposure to *P. perniciosus* in natural canine populations from endemic areas.

An important remark when analysing the longitudinal dynamics of anti-sand fly saliva IgG in the study dog

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**Table 2** Median values of normalized OD readings for SGH and rSP03B obtained per sampling location at all time points

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (Range)</th>
<th>Geographical coordinates</th>
<th>SGH Median (Range)</th>
<th>rSP03B Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordis</td>
<td>8 (7–9)</td>
<td>42°13′37.7″N, 2°54′24.1″E</td>
<td>9.14 (6.45–45.05)</td>
<td>15.16 (8.35–54.50)</td>
</tr>
<tr>
<td>Madremanya</td>
<td>14 (12–15)</td>
<td>41°58′47.0″N, 2°58′7.2″E</td>
<td>11.22 (6.79–49.84)</td>
<td>14.49 (8.95–43.99)</td>
</tr>
<tr>
<td>Vidreres</td>
<td>8 (7–9)</td>
<td>41°47′27.4″N, 2°45′0.4″E</td>
<td>10.59 (7.80–16.86)</td>
<td>13.46 (8.58–40.23)</td>
</tr>
<tr>
<td>Massanes</td>
<td>21 (20–23)</td>
<td>41°45′15.3″N, 2°38′44.0″E</td>
<td>9.31 (5.67–62.59)</td>
<td>16.35 (7.82–55.81)</td>
</tr>
<tr>
<td>Hostalnou de Biarnya</td>
<td>12 (11–14)</td>
<td>42°13′26.0″N, 2°26′9.7″E</td>
<td>8.75 (5.35–33.16)</td>
<td>13.19 (6.27–46.82)</td>
</tr>
<tr>
<td>Montagut</td>
<td>13 (7–15)</td>
<td>42°51′7.7″N, 2°35′57.6″E</td>
<td>12.01 (3.94–72.61)</td>
<td>17.87 (8.39–112.54)</td>
</tr>
<tr>
<td>St. Esteve de Llímena</td>
<td>9 (9–10)</td>
<td>42°3′35.1″N, 2°37′1.4″E</td>
<td>9.49 (6.23–22.40)</td>
<td>14.18 (9.12–22.46)</td>
</tr>
<tr>
<td>Canet d’Adri</td>
<td>8 (4–10)</td>
<td>42°15′3.7″N, 2°44′15.3″E</td>
<td>10.61 (6.52–100.58)</td>
<td>14.03 (7.36–124.01)</td>
</tr>
<tr>
<td>Aiguaviva</td>
<td>19 (16–22)</td>
<td>41°54′27.2″N, 2°46′19.0″E</td>
<td>9.11 (5.25–20.57)</td>
<td>12.71 (7.53–64.44)</td>
</tr>
<tr>
<td>St. Feliu de Guxols</td>
<td>4</td>
<td>41°54′23.1″N, 2°59′58.7″E</td>
<td>14.14 (7.44–55.45)</td>
<td>16.73 (8.57–65.97)</td>
</tr>
<tr>
<td>Riells i Viabrea</td>
<td>20 (18–21)</td>
<td>41°43′59.3″N, 2°33′39.3″E</td>
<td>10.02 (6.07–66.23)</td>
<td>13.43 (8.59–35.31)</td>
</tr>
<tr>
<td>Vilobi d’Onyar</td>
<td>23 (22–23)</td>
<td>41°53′3.2″N, 2°43′38.6″E</td>
<td>9.13 (5.17–16.49)</td>
<td>13.05 (6.15–38.07)</td>
</tr>
</tbody>
</table>

Abbreviation: n mean number of dogs sampled in each location

**Fig. 3** Dynamics of dogs’ IgG recognizing SGH (a) and rSP03B protein (b) in the different sampling locations during a sand fly activity season. Values presented refer to the normalized OD medians obtained at each sampling month.
population is that there was a clear basal antibody level before the transmission season. After the expected rise in humoral response during summer months, IgG levels returned again to basal levels. These results show that, though exposed to repetitive bites during several months, dogs from endemic areas do not sustain high anti-saliva IgG levels throughout the year, allowing the detection of recent exposure to sand flies in natural populations. Similar results were recently reported in a longitudinal field study in Brazil, where canine IgG against *Lutzomyia longipalpis* saliva were evaluated [34]. Our study identified the same trends for both SGH and rSP03B, which reinforces the suitability of recombinant antigens in detecting recent exposure to phlebotomine vectors in endemic settings, particularly when considering the use of these tests in large-scale studies for vector control interventions [35, 36].

Antibodies recognizing both SGH and rSP03B followed similar dynamics throughout the field study. However, the correlation between the two antigens was only moderate ($r_S = 0.54; 95\% CI: 0.48–0.60, P < 0.001$). Even so, available studies show that rSP03B is the most promising surrogate for SGH as a marker of exposure to *P. perniciosus* in the canine host. It has presented high levels of correlation with SGH in both experimentally [25] and naturally bitten dogs [16, 17, 26]. Two apyrase proteins (rSP01B and rSP01) have also shown a good correlation with SGH [25]. However, in a study where these three recombinant proteins presented similarly high correlations with SGH, rSP03B presented the lowest data dispersion and was considered a better option [16]. These results were confirmed in a field trial, where single rSP03B demonstrated a higher correlation coefficient with SGH than the combination of rSP01B and rSP01 [17].

A similar correlation between SGH and rSP03B to the one obtained in the present study has been observed before in Umbria region (central Italy) ($r_S = 0.56; 95\% CI: 0.38–0.71, P < 0.001; n = 96$), in a screening study of dog exposure to *P. perniciosus* across European CanL endemic foci [26]. A possible reason for these discordant results may be the presence of other closely related phlebotomine species which could induce cross-reactivity with the SGH [22]. In some parts of Catalonia, *P. perniciosus* is sympatric with *P. ariasi*, also a proven vector of *L. infantum* [21].

### Table 3: Estimates of the multilevel linear regression model of the relationship between log transformed normalized SGH OD values and sampling time, location and dog seropositivity to *L. infantum*. **"Dog"** was included as a random effects variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
<th>Estimate</th>
<th>SE</th>
<th>$P$-value$^{a}$</th>
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<td></td>
</tr>
<tr>
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<tr>
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### L. infantum seropositivity

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<tr>
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Abbreviation: SE standard error

*Level of significance of $P$-value < 0.05 was used*

### Table 4: Estimates of the multilevel linear regression model of the relationship between log transformed normalized rSP03B OD values and sampling time, location and dog seropositivity to *L. infantum*. **"Dog"** was included as a random effects variable.

<table>
<thead>
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### L. infantum seropositivity

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<th>Levels</th>
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<th>$P$-value$^{a}$</th>
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<td>Seronegative</td>
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<tr>
<td>Seropositive</td>
<td>0.07</td>
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<td>0.032</td>
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</tbody>
</table>

Abbreviation: SE standard error

*Level of significance of $P$-value < 0.05 was used*
expected that they share similar salivary antigens [37]. When comparing the percentage of seropositive dogs detected by both methods during the study, results for SGH are higher (49.43%) than for rSP03B (28.98%). Also, median results per sampling location show differences between SGH and rSP03B: in some cases, the trend between antigens is very similar (e.g. sera from Sant Feliu de Guíxols); in other cases, there is a recognizable peak in anti-SGH IgG, while anti-rSP03B IgG shows no change (e.g. sera from Madremanya). These differences can also be observed over time in the same location, with humoral responses to SGH and rSP03B peaking in different months along the transmission season (e.g. Canet d’Adri).

We may hypothesize that SGH, because it contains more proteins than the single-antigen rSP03B, will more likely cross-react with antibodies against *P. ariasi*, inducing a stronger unspecific reaction to this vector species. It would also mean that the prevalence of sand fly species responsible for *L. infantum* transmission in the province varies according to the location, and possibly in the same location throughout the transmission season, for which it would be interesting to perform further entomological studies in the region.

Correlation indexes between levels of antibodies against both salivary antigens and *L. infantum* infection were low [SGH: $r_S = 0.27$ (95% CI: 0.19–0.35, $P < 0.001$); rSP03B: $r_S = 0.25$ (95% CI: 0.18–0.32, $P < 0.001$)]. Similar low correlations have been described before between sand fly bites and human visceral leishmaniasis (VL), while stronger correlations are reported between human cutaneous leishmaniasis (CL) and recent vector exposure (reviewed in [23]). This can be explained by VL’s longer incubation period and/or the differences in host immune responses to cutaneous and visceral infection [38]. Results from some studies in human populations also suggest that the repeated contact with non-infected sand flies could be correlated with markers of protection for VL [39]. Partial protection against *L. major*, an agent of CL, has also been achieved in immunized mice by the bites of uninfected sand flies [40]. However, another study with BALB/c mice demonstrated that this type of immunity is limited to short-term exposure and questioned the efficacy of sand fly saliva-induced protection against *Leishmania* infection in CL endemic areas [41]. CanL follows a pattern which is more similar to VL than to CL, therefore a low correlation between humoral responses to sand fly saliva and Leishmania would be expected [15]. However, results of the multilevel linear regression model show a positive and statistically significant relationship between *P. perniciosus* bites and a seropositive status for *L. infantum*, both for SGH and rSP03B. Similar results have been described in other longitudinal field studies on both canine anti-*P. perniciosus* and anti-*L. longipalpis* IgG dynamics [17, 34]. Unlike cross-sectional surveys, longitudinal studies are able to detect the relationship between a higher number of sand fly bites at a given time point and a subsequent *L. infantum* infection. Therefore, this type of study is likely to better explain the relationship between these two events, which can take place several months apart.

**Conclusions**

The results of this study confirmed the applicability of both anti-*P. perniciosus* SGH and rSP03B IgG as markers of exposure to *L. infantum* vectors in natural dog populations from an endemic area. Canine humoral response to both antigens is compatible with the annual sand fly activity dynamics expected for the region. Significantly lower IgG levels were observed during the non-transmission season; despite the repeated exposure to sand flies during the summer months, there is a return to basal IgG levels in these dog populations during the winter. The comparative performance of SGH and rSP03B showed a moderate correlation, which might be explained by the occurrence of cross-reactions of SGH with other closely related sympatric sand flies. Further longitudinal studies in natural canine populations from endemic areas, together with entomological studies, should be carried out in order to corroborate this hypothesis. Nevertheless, both antigens are expected to detect only vectors of *L. infantum*, confirming their suitability for host-vector-parasite studies. Finally, the overall results support the CanL endemicity status for the study region, which had already been suggested by previous studies [33].

**Methods**

**Experimental design**

The study included a heterogeneous population of 176 dogs distributed by 12 locations in Girona Province (Catalonia, northeast of Spain), an area endemic for CanL [33]. These dogs were enrolled in a canine leishmaniosis vaccine field trial, but no statistically significant differences in *L. infantum* infection between groups were observed during or at the end of the trial. These were all owned dogs, used mainly for hunting, but some breeding and racing individuals were also included. All animals were kept in large packs in open-air facilities, mostly in rural and periurban areas. Furthermore, no specific anti-sand fly insecticide treatments were applied, providing conditions for dog exposure to the vector. Dog density per study location varied between 4–23. The dogs were followed from February 2016 to April 2017 and blood samples, obtained by venepuncture and placed in 5 ml EDTA tubes, were collected at 5 pre-determined time points (Table 1). Plasma was obtained and stored at -40 °C until processing.

**Sand flies and salivary proteins**

A colony of *P. perniciosus* was reared under standard conditions as described previously [42]. Salivary glands were
dissected from 4–6 day-old females, pooled at a concentration of 1 salivary gland per 1 μl of 20 mM Tris buffer with 150 mM NaCl and stored at -80 °C. The P. perniciosus 43 kDa yellow-related recombinant protein (rSP03B, Genbank accn. DQ150622) was obtained from Apronex s.r.o. (Prague, Czech Republic) and quantified by the Lowry method (Bio-Rad, Hercules, California, USA) following the manufacturer’s protocol.

Serological detection of dog exposure to sand flies

Anti-P. perniciosus IgG was measured by an in-house enzyme-linked immunosorbent assay (ELISA) as described previously [17]. All samples from a single dog were processed in the same plate. Briefly, microtiter plates were coated either with salivary gland homogenate (SGH) (40 ng per well, equivalent to 0.2 salivary gland) or with rSP03B (5 μg/ml) in 20 mM carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4 °C. Plates were then blocked with 6% (w/v) low fat dry milk in PBS with 0.05% Tween 20 (PBS-Tw). Canine plasma were diluted 1:200 for SGH and 1:100 for rSP03B in 2% (w/v) low fat dry milk/PBS-Tw. Secondary antibodies (anti-dog IgG, Bethyl laboratories) were diluted 1:9000 in PBS-Tw. The reaction was stopped with 10% H2SO4 and absorbance was measured at 492 nm using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Each sample was tested in duplicate and positive and negative controls were included in each plate. To account for the variability between plates, sample OD readings were normalized by dividing them by the mean OD of positive controls run in the same plate [43]. The normalized OD values were multiplied by 100. Positivity cut-offs were calculated as the mean plus 3 standard deviations from 14 dog samples from a non-endemic area.

Serological detection of L. infantum infection

All samples were tested for the presence of IgG against L. infantum through an in-house enzyme-linked immunosorbent assay (ELISA), using a technique described previously [44, 45]. Again, serial samples from a single dog were tested in parallel on the same plate. Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar® Corning®, New York, USA) previously coated with sonicated whole promastigotes at a protein concentration of 20 μg/ml at 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase (1:30,000, Sigma-Aldrich®, St. Louis, Missouri, USA) was used as conjugate and reactions were stopped with H2SO4 3M when a pre-determined calibrator control serum reached an optical density of 450 at 450 nm. Sample optical densities were read at 492 nm. All samples were run in duplicate and the calibrator, positive and negative sera were included in all plates. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The positivity cut-off was established at 24U.

Statistical analysis

Statistical analyses were performed using R software (http://cran.r-project.org/) and StatA 15 software (StataCorp LP, College Station, TX, USA).

Correlations between IgG responses to P. perniciosus SGH and rSP03B and between each one of the salivary antigens and anti-L. infantum IgG levels were tested by the Spearman rank correlation test. Median OD values between time points were compared using the Wilcoxon signed rank sum test.

The relationship between anti-SGH and anti-rSP03B antibodies and sampling month, L. infantum infection status and location was tested by fitting multilevel linear regression models, taking into account the correlation between repeated measures of the same dogs over time. In the models, log-transformed anti-saliva or rSP03B normalized OD values were considered as continuous dependent variables and sampling month, L. infantum infection and location as categorical predictor variables. In order to assess variations in OD between the first sampling month and those following, “February 2016” was set as reference level for this variable. Likewise, the locality with the lowest median OD (“Aiguaviva”) was considered to be the reference for the variable location. Finally, “seronegative” was set as the reference level for the variable L. infantum infection. The random component included dog and time to allow for variation at the intercept (between dogs) and the slope (over time). The inclusion of “dog” as a random effects variable significantly improved both models, with a between dog variance of 48% for SGH and of 47% for the rSP03B model. A P-value of < 0.05 was considered to indicate statistical significance.

Abbreviations

CanL: Canine leishmaniosis; CL: Human cutaneous leishmaniasis; ELISA: Enzyme-linked immunosorbent assay; IgG: Immunoglobulin G; OD: Optical density; rSP03B: 43 kDa yellow-related recombinant protein; SGH: Salivary gland homogenate; VL: Human visceral leishmaniasis

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We thank all dog owners, for providing us access to their dogs. We also thank the reviewers for their helpful suggestions and comments, and Trelawny Bond-Taylor for language revision and editing of the final manuscript.

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Programme, Generalitat de Catalunya. The supporters had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding authors upon reasonable request.

Authors’ contributions
RV, LW, JC, PV and MG designed the study; RV, ED and MG performed the fieldwork; RV, TS and LW performed the lab work; TS and RV analysed and interpreted the data; RV and MG wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The research protocol was submitted to the Ethics Committee on Animal Experimentation (CEEA) of University of Barcelona, which considered that an ethical approval was not required for this study. The project was also interpreted the data; RV and MG wrote the manuscript. All authors read and

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References


Evaluation of the rSP03B sero-strip, a newly proposed rapid test for canine exposure to *Phlebotomus perniciosus*, vector of *Leishmania infantum*

Evaluation of the rSP03B sero-strip, a newly proposed rapid test for canine exposure to Phlebotomus perniciosus, vector of Leishmania infantum

Laura Willen1*, Pascal Mertens2, Petr Volf1

1 Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, 2 Coris BioConcept, Crealys Park, Gembloux, Belgium

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Abstract

Background

Canine leishmaniasis (CanL) is a zoonotic disease, caused by Leishmania infantum and transmitted by Phlebotomus perniciosus in the Mediterranean basin. Previously, an ELISA based on the P. perniciosus salivary protein SP03B was proposed as a valid tool to screen for canine exposure to sand fly bites across regions endemic for CanL. Although this approach is useful in laboratory settings, a practical tool for immediate application in the field is needed. In this study we propose the rSP03B sero-strip, the first immunochromatographic test (ICT) in the field of vector exposure able to rapidly screen dogs living in endemic areas for the presence of P. perniciosus and to aid in the evaluation of vector control programs.

Methodology/Principal findings

The ICT was prepared using the bacterially expressed recombinant protein rSP03B as antigen. For test optimization, pre-immune sera from non-bitten laboratory-bred Beagles were used as negative controls. In order to validate the test, sera from laboratory-bred Beagles experimentally exposed to P. perniciosus bites were used as positive controls. Additionally, all samples were tested by ELISA using whole salivary gland homogenate (SGH) and the rSP03B protein as antigen. An almost perfect degree of agreement was found between the ICT and the SGH-ELISA. Furthermore, the newly proposed rSP03B sero-strip showed a sensitivity of 100% and a specificity of 86.79%.

Conclusions/Significance

We developed a simple and rapid ICT based on the P. perniciosus rSP03B salivary protein, able to replace the standard ELISA used in previous studies. Our rSP03B sero-strip showed to be highly sensitive and specific in the detection of antibodies (IgG) against P. perniciosus saliva. In the future, this test can be employed during large-scale epidemiological studies of CanL in the Mediterranean area to evaluate the efficacy of vector control programs.
The sand fly *Phlebotomus perniciosus* is the principle vector of *Leishmania infantum*, causing canine leishmaniasis in the Mediterranean basin. While the sand fly female takes a blood meal, it injects saliva into the host skin, evoking a specific antibody response in the host. The antibody level in the host correlates with the intensity of exposure to sand flies. Previously, the specific antibody response (IgG) against a salivary protein of *P. perniciosus*—SP03B—has been proposed as a valid biomarker to estimate dog exposure to *P. perniciosus* in the Mediterranean area. Since standard serological methods are impractical and time-consuming in field conditions, we propose the rSP03B sero-strip—a rapid test that can be immediately applied to screen large cohorts of dogs for the presence of anti-*P. perniciosus* antibodies. Our test is the first rapid test in the field of vector exposure, it is highly sensitive and specific and shown to be a valid replacement for standard serological assays. In addition, this test could be used as an evaluation tool for vector control programs.

### Introduction

Canine leishmaniasis (CanL) is a widespread zoonotic disease present in several countries in Latin-America, Europe and Asia [1,2]. It is a severe multi-systemic disease of dogs caused by the protozoan parasite *Leishmania infantum*. The disease manifests itself in variable clinical signs, with the majority of dogs experiencing poor body condition, generalized muscular atrophy, lymphadenomegaly and excessive skin scaling (reviewed in [3]). CanL is endemic across the Mediterranean basin [1,2], with seroprevalences varying from region to region depending on ecological aspects [4]. Overall, 2.5 million dogs are estimated to be infected in southern Europe [4]. Since dogs suffering from the disease are extremely difficult to treat, it is not surprising that the high incidence of CanL in southern Europe represents the main cause of deaths amongst dogs in the region [5]. However, recent occurrence of autochthonous cases in Romania, Hungary and northern Italy suggests that the disease is not limited anymore to the Mediterranean region, but confirms its spread to more northern areas (reviewed in [6]). It is noteworthy that less than 50% of infected dogs develop the disease [7]. However, both sick and asymptomatic dogs represent the main reservoir of the parasite and form a risk for human disease, zoonotic visceral leishmaniasis (ZVL) [3,5]. In the Mediterranean region, the annual incidence of ZVL is estimated to range from 1,200 up to 2,000 [8].

CanL endemicity is associated with the distribution and abundancy of its vectors, phlebotomine sand flies. In southern Europe, 5 species are proven vectors of CanL [9], of which *Phlebotomus perniciosus* is the most important. During the bite, the sand fly injects saliva containing a cocktail of bio-active molecules with anti-hemostatic, anti-inflammatory and immune-modulatory activities into the host skin (reviewed in [10]). These molecules facilitate the blood-feeding process of the sand fly and trigger a humoral immune response in the host. It is well-known that the amount of host anti-saliva IgG antibodies (Abs) correlates with the level of exposure to sand flies [11,12]. Furthermore, previous studies showed a clear fluctuation of the Ab response during longitudinal sampling of dogs over two transmission seasons [26], suggesting that proteins present in sand fly saliva can be a useful tool to evaluate the efficacy of vector control programs. For example, previous studies on mosquitoes [13–15] and triatomine bugs [16] have shown that a reduction in vector density observed after the implementation of insecticide treated nets (ITNs) correlates with a reduction in anti-vector salivary Ab-response. With regard to sand flies, only one study performed in India and Nepal measured anti-*P. argentipes* Abs to evaluate the use of ITNs [17]. Performing large-scale serological studies to...
detect host exposure to sand fly bites was limited in the past due to the fact that dissecting large amounts of sand fly salivary glands is a demanding and labour-intensive process. Besides, the use of whole SGH is subject to protein content variability, dependent on the age of the sand fly at the time of dissection [18] and might antigenically cross-react with taxonomically closely related sand fly species (reviewed in [19]). Therefore, using specific antigenic recombinant sand fly salivary proteins as a replacement to the use of whole SGH has gained more attention [20–23].

Previously the specific antibody response (IgG) against the salivary protein SP03B from P. perniciosus was proposed as a valid exposure marker across regions endemic for CanL [22]. This study demonstrated the presence of similar antigenic epitopes in the recombinant SP03B protein compared to its native form, and indicated a substantial antigenic cross-reactivity amongst P. perniciosus populations from Campania, Umbria and the metropolitan Lisbon region [22]. The SP03B salivary protein belongs to the family of yellow-related proteins [24] and was previously shown to possess binding activity for pro-haemostatic and pro-inflammatory biogenic amines in Lutzomyia longipalpis [25]. Recently, recombinant yellow-related proteins were subject of epidemiological studies to determine the levels of specific anti-vector salivary Abs in naturally bitten hosts [20,21,26,27]. All of these studies used indirect enzyme-linked immunosorbent assays (ELISA). Although this approach is useful in laboratory settings, a practical tool that can immediately be used in the field and that allows a fast screening of hosts living in endemic areas is called for.

In this study, we evaluated our newly proposed colloidal gold immunochromatographic test (ICT)—the rSP03B sero-strip—against the standard ELISA method. The use of colloidal gold ICTs has first been described by Osikowicz and Beggs for the qualitative detection of human chorionic gonadotropin (hCG) [28] and since then deployed in a broad range of fields. However, with regard to the detection of IgG Abs against arthropod saliva, no such test has been described yet. Therefore, we propose the first colloidal gold ICT in the field of vector exposure. Since our test is very straightforward—no special equipment or skill is required—it can be easily operated in non-laboratory settings to rapidly screen large cohorts of dogs for exposure to P. perniciosus.

Methods

Ethics statement

Reuse of canine sera samples obtained during previous studies [11,26] was approved by the Ethical Board of Charles University. Ethical approval of sera samples collected from non-bitten laboratory-bred Beagles housed at the University of Zaragoza, Spain (UNIZAR) was obtained during another ongoing study, protocol PI44/17. All sampling complied with the European guidelines on the protection of animals (Directive 2010/63/UE).

Sources of sera

Forty-two sera samples from laboratory-bred Beagles experimentally exposed to P. perniciosus were used to prepare the first prototype of the test. These dogs were individually exposed to approximately 200 P. perniciosus females during a previous study. The sampling protocol is described in more detail in [11]. Negative control sera were collected from 29 non-bitten laboratory-bred Beagles housed at the University of Zaragoza, Spain (UNIZAR). Furthermore, sera samples from 24 laboratory-bred Beagles born in a breeding facility located in northern France were used as negative controls.
The antigen (Ag) used for the preparation of the rSP03B sero-strip is a bacterially expressed 43kDa yellow-related recombinant protein of *P. perniciosus* (rSP03B, Genbank accn. DQ150622). The recombinant protein was obtained from Apronex s.r.o. (Prague) as described in [29] and was expressed with the *Escherichia coli* BL21 (DE3) expression system in the pET28b vector (Novagen) with a poly-His tag (6 histidines). The protein was isolated under denaturing conditions with 8M urea (50mM Tris, pH 8, 300mM sodium chloride) and prepared for usage in the rSP03B sero-strip by gradually dialyzing it to a final concentration of 0M urea (PBS 1x, pH 6) using Slide-A-Lyzer mini dialysis units (10K MWCO, 0.1mL), following the manufacturer’s protocol. The UV absorbance value of the protein was determined by Nanodrop at 280nm. The protein concentration was then quantified by means of the known molar extinction coefficient of the protein.

The Ags used for the ELISA include the rSP03B salivary protein and the whole salivary gland homogenate (SGH) from *P. perniciosus*. A colony of *P. perniciosus* was reared under standard conditions as described in [30] and salivary glands were dissected from 4–6 days-old female sand flies, pooled in 20mM Tris buffer with 150mM NaCl and stored at -20˚C. Before use, the SGH was prepared by disrupting the salivary glands during 3 freeze-and-thaw cycles in liquid nitrogen.

### Indirect enzyme-linked immunosorbent assay (ELISA)

All sera samples were analyzed by an indirect enzyme-linked immunosorbent assay (ELISA) that measures anti-*P. perniciosus* IgG. The ELISA was performed in accordance with previous studies [26], with minor modifications. Briefly, flat bottom microtiter plates (Immulon) were coated with *P. perniciosus* salivary gland homogenate (SGH) (0.2 salivary gland per well) or with rSP03B (5μg/ml) in 20mM carbonate-bicarbonate buffer (pH 9, 100μl/well) and incubated overnight at 4˚C. The plates were washed with PBS + 0.05% Tween 20 (PBS-Tw) and blocked with 6% (w/v) low fat dry milk diluted in PBS-Tw. Canine sera diluted in 2% (w/v) low fat dry milk/PBS-Tw was added to the wells (100μl/well). Sera were diluted at 1/200 and 1/100 for SGH and rSP03B, respectively. After 90min incubation at 37˚C, the plates were incubated at 37˚C for 45min with secondary Abs (polyclonal anti-dog IgG-horseradish peroxidase (HRP), Bethyl laboratories, 100μl/well) diluted 1:9000 in PBS-Tw. The ELISA was developed using an orthophenyldiamine (OPD) solution in a phosphate-citrate buffer (pH 5.5) with 0.1% hydrogen peroxide. The reaction was stopped after 5min with 10% sulfuric acid and absorbance (OD value) was measured at 492nm using a Tecan Infinite M200 microplate reader (Schoeller). Each serum was tested in duplicate.

### Preparation of the rSP03B sero-strip

The rSP03B sero-strip is composed of a lower absorbent pad and an upper absorbent pad that both overlap a nitrocellulose (NC) membrane located in the middle of the test (Fig 1). The lower absorbent pad is impregnated with a colloidal gold-conjugate consisting of a mixture of one conjugate for the test line and one for the control line. The conjugate for the test line was prepared by coupling a polyclonal anti-dog IgG Ab (Bethyl laboratories) to colloidal gold nanoparticles. Secondly, the control conjugate was prepared by coupling a chicken Ab from non-immunized chickens to colloidal gold nanoparticles. The coupling of both conjugates was followed by a saturation step (gold blocking buffer, Coris BioConcept). On the NC membrane 3 lines were coated. The first line consists of sample deposition line and enables a complete migration of the sample. The second line is the test line on which the dialyzed rSP03B protein
is coated (0.6mg/ml, 0.1μl/mm) and the third line (migration control) consists of a goat anti-chicken Ab (GAC) that binds to the colloidal gold ‘control’ conjugate.

**Principle of the rSP03B sero-strip**

In order to launch the test, a buffer (3μl; HC dilution buffer, Coris BioConcept) is applied to the sample line (blue) on the NC membrane (step 1 in Fig 2). Immediately after applying the buffer, the serum sample (1μl) is deposited on the same spot (step 2 in Fig 2). This will cause the sample to start migrating to the upper part of the strip, where the anti-rSP03B Abs present in the serum sample will be captured by the rSP03B Ag coated on the test line (step 3 and 4 in Fig 2). Directly after deposition of the sample, the strip is dipped into the migration buffer solution (step 4 in Fig 2; Ly-B dilution buffer, Coris BioConcept). The colloidal gold conjugate,
consisting of the test- and control-conjugate mixture, gets hydrated and starts migrating upwards together with the moving liquid (step 5 in Fig 2). Once arrived at the test line, the conjugate will recognize dog IgG Abs bound to the rSP03B Ag coated at the test line (step 6 in Fig 2). While moving further up the NC membrane, the GAC Abs present at the control line will capture the colloidal gold ‘control’ conjugate, resulting in the appearance of a purple color. This is an essential part of the rSP03B sero-strip as it ensures that the migration went well and the strip is functioning properly. The test is run for 15min and excess buffer solution is absorbed by the upper absorption pad. Intensity of the purple color at the test line relates to the amount of target Ab present in the sample and is visually inspected (Fig 3). The test is only valid if the migration control line is visible. A positive test result is observed when two lines (test and control) are visible on the NC membrane. The test is considered negative when only the control line is present (Fig 4).

Statistical analyses

All samples tested in ELISA were tested in duplicate. The coefficient of variation (CoV) was calculated for each duplicate sample. If the CoV was more than 15% the sample was retested. Since plates were ran on multiple days, positive control sera (PC, n = 2) and negative control sera (NC, n = 2) were included in each plate. In order to correct for interplate variability, the obtained OD values were standardized (SOD) according to the following formula:

\[
SOD (\%) = \frac{OD_{sample}}{Average\ OD\ PC} \times 100
\]

ELISA cut-offs were determined by the mean of the OD values plus 3 standard deviations (SD) obtained from non-bitten negative controls. Graphical representation of the distribution of the SOD (%) was visualized using the “beeswarm” package in R software [31,32]. The results of the rSP03B sero-strip were classified according to the intensity of the observed band, starting from (0) a negative test result; (1) a very faint signal; (2) a low positive signal; (3) a positive but less intense signal than the control band and (4) a strong positive signal, same intensity as the control band. All samples classified in categories (1), (2), (3) and (4) were considered positive. The data was graphically represented using the “ggplot2” package in R software [31,33]. The degree of agreement between SGH-ELISA, rSP03B-ELISA and the rSP03B sero-strip was measured by Cohen’s Kappa according to the methods of Jacob Cohen [34]. Furthermore, the percentage of agreement between different serological methods was measured and the McNemar’s \( \chi^2 \) test in R software [31] was used to test for significant differences in agreement between the golden standard SGH-ELISA, the rSP03B-ELISA and the rSP03B sero-strip. Correlation between both ELISA tests was analyzed using Pearson’s r correlation coefficient and graphically visualized using the “ggplot2” package in R software [31,33].

Results

Sensitivity and specificity of each serological method

The specificity and sensitivity of the 3 serological methods used were calculated based on the results from experimentally exposed dogs and negative control sera (Table 1). The distribution of the results in SOD (%) is shown in Fig 5. Results from the SGH-ELISA and rSP03B-ELISA were first classified as being positive or negative according to their respective cut-off value (Fig 5A and 5B). All 42 sera of dogs experimentally exposed to P. perniciosus appeared positive on the SGH-ELISA. However, only 29 out of these 42 samples were classified as being positive in the rSP03B-ELISA. Additionally, a single serum sample out of 53 negative control sera was
found to be positive in the SGH-ELISA, and rSP03B-ELISA. These observations resulted in a sensitivity of 100% and 69% for the SGH-ELISA and rSP03B-ELISA, respectively, and a specificity of 98.11% for both methods. Results obtained from our newly prepared rSP03B sero-strip were classified according to the intensity of the observed band. The distribution of the

Fig 3. Intensity of test line relates to amount of target Ab deposited. Dilution series of four highly positive sera samples indicate that the intensity of the purple color at the test line decreases when a lower amount of target Ab is deposited on the strip. The sample appears negative when a dilution of 1/50 is used. A: undiluted sample; B: 1/10 sample dilution; C: 1/20 sample dilution; D: 1/30 sample dilution; E: 1/40 sample dilution; F: 1/50 sample dilution.

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samples tested by the rSP03B sero-strip is shown in Fig 5C. All 42 sera samples of experimentally exposed dogs presented 2 purple bands—one on the test line and one on the control line—resulting in a sensitivity of 100%. On the other hand, 7 out of 53 sera from non-bitten negative controls were also found positive with our rSP03B sero-strip, giving it a specificity of

Table 1. Sensitivity and specificity of each serological method (SGH-ELISA, rSP03B-ELISA and the rSP03B sero-strip).

<table>
<thead>
<tr>
<th></th>
<th>True positives</th>
<th>True negatives</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGH-ELISA</td>
<td>42/42</td>
<td>52/53</td>
<td>100% (95% CI [89.56%–100%])</td>
<td>98.11% (95% CI [88.62%–99.90%])</td>
</tr>
<tr>
<td>rSP03B-ELISA</td>
<td>29/42</td>
<td>52/53</td>
<td>69% (95% CI [52.76%–81.89%])</td>
<td>98.11% (95% CI [88.62%–99.90%])</td>
</tr>
<tr>
<td>rSP03B sero-strip</td>
<td>42/42</td>
<td>46/53</td>
<td>100% (95% CI [89.56%–100%])</td>
<td>86.79% (95% CI [74.05%–94.09%])</td>
</tr>
</tbody>
</table>

The predictive values for each method were calculated based on results from experimentally exposed dogs and negative control sera. CI, Confidence Interval.
Fig 5. Distribution of results from ELISA and the rSP03B sero-strip. SOD (%) distribution of positive sera samples (experimentally exposed dogs) and negative sera samples (non-bitten dogs) tested by SGH-ELISA (A) and rSP03B-ELISA (B) is shown. The cut-off for each ELISA was calculated by the mean of non-bitten negative control sera +3SD. Results from the rSP03B sero-strip were classified according to the intensity of the observed band, starting from (0) a negative test result; (1) a very faint signal; (2) a low positive signal; (3) a positive but less intense signal than the control band and (4) a strong positive signal, same intensity as the control band. All samples classified in categories (1), (2), (3) and (4) were considered positive. Distribution of positive sera samples and negative sera samples is shown in (C). Correlation between SOD (%) values of SGH-ELISA and rSP03B-ELISA was performed using Pearson’s r correlation and is shown in (D). SD, Standard Deviation; SOD, Standardized Optical Density; SGH, Salivary Gland Homogenate; r, Correlation index; CI, Confidence Interval.

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86.79%. Importantly, raising the detection limit of the rSP03B sero-strip until category (1) will be considered negative results in a specificity of 96.23%, without changing the sensitivity.

### Comparison of rSP03B-ELISA and rSP03B sero-strip with the SGH-ELISA

Since the SGH-ELISA showed the highest sensitivity and specificity amongst all 3 methods, it was set as the golden standard against which the rSP03B-ELISA and the rSP03B sero-strip were evaluated (Table 2). The sensitivity and specificity of the rSP03B sero-strip as compared to the SGH-ELISA were 97.67% and 87%, respectively. A percentage of agreement of 91.58% was obtained with no systematic difference between the proportions of positive responses from these two methods (McNemar $\chi^2 P > 0.05$). Furthermore, the Cohen’s kappa value between the rSP03B sero-strip and the SGH-ELISA was 0.83, suggesting an almost perfect strength of agreement between these two methods. With regard to the rSP03B-ELISA a sensitivity and specificity of 67.44% and 98% were measured, respectively. The percentage of agreement between the rSP03B-ELISA and the SGH-ELISA was set at 84.21% with a Cohen’s kappa value of 0.67, suggesting a substantial strength of agreement. However, McNemar $\chi^2$ tested a significant systematic difference between the proportions of positive responses from the rSP03B-ELISA and the SGH-ELISA ($P < 0.05$). Interestingly, when raw OD-values of both ELISA methods were compared, a correlation of 83.43% was obtained (95% CI [76.06%–88.67%], $P < 0.001$) (Fig 5D).

### Discussion

Previous studies addressing the level of host exposure to sand fly bites consistently used ELISA methods to determine the levels of specific anti-vector salivary Abs. Although this method is useful in laboratory settings, a rapid test that can aid in vector control by allowing a consistent screening of hosts in the field has not yet been described. Here, we developed a new rapid test that can be immediately used in the field to screen dogs living in endemic CanL areas for the presence of anti-\(P. \text{perniciosus}\) IgG Abs. In the proposed rSP03B sero-strip, the yellow-related rSP03B \(P. \text{perniciosus}\) salivary protein was used as Ag, previously proposed as a valid exposure marker for \(P. \text{perniciosus}\) across its entire area of distribution [22]. The principle of the rSP03B sero-strip is similar to an indirect ELISA; first specific canine Abs present in the sample bind to the \(P. \text{perniciosus}\) salivary Ag immobilized on the NC membrane of the sero-strip, after which the interaction is visualized by an anti-dog IgG Ab gold-conjugate.

In this study, 3 serological methods to define the level of \(P. \text{perniciosus}\) salivary IgG Abs were compared: the golden standard SGH-ELISA, the previously described rSP03B-ELISA [22] and our newly proposed rSP03B sero-strip. The results highlight the SGH-ELISA as being the most sensitive (100%) and most specific (98.11%) amongst all 3 methods. When
comparing the performance values of the rSP03B-ELISA and the rSP03B sero-strip, the rSP03B sero-strip appears to have the highest sensitivity (69% vs. 100%, respectively) whereas the rSP03B-ELISA shows the highest specificity (98.11% vs. 86.79%, respectively). Finally, the rSP03B sero-strip was shown to have an almost perfect agreement with the SGH-ELISA without any significant differences, overall suggesting that the sero-strip performs better than the rSP03B-ELISA. Moreover, the performance of the sero-strip can be further improved as was shown by an increased specificity (up to 96%) without a loss in sensitivity when very faint results would be considered negative. This can be achieved by the use of a strip reader which will classify the results according to a pre-defined cut-off value for the sero-strip.

Although our results indicate that our newly proposed rSP03B sero-strip is a valid replacement for the rSP03B-ELISA, the performance values of the rSP03B-ELISA should be taken with caution. When raw OD-values of both ELISA methods were compared, a correlation of 83.43% was achieved. This high correlation between the rSP03B-ELISA and the SGH-ELISA suggests that classifying the samples according to positivity might be the reason for the high number of false negatives in the rSP03B-ELISA. The higher cut-off value observed in the rSP03B-ELISA could be explained by a non-specific reaction that takes place in the ELISA between the negative control samples and bacterial proteins that possibly co-purified with the rSP03B protein [35] and might be overcome by producing the protein in a different expression system (e.g. mammalian cells). The reason why these false negative results do not occur to the same extent with the rSP03B sero-strip is explained by the fact that this is a qualitative method and is therefore not dependent on a specific cut-off value. Thus, any observed signal will be classified as positive.

In summary, we developed a simple and rapid colloidal gold ICT based on the bacterially expressed recombinant protein rSP03B that is able to replace the ELISA method used in numerous previous studies [11,20–22,26]. Our rSP03B sero-strip showed to be highly sensitive (100%) and specific (86.79%) in the detection of IgG Abs against P. perniciosus saliva. The test is easily operated with no requirements for skilled personnel or specialized equipment. However, in order to confirm the field detection accuracy and applicability of the test, further evaluation of canine populations exposed to various frequencies of sand fly bites and validation of the test with whole canine blood is required. Additionally, it is worth to mention that potential cross-reactivities between Abs recognizing salivary proteins of closely related sand fly species has been observed in previous studies [36]. Therefore, a cross-reaction between the rSP03B protein and Abs against salivary proteins of closely related sand fly species of subgenus Larroussius is likely to occur, hence rendering the sero-strip useful for estimating exposure to other vectors of CanL in the Mediterranean basin. However, due to the lack of colonies of sand fly species co-occurring with P. perniciosus [37] this cross-reaction cannot be tested at the moment. Unfavorable cross-reactions with other haematophagous insects are very unlikely but we suggest to reflect on this during further studies.

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Field study of the improved rapid sand fly exposure test in areas endemic for canine leishmaniasis

Field study of the improved rapid sand fly exposure test in areas endemic for canine leishmaniasis

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Background: Canine leishmaniasis (CanL) is a severe chronic disease caused by *Leishmania infantum* and transmitted by sand flies; the main vector in the Western part of the Mediterranean basin is *Phlebotomus perniciosus*. Previously, an immunochromatographic test (ICT) was proposed to allow rapid evaluation of dog exposure to *P. perniciosus*. In the present study, we optimized the prototype and evaluated the detection accuracy of the ICT in field conditions. We also studied possible cross-reactions with other hematophagous arthropods.

Methodology/Principal Findings: The ICT was optimized by expressing the rSP03B protein in a HEK 293-cell line, which delivered an increased specificity (94.92%). The ICT showed an excellent inter-rater and inter-performer agreement and a good reproducibility. Field detectability of the ICT was assessed by screening 186 dogs from different CanL endemic areas and 154 longitudinally sampled dogs with the SGH-ELISA and the ICT. The ICT results corresponded to the SGH-ELISA for most areas, depending on the statistical measure used. Furthermore, a clear seasonal fluctuation was obtained by both methods, with the peaks in antibody response corresponding to higher signal intensities observed in the ICT. We excluded cross-reactions between non-vectorial species and confirmed favorable cross-reactions with other *L. infantum* vectors belonging to the subgenus *Larroussius*. Finally, the ICT was optimized for use with whole canine blood rendering a high degree of agreement with the use of serum.

Conclusions/Significance: We have successfully optimized the rSP03B Sero-Strip, now also suitable to be used with whole canine blood. The ICT is able to distinguish recent from past exposure and showed a good detectability in a field population of naturally exposed dogs, particularly in areas with a high prevalence of bitten dogs. Furthermore, our study showed the existence of favorable cross-reactions with other sand fly vectors thereby expanding its use in the field.
Author summary

Canine leishmaniasis (CanL) is a widespread severe chronic disease in dogs, caused by the protozoan parasite *Leishmania infantum*. *Leishmania* parasites are transmitted by the bite of phlebotomine sand flies, of which the most important one in southwestern Europe is *Phlebotomus perniciosus*. A sand fly bite is accompanied by the inoculation of saliva, which elicits a specific antibody response in the host. Past studies have used these antibodies as markers of exposure to sand flies. Recently, a rapid sand fly exposure test was prepared using one salivary protein of *P. perniciosus*, the SP03B protein. This rapid test possessed a high sensitivity and specificity when screening experimentally exposed versus non-bitten dogs. However, since naturally exposed dogs possess a higher variability of antibodies against sand fly saliva, we have optimized the previously proposed rapid test and evaluated its detectability on field samples. In this study we present the optimized rSP03B Sero-Strip reaching a higher specificity and ability to detect seropositive dogs from a naturally exposed dog population in a similar fashion as standard serological methods. Furthermore, we have optimized it with whole canine blood which renders it suitable for use in field conditions.

Introduction

*Leishmania infantum* is a protozoan kinetoplastid parasite (Trypanosomatida, Trypanosomatidae) that annually accounts for approximately 1200 to 2000 new cases of human visceral leishmaniasis (VL) in the Mediterranean basin [1]. It is a zoonotic disease transmitted by sand flies (Diptera, Psychodidae), with the most important vector in the western part of the Mediterranean basin being *Phlebotomus perniciosus* [2]. Domestic dogs represent the primary reservoir; most infected dogs will appear asymptomatic, others may suffer from a severe chronic disease known as canine leishmaniasis (CanL) [3]. Importantly, both clinical and subclinical infections contribute to the transmission of CanL [3,4]. Across the Mediterranean, canine seroprevalences of the infection vary greatly according to region,
going from 3.7 - 34.6% in Spain [5] and from 3.9 - 24% in Italy [6–8]. However, these numbers underestimate the real amount of infected dogs in the area as has been confirmed by PCR-based screening of seronegative dogs [9,10]. Importantly, municipal kennels housing dogs throughout their life are frequently found in certain European countries and contribute to the persistence of focal hot spots of CanL, thereby forming a major barrier to the control of the disease [11].

The zoonotic nature of the disease together with the severe clinical symptoms it may cause in dogs calls for efforts to control the vector and the spread of the infection. Hence, information on sand fly population dynamics is fundamental and can be obtained by measuring vector exposure in hosts living in endemic areas. This has already been achieved in numerous previous studies by using whole sand fly salivary gland homogenate (SGH) [12,13] or specific salivary proteins [14–17]. The IgG response against the *P. perniciosus* salivary protein SP03B has been shown to relate to the seasonal abundance of this sand fly species [14] and to be highly reactive in dogs bitten by different *P. perniciosus* populations across countries in the Mediterranean area [18,19]. All these studies together verify the use of the IgG response against this protein as a marker of exposure capable to replace the use of SGH.

In order to allow rapid evaluation of vector control programs, our recent study focused on using the specific IgG response against the *P. perniciosus* SP03B salivary protein as the detectable agent in a rapid vector exposure test [20]. The serological immunochromatographic test (Sero-Strip ICT) was prepared with the recombinant SP03B protein purified from overexpression in *E. coli* and showed to be a valid replacement for the ELISA method. Even though the predictive values of this Sero-Strip were already in the excellent range – with a sensitivity of 100% and a specificity of 86.8% – further efforts to increase the specificity of the test were undertaken. In the present study we have optimized the Sero-Strip by using the rSP03B recombinant salivary protein expressed in the Human Embryonic Kidney (HEK)
293S GnTI cell line, which enables proper protein folding and introduces post-translational modifications which are essential for complete biological activity of the protein [21,22]. Moreover, we assessed the detection accuracy of the rSP03B Sero-Strip in field conditions by screening canine populations exposed to various frequencies of sand fly bites in different areas in the Mediterranean basin and collected on different time points. In order to exclude unfavorable cross-reactions between the proposed rSP03B Sero-Strip and non-vector sand fly species together with other hematophagous arthropods present in the Mediterranean basin, we tested the cross-reactivity between salivary antigens of *P. perniciosus* (whole SGH and rSP03B protein) and sera of animals that were experimentally bitten by *Ixodes ricinus*, *Ctenocephalides felis*, *Culex molestus*, *Phlebotomus papatasi*, *P. sergenti* or *P. tobbi*. Lastly, in order to facilitate the use of the rSP03B Sero-Strip in the field, the Sero-Strip was validated with whole canine blood.

**Methods**

**Ethics statement**

Ethical approval for the reuse of canine sera samples from previous studies [13,14,18] was granted by the Ethical Board of Charles University (Prague, Czech Republic). The use of canine sera samples obtained from non-bitten laboratory-bred Beagles housed at the University of Zaragoza, Spain (UNIZAR) was approved during another ongoing study, protocol PI44/17. Collection of blood samples of naturally exposed field dogs was performed by a trained veterinarian and did not include additional or unnecessary invasive procedures. Consent was obtained from the dog owners. International animal experimentation guidelines were followed. All sampling complied with the European guidelines on the protection of animals (Directive 2010/63/UE). BALB/c mice were maintained and handled in the animal facility of Charles University (Prague, Czech Republic) following institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on the Protection of Animals against
Cruelty in present statutes at large), complying with all relevant European Union and international guidelines for experimental animals. All experiments were approved by the Committee on the Ethics of Laboratory Experiments of the Charles University (Prague, Czech Republic) (permission no. MSMT-10270/2015-6 of the Ministry of the Environment of the Czech Republic).

**Sources of samples**

In order to assess the predictive values of the optimized rSP03B Sero-Strip, the test was run with 60 sera samples from laboratory-bred Beagles experimentally exposed to *P. perniciosus*, 30 sera samples from non-bitten laboratory-bred Beagles housed in a breeding facility in northern France, and 29 sera samples from non-bitten laboratory-bred Beagles housed at the University of Zaragoza, Spain (UNIZAR). The sampling protocol of canine sera samples obtained from dogs individually exposed to approximately 200 *P. perniciosus* females is described in more detail in [13].

Furthermore, to evaluate the field detection capability of the rSP03B Sero-Strip, sera samples from naturally bitten dogs in regions endemic for CanL were used. More specifically, sera samples from naturally-exposed dogs housed in open-air kennels in rural areas in Montagut and Canet d’Adri (province of Girona, Catalonia, Spain) collected in July (n = 29 and n = 12) and September 2017 (n = 26 and n = 12), respectively were evaluated. Furthermore, canine sera samples from 12 naturally exposed dogs of different breeds living in domestic gardens in Colònia de Sant Jordi (Ses Salines District, Mallorca, Spain) were analyzed. Additionally, 38 sera samples from dogs living in Campania (Italy) collected in July 2012 were tested, as well as a total of 57 canine sera samples collected in Umbria (Italy) at different points in time (October 2013 (n = 27), August 2013 (n = 30)). The sampling protocol of the samples derived from Campania and Umbria is explained in more detail in [18]. For all samples listed above, no pre-selection of dogs took place in order to determine the
performance of the test in the field as close as possible. Dogs from both Spain and Italy comprised a mixture of hunting breeds and mongrels. Furthermore, sera samples from 154 naturally exposed dogs housed in an open-air kennel in a rural municipality of Naples province (Campania region, southern Italy) sampled multiple times during two transmission seasons were used [14] to assess the seasonal fluctuation of the Ab-response with the Sero-Strip.

A blood sample collected from a healthy unexposed dog living in the Czech Republic was spiked with 30 sera samples from experimentally exposed laboratory-bred Beagles and 29 non-bitten laboratory dogs from Zaragoza (Spain) in order to define the predictive values of the test when whole canine blood is used compared to when the test is performed with the same sera. The blood sample was also spiked with sera samples collected in Montagut and Canet d’Adri (province of Girona, Catalonia, Spain) and Colònia de Sant Jordi (Ses Salines District, Mallorca, Spain) in order to compare the functionality of the test when sera or blood samples are used in CanL endemic settings.

Finally, to assess potential cross-reactions with other hematophagous arthropods, sera samples from mice or dogs experimentally exposed to *C. felis, I. ricinus, Cu. molestus, P. papatasi, P. sergenti, P. tobbi, and P. perniciosus* were used. More specifically, canine sera samples from laboratory-bred Beagles six times experimentally exposed to several hundreds of *C. felis* were used to screen for cross-reactions between specific anti-*C. felis* IgG and salivary proteins (SGH and rSP03B) from *P. perniciosus*. Furthermore, sera samples from mice exposed twice to ten *I. ricinus* nymphs were used to test for a reaction with specific anti-*I. ricinus* IgG. Lastly, sera samples positive for anti-*Culex* and anti-*Phlebotomus* antibodies were collected from BALB/c mice used for regular blood feeding of mosquito (*Cu. molestus*) and sand fly (*P. papatasi, P. sergenti, P. tobbi, P. perniciosus*) colonies maintained in the
insectary at Charles University (Prague, Czech Republic) according to [23]; all of them being exposed more than ten times. Sera from non-bitten mice were used as negative control.

Antigens
The antigen used for the preparation of the optimized rSP03B Sero-Strip was the recombinant 43 kDa yellow-related protein of *P. perniciosus* (SP03B, GenBank accn. DQ150622) expressed in a human cell line. Cloning, production and purification of this protein was performed by isolating the mRNA coding for SP03B from one-day-old *P. perniciosus* females by the High Pure RNA Tissue Kit (Roche), after which it was transcribed into the cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche). The DNA fragment was amplified by PCR and subcloned into the pTW5sec expression plasmid, a derivative of pTT5 [22,24]. Proteins expressed from this plasmid contain amino acid residues ITG at their N-terminus and GTHHHHHHHHG, i.e. a histidine tag, at their C-terminus. The rSP03B protein was transiently expressed in HEK293S GnTI- cells (ATCC CRL-3022) as previously described in [22]. Briefly, suspension adapted HEK293S GnTI- cells were grown in EX-CELL293 medium (square-shaped glass bottles) supplemented with 4 mM L-glutamine (Sigma) and shaken at 135 rpm in a humidified incubator at 37°C with 5% CO₂. For transient transfection, the cell culture was freshly transferred into EX-CELL293 medium at 20 × 10⁶ cells/ml cell density. The expression plasmid DNA (diluted in PBS; 1 µg of DNA per 1 × 10⁶ cells) and the 25 kDa linear polyethylenimine were added directly in a 1:4 weight ratio into the high-density cell culture. Following 4 hours of incubation, the culture was diluted with EX-CELL293 medium to 2 × 10⁶ cells/ml. Culture medium was harvested five to seven days post-transfection by centrifugation (10000 × g, 30 min) and filtered thereafter (0.22 µm Steritop filter; Millipore, USA). Before purification, the harvested medium was diluted with an equal volume of buffer (50 mM Na₂HPO₄, 300 mM NaCl, 10 mM NaN₃, pH 7.5). The histidine-tagged product was then recovered by IMAC chromatography on HiTrap TALON crude columns (GE Healthcare,
USA) and further purified by size exclusion chromatography on Superdex 200 10/300 GL column (GE Healthcare, USA). Finally, the protein identity was verified by mass spectrometry. The UV absorbance value of the protein was measured using a nanospectrophotometer at 280 nm. Protein concentration was then quantified by means of its known theoretical molar extinction coefficient.

The whole SGH from *P. perniciosus* was used as antigen in ELISA to serve as the gold standard against which the rSP03B Sero-Strip is evaluated. A colony of *P. perniciosus* was reared under standard conditions as described in [23]. Four-to-six day-old female sand flies were dissected; salivary glands were collected and pooled in 20 mM Tris buffer with 150 mM NaCl (TBS). The SGH were stored at -20°C and prepared before use by disrupting the salivary glands during three freeze-and-thaw cycles in liquid nitrogen.

**Indirect enzyme-linked immunosorbent assay**

An indirect enzyme-linked immunosorbent assay (ELISA) was performed on all sera samples to screen for anti-*P. perniciosus* IgG. The ELISA was carried out analogously to a previous study [20]. In short, *P. perniciosus* salivary gland homogenate (SGH) was coated on flat bottom microtiter plates (Immuron; 0.2 salivary gland per well) in 20 mM carbonate-bicarbonate buffer (pH 9, 100 µl/well) and incubated overnight at 4°C. After blocking the plates with 6% (w/v) low fat dry milk diluted in PBS + 0.05% Tween 20 (PBS-Tw), canine sera diluted 1/200 in 2% (w/v) low fat dry milk/PBS-Tw were added to the wells (100 µl/well). Further, the plates were incubated with secondary antibodies (polyclonal anti-dog IgG-horseradish peroxidase (HRP), Bethyl laboratories, 100 µl/well) diluted 1:9000 in PBS-Tw. The ELISA was developed with orthophenylendiamine (OPD) in a phosphate-citrate buffer (pH 5.5) and 0.1% hydrogen peroxide. The reaction was stopped after 5 min with 10% sulfuric acid and the absorbance (OD value) was measured at 492 nm using a Tecan Infinite M200 microplate reader (Schoeller). Each serum was tested in duplicate.
The principle of the rSP03B Sero-Strip has been described in our previous study [20]. In essence, the serum sample to be tested is deposited (1µl) on the sample line of the Sero-Strip immediately after a blocking buffer is applied on the same line. Both the buffer and the sample migrate to the upper part of the strip, where the anti-rSP03B antibodies present in the sample bind to the rSP03B coated on the test line. After dipping the strip into the migration buffer, the colloidal gold conjugated to anti-dog IgG (gold-conjugate) starts migrating upwards, eventually leading to labelling of the dog IgG captured on the test line by immobilized rSP03B. The control colloidal gold conjugate (chicken antibody) will bind to goat-anti chicken antibodies present at the control line. In case of a positive sample, two purple lines will appear on the nitrocellulose (NC) membrane, whereas for negative samples only the control line will be visible. In the present study the ICT was further optimized for use with whole canine blood. In order to do so, a blood sample from a healthy non-exposed dog living in the Czech Republic was spiked (2 units cells: 1 unit serum) with serum samples from (a) experimentally exposed laboratory-bred beagles, (b) non-bitten laboratory dogs, (c) dogs living in an open-air kennel in Girona province (Spain), and (d) dogs living in domestic gardens in Colonia de Sant Jordi in Mallorca (Spain). For optimal performance of the strip, small adjustments in the lay-out of the strip had to be made; (1) the blood sample (1 µl) is deposited immediately on the sample line without the need of saturating the NC membrane with the blocking buffer, and (2) the anti-dog IgG gold-conjugate was impregnated at a slightly higher concentration compared to when serum is used.

Detection of anti-salivary antibodies in sera of experimentally bitten hosts

A dot blot was performed in order to verify the presence of specific anti-saliva antibodies in the sera samples of hosts experimentally bitten by various hematophagous arthropods. Salivary glands from *C. felis, I. ricinus, Cu. molestus, P. papatasi, P. sergenti, P. tobbi,* and
*P. perniciosus* were collected, stored at -20°C and subjected to three freeze and thaw cycles in liquid nitrogen prior to use. After spotting 3 µl of each SGH on a section of NC membrane (Serva, pore size 0.2 µm), it was dried at RT in order to stabilize the antigen. The spotted volume corresponds to a total of three salivary glands for *C. felis*, *Cu. molestus*, and all tested sand fly species; whereas it equals 2.6 µg of salivary gland extract proteins from *I. ricinus*. Consequently, the NC membrane was blocked using 5% (w/v) low fat dry milk in TBS + 0.05% Tween 20 (pH 7.2, TBS-Tw) and incubated overnight at 4°C. The sera were diluted 1:50 in TBS-Tw in case of sera from animals exposed to *C. felis* and *I. ricinus*, or 1:100 for sera from animals exposed to *P. perniciosus*, *P. papatasi*, *P. sergenti*, *P. tobbi* and *Cu. molestus*. The NC membrane was then washed four times with TBS-Tw and incubated with the diluted sera for 1 hour at RT. After washing the NC membrane for four times in TBS-Tw, secondary antibodies conjugated to horseradish peroxidase (polyclonal anti-mouse or anti-dog IgG-HRP, AbD Serotec/ Bethyl laboratories) were diluted at 1:1000 in TBS-Tw and added to each respective NC membrane. After incubating for 1 hour at RT, the reaction was developed using diaminobenzidine (DAB) in TBS (pH 7.2) with 0.03% H₂O₂. The reaction was stopped by adding distilled water to each well. Sera coming from non-exposed animals were used as negative control.

**Assessment of cross-reactivity by western blot**

A western blot analysis was performed to study cross-reactions between the salivary proteins of all previously mentioned arthropods and *P. perniciosus*. Both *P. perniciosus* SGH (15 glands per well; 0.22 µg of protein per gland) and the rSP03B protein (46 µg/ml) were separated on a 12% SDS–PAGE gel under non-reducing conditions using the Mini-Protean III apparatus (BioRad) and blotted on an NC membrane. Subsequently, the NC membrane was cut into strips which were blocked with 5% low fat dry milk in TBS-Tw and incubated for 1 hour with sera diluted at 1:50 in TBS-Tw for sera positive for *I. ricinus* and *C. felis*; at 1:200
for sera positive for *P. papatasi*, *P. sergenti*, *P. tobbi*, *Cu. molestus* and for the negative control sera; and at 1:500 for sera positive for *P. perniciosus*. The strips were then incubated for 1 hour with secondary antibodies (polyclonal anti-mouse or anti-dog IgG-HRP, AbD Serotec/ Bethyl laboratories) – diluted at 1:1000 in TBS-Tw – after which the chromogenic reaction was developed using DAB in TBS with 0.03% H$_2$O$_2$. Distilled water was added to the strips to stop the reaction.

**Statistical analyses**

In ELISA, all samples were run in duplicate and retested when a coefficient of variation (CoV) of more than 15% was obtained. Sera of positive (PC) and negative control (NC) dogs were included in each plate to correct for interplate variability. OD values were standardized (SOD) according to the following formula:

$$SOD (\%) = \frac{\text{OD sample}}{\text{Average OD PC} - \text{average OD NC}} \times 100$$

In all subsequent analysis the SGH-ELISA was set as the gold standard to which the results of the rSP03B Sero-Strip were evaluated. The prevalence of bitten dogs per region and per month was calculated from the SOD (%) SGH-ELISA results using a cut-off value for positivity according to the mean SOD (%) values from non-bitten negative control dogs plus two standard deviations (SD). Thereafter, expected positive and negative predictive values (PPV and NPV, respectively) per region were computed using the known sensitivity and specificity of the ICT. Consequently, all samples were also analyzed with the ICT. The resulting signal was classified into four categories according to the intensity of the observed band, analogous to what has been proposed previously [20]. Samples classified in category (0) were considered negative, the ones classified in categories (2), (3), and (4) were considered positive. Since samples in category (1) merely showed a faint background signal, they were considered either positive or negative. The results of all samples when screened with the ICT.
were converted into a proportion of dogs classified as positive per region or per time point and graphically visualized using the “ggplot2” package in R software [25,26].

The degree of agreement between the SGH-ELISA and the rSP03B Sero-Strip was measured per region by Cohen’s Kappa according to the methods of Jacob Cohen [27]. The McNemar’s $\chi^2$ test in R software [25] was applied to test for significant differences in agreement between these two methods. The Wilcoxon rank sum test was used to test for significant differences between the mean values of the SOD (%) ELISA results obtained on different time points. In order to assess the consistency in the ratings of signal intensity across samples between different readers, the inter-reader reliability (IRR) was calculated. Similarly, the reproducibility of the ICT was assessed so to assure consistency in ratings over time. Both IRR and reproducibility were computed using a two-way mixed, consistency, single-measures intraclass correlation coefficient (ICC) [28]. Moreover, the random effects single-measures model was used in order to allow generalization of the results rated by a single reader. Finally, the ICC was also computed to compare the performance of the ICT when run with serum or with whole canine blood.

**Results**

**Inter-rater reliability and reproducibility of the rSP03B Sero-Strip**

To verify if the person rating the ICT does not have an influence on the test result, 30 randomly chosen samples were read by two different readers. Similarly, all 30 samples were run by two different performers so to exclude any bias in the person executing the ICT. In order to do so, the intra-class correlation coefficient (ICC) was calculated for all combinations and is shown in Table 1. When the results of the two readers were compared, ICC values of 0.966 and 0.956 were obtained. Furthermore, comparing two different performers resulted in ICC values of 0.865 and 0.881. The reproducibility of the rSP03B Sero-Strip was ascertained
by testing 30 sera samples on two different days. The ICC between both measurements was 0.68 (95% CI: 0.42 - 0.835; P-value: 1.92E-05).

**Table 1. Intraclass Correlation Coefficients for inter-rater reliability**

<table>
<thead>
<tr>
<th>Performer A vs. B</th>
<th>Rater A vs. B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ICC</strong></td>
<td><strong>95 CI</strong></td>
</tr>
<tr>
<td>Constant Rater A</td>
<td>0.865</td>
</tr>
<tr>
<td>Rater B</td>
<td>0.881</td>
</tr>
</tbody>
</table>

The ICC values obtained after comparing the results of the Sero-Strip when two different performers were used are shown on the left; the ICC values obtained when the results of two different raters are compared are shown on the right. ICC, Intraclass Correlation Coefficient; CI, Confidence Interval.

**Predictive values of the optimized rSP03B Sero-Strip**

The sensitivity and specificity of the optimized ICT were determined based on the test results of dogs experimentally exposed to *P. perniciosus* female sand flies and samples coming from non-bitten laboratory-bred Beagles. In 3 out of 60 dogs experimentally exposed to *P. perniciosus* no band appeared on the test line when run by the ICT, allocating a sensitivity of 95% (95% CI 85.18% - 98.70%) to this optimized ICT. Furthermore, in 3 out of 59 unexposed dogs a band appeared on the test line, giving it a specificity of 94.92% (95% CI 84.94% - 98.68%). Seroprevalence of bitten dogs per region were defined based on the SOD (%) obtained by SGH-ELISA and are given in Table 2. The cut-off for ELISA was set at 34.74 SOD (%) and represented the mean SOD (%) of non-bitten laboratory-bred Beagles plus two SD, respectively. The positive and negative predictive values (PPV and NPV, respectively) of the ICT were then calculated per region based on the previously calculated prevalence of bitten dogs and the known sensitivity and specificity of the Sero-Strip and are also shown in Table 2.
Table 2. Seroprevalence of bitten dogs and predictive values of the ICT per region.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Seroprevalence of bitten dogs (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campania July 2012</td>
<td>8.57</td>
<td>62.87</td>
<td>99.51</td>
</tr>
<tr>
<td>Umbria August 2013</td>
<td>14.29</td>
<td>75.06</td>
<td>99.13</td>
</tr>
<tr>
<td>Umbria Oct 2013</td>
<td>15.38</td>
<td>76.66</td>
<td>99.05</td>
</tr>
<tr>
<td>Montagut July 2017</td>
<td>33.33</td>
<td>90.03</td>
<td>97.43</td>
</tr>
<tr>
<td>Montagut Sept 2017</td>
<td>34.62</td>
<td>90.53</td>
<td>97.28</td>
</tr>
<tr>
<td>Canet d’Adri July 2017</td>
<td>75.00</td>
<td>98.19</td>
<td>86.33</td>
</tr>
<tr>
<td>Canet d’Adri Sept 2017</td>
<td>9.09</td>
<td>64.36</td>
<td>99.48</td>
</tr>
<tr>
<td>Colonia de Sant Jordi Oct 2017</td>
<td>41.67</td>
<td>92.81</td>
<td>96.37</td>
</tr>
</tbody>
</table>

The seroprevalence of bitten dogs per region was calculated based on the SOD (%) obtained by SGH-ELISA and was used together with the sensitivity and specificity of the ICT to calculate the PPV and NPV per region. PPV, Positive Predictive Value; NPV, Negative Predictive Value.

Field detectability of the rSP03B Sero-Strip

Both the seroprevalence calculated based on the SGH-ELISA results as well as the results from the rSP03B Sero-Strip were plotted per region and are shown in Figure 1. When including samples from category (1) into our analysis, no significant systematic differences were observed between the proportions of positive responses from these two methods (McNemar χ² P > 0.05) for a total of four out of eight regions tested. The Cohen’s kappa value between the ICT and the SGH-ELISA was calculated for each region and suggests an almost perfect strength of agreement between these two methods for one out of the eight regions tested. A fair degree of agreement was obtained for an additional two regions; for the other regions only a slight or no degree of agreement was obtained. However, when we considered samples in category (1) to be negative, the Cohen’s kappa value between the ICT and the SGH-ELISA suggested a substantial degree of agreement between these two methods for one region, a fair degree of agreement for four out of eight regions, and only a slight or no degree of agreement for the other regions. Even more so, McNemar χ² tested no systematic difference between the proportions of positive responses between SGH-ELISA and the ICT.
for all regions tested. The outcome of the McNemar’s $\chi^2$ test and the Cohen’s kappa values for both analyses are shown in Table 3.

**Figure 1. Graphical representation of results obtained per region.** (A) Seroprevalence (%) calculated based on the SGH-ELISA results are plotted per region. (B) Results obtained by the rSP03B Sero-Strip are visualized per region and plotted according to the different categories of the ICT. The size of the plotted circle represents the proportion of dogs classified per category.

**Table 3. Comparison of the rSP03B Sero-Strip with SGH-ELISA per region.**

<table>
<thead>
<tr>
<th>Region</th>
<th>Category (1) is positive</th>
<th>Category (1) is negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kappa</td>
<td>McNemar</td>
</tr>
<tr>
<td><strong>Campania July 2012</strong></td>
<td>0.196</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Umbria August 2013</strong></td>
<td>0.195</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Umbria October 2013</strong></td>
<td>0.126</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Montagut July 2017</strong></td>
<td>0.093</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Montagut Sept 2017</strong></td>
<td>0.210</td>
<td><strong>0.065</strong></td>
</tr>
<tr>
<td><strong>Canet d’Adri July 2017</strong></td>
<td><strong>1.000</strong></td>
<td><strong>1.000</strong></td>
</tr>
<tr>
<td><strong>Canet d’Adri Sept 2017</strong></td>
<td>0.214</td>
<td><strong>0.125</strong></td>
</tr>
<tr>
<td><strong>Colonia de Sant Jordi October 2017</strong></td>
<td>NA</td>
<td><strong>1.000</strong></td>
</tr>
</tbody>
</table>

Degree of agreement between the rSP03B Sero-Strip and the SGH-ELISA is shown. Two analyses were performed based on classifying category (1) as positive or negative. For both analyses the Cohen’s kappa value was calculated per region and the McNemar $\chi^2$ test was performed. A fair degree of agreement according to Cohen’s kappa is highlighted in light grey (0.21 – 0.40), a substantial degree of agreement is highlighted in darker grey (0.61 – 0.80), and an almost perfect degree of agreement is highlighted in dark grey (0.81 – 1.00). P-values for areas for which McNemar $\chi^2$ test did not detect a significant systematic difference between the outcome of the rSP03B Sero-Strip and the SGH-ELISA are highlighted in bold. NA, Not Applicable.
Longitudinal assessment of the rSP03B Sero-Strip compared to the SGH-ELISA

Results of naturally exposed dogs sampled multiple times during two transmission seasons are visualized in Figure 2 and show a clear fluctuation for seroprevalence of bitten dogs as well as for the results of the rSP03B Sero-Strip, with a peak at months August – September of the first year of sampling and a second more obvious one at July – August of the second year. Differences between subsequent sampling months were calculated for the SOD (%) SGH-ELISA results and showed to be significant for July and August ($P = 0.001$), August and September ($P = 0.016$), and September and October ($P = 0.006$) of the first year of sampling, and for March and July ($P = 0.006$) of the second year of sampling. When considering the results of the ICT, the highest proportion of positive dogs were observed during October (58.34%) of the first year and July (82.34%) and August (82.34%) of the second year. However, when considering samples in category (1) as negative, the highest proportion of positive dogs is observed for September (41.17%) of the first year and for July (76.46%), August (52.93%) and September (62.5%) of the second year. Even more so, these results also show a clear drop in the total proportion of positive dogs from December to March, reaching zero percent in December and slowly increasing during January and March up to 5.88% for both months.

Figure 2. Graphical representation of results obtained during longitudinal sampling. (A) Seroprevalence (%) calculated based on the SGH-ELISA results are plotted per month. (B) Results obtained by the rSP03B Sero-Strip are visualized per month and plotted according to the different categories of the ICT. The size of the plotted circle represents the proportion of dogs classified per category.

Cross-reactions with other hematophagous arthropods

The presence of specific anti-saliva IgG antibodies in the sera samples of mice and dogs experimentally exposed to different blood-feeding arthropods was verified by performing a
dot blot. Results are shown in Figure 3 (indicated by a (+) sign) and confirm the presence of specific IgG in all sera samples, hence rendering them useful for further cross-reactivity testing. Additionally, results from the negative control sera validate that there are no unspecific interactions between the respective salivary proteins of most arthropods tested and the IgG of non-bitten animals, the only exception being *I. ricinus* and *Cu. molestus* for which a weak background reaction is observed. However, this can be explained by the presence of Ig-binding proteins present in the saliva of ixodid ticks and mosquitoes [29,30]. Results of the negative control sera are indicated by a (-) sign in Figure 3.

In order to study possible cross-reactions between anti-*P. papatasi*, anti-*P. sergenti*, anti-*P. tobbi*, anti-*Cu. molestus*, anti-*C. felis* and anti-*I. ricinus* IgG and the salivary proteins of *P. perniciosus*, a western blot was performed with both the SGH of *P. perniciosus* and the rSP03B protein. The results are shown in Figure 4 and indicate that most sera samples do not interact with *P. perniciosus* salivary proteins located within the 15–80 kDa range. Only for mice experimentally exposed to *P. tobbi* a cross-reaction was observed between anti-*P. tobbi* IgG Abs and the tested salivary antigens from *P. perniciosus*

**Figure 3. Dot Blot.** Results of a dot blot to verify the presence of specific anti-saliva IgG in sera samples of animals experimentally bitten by different blood-feeding arthropods are shown (indicated by a (+) sign). The (-) sign indicates dot blots performed with sera samples from negative control sera.

**Figure 4. Western Blot.** Results of a Western Blot performed with the SGH and the rSP03B protein of *P. perniciosus*. The location of the SP03B protein is indicated with a star sign. (-), negative control sera; STD, Standard; AB, Amidoblack stain.
Validation with whole canine blood

All 30 spiked blood samples from experimentally exposed dogs appeared positive on the test together with 4 out of 29 spiked blood samples from non-bitten laboratory dogs, giving the test with whole canine blood a sensitivity of 100% (95% CI 85.87% - 100%) and a specificity of 86.21% (95% CI 67.43% – 95.49%). The ICC between the results of sera collected in different regions endemic for CanL and whole canine blood spiked with the same sera samples was calculated and equals 0.866 (95% CI 0.822 – 0.9; P-value: 6.56E-51).

Discussion

We present a significant improvement of the rSP03B Sero-Strip previously demonstrated to be a valid replacement for the standard SGH-ELISA to measure levels of specific anti-vector salivary antibodies during epidemiological studies [20]. In order to ascertain proper functionality of the test in field conditions we have addressed additional specifications of the ICT. First, the ICT was optimized using the rSP03B recombinant protein expressed in a human cell line. In order to do so we used the previously optimized protocol for high-density transfection of the suspension adapted HEK293S GnTI- cell line [22]. The succeeding rSP03B Sero-Strip rendered an increased specificity (94.92%) compared to the first proposed ICT while maintaining its high sensitivity value (100%). The increase in specificity of this optimized ICT is most likely due to the proper protein folding and post-translational modifications introduced by producing the protein in HEK293 cells.

In a second step, the inter-rater reliability and reproducibility of the ICT were evaluated by computing the ICC for both measurements. The obtained ICC values were evaluated according to the rules of Cicchetti et al [31]. When inter-rater reliability ICC values were considered, the results were in the excellent range indicating that different individuals rating or performing the ICT have a high degree of agreement. These results indicate that only a minimal amount of measurement error is introduced by independent raters. Consequently,
signal intensity ratings were considered to be suitable for use in the present study. Furthermore, when samples were run on different days a good degree of agreement was acquired according to the obtained ICC, assuring that also statistical power for sequential analyses performed on different time points was not substantially reduced.

In a third part of this study, we have made a first attempt to address the field detection accuracy of the optimized test by (a) screening serum samples from canine populations exposed to various frequencies of sand fly bites with both the optimized ICT and the SGH-ELISA and (b) testing possible cross-reactions with other hematophagous arthropods present across the Mediterranean area using sera of experimentally bitten hosts.

Since in our previous study we confirmed that the SGH-ELISA is the most reliable method to evaluate the extent to which dogs have been bitten by *P. perniciosus* – with sensitivity and specificity values equaling 100% and 98.11%, respectively [20] – we continued to use this method as the gold standard against which the rSP03B Sero-Strip is evaluated. Therefore, the results obtained by the SGH-ELISA were used to estimate the prevalence of bitten dogs per screened area. These results indicate that only four areas have a prevalence of bitten dogs (>30%) high enough in order to allow an optimal representation of the predictive values of the ICT in the field, as is indicated by higher PPVs for these areas. Results obtained by the ICT were translated into a proportion of bitten dogs and compared to the prevalence per region. The results indicate that samples classified in category (1) represent a grey zone, comprising both true positive dogs (in areas with a high prevalence) as well as negative dogs (in areas with a low prevalence). When excluding these samples from the analysis, all areas showed an agreement between the ICT and the SGH-ELISA (McNemar $\chi^2$) and for six out of eight areas a certain degree of agreement was observed (Cohen’s kappa). Interestingly, the degree of agreement according to Cohen’s kappa increases for areas with a low prevalence of bitten dogs, whereas it decreases for areas with a high prevalence of bitten
dogs. This can be explained by the high proportion of dogs classified in category (1). Furthermore, longitudinally collected canine sera samples showed a clear fluctuation for both the SGH-ELISA results and the ICT. For the SGH-ELISA, significant differences between months were observed from July until October in the first year of sampling and from March until July in the second year of sampling, suggesting that during these months the dogs experience an increased biting rate which is in line with the peak of sand fly abundance and has already been extensively discussed by Kostalova et al [14]. In the present study, however, we were able to relate these peaks in antibody response to higher signal intensities observed in the ICT represented by a higher proportion of dogs classified as being positive. A more pronounced fluctuation in signal intensities was observed when the grey zone – as explained before – was excluded from the analysis. Taken together, these results indicate that the high sensitivity and specificity of the ICT enable it to distinguish recent from past exposure to *P. perniciosus* and hence support its use in the field, particularly in areas with a high prevalence of bitten dogs. However, bearing in mind that for samples which show a weak background signal on the ICT, re-evaluation of the results by another method or the use of an automated strip reader is recommended (reviewed in [32]).

A second step in confirming the field detection accuracy of the optimized test was to evaluate possible cross-reactions between the anti-saliva antibodies of other bloodsucking arthropods and salivary proteins of *P. perniciosus*. It is believed that salivary proteins from phylogenetically closely related sand fly species will likely cross-react with each other, as has been described in previous studies [33,34]. Vectors of canine leishmaniasis co-occurring with *P. perniciosus* in the western part of Mediterranean Europe comprise *P. ariasi*, *P. perfiliewi* and *P. neglectus* [2], all being members of the *Larroussius* subgenus. Due to a lack of colonies of most of these sand fly species [35] testing their cross-reactivity is challenging. Therefore, in order to get an idea on these favorable cross-reactions *P. (Larroussius) tobbi*
was included in our study; a vector of *L. infantum* in the eastern part of the Mediterranean basin, of which the distribution does not overlap with *P. perniciosus* [36,37]. This study confirms the presence of cross-reactions between salivary proteins across members of the subgenus *Larroussius*, vectors of canine leishmaniasis. On the contrary, we confirmed the absence of unspecific interactions between *P. perniciosus* and other hematophagous arthropods, including two sand fly species – *P. papatasi* and *P. sergenti* – which are refractory to *Leishmania infantum* [38,39] thereby affirming the use of salivary antigens from *P. perniciosus* to detect canine vector exposure without any background signal caused by unspecific interactions with non-vectorial species.

The final step towards field optimization of the ICT comprised validation of the test with whole canine blood, for which minor adjustments were made to the ICT. Results showed that when the ICT was run with sera compared to whole canine blood spiked with the same sera an excellent ICC value was obtained, designating a high degree of agreement between the use of serum and whole canine blood.

In summary, we have successfully optimized the previously proposed rSP03B Sero-Strip by using the rSP03B protein expressed in a human cell line which delivered a higher specificity of the test. Additionally, signal intensity ratings were equal across performers as well as raters and the reproducibility of the ICT was high, proving that future use of the ICT will not be influenced by these factors. The ICT has been optimized for use with whole canine blood and results from the field assessment confirm its functionality in the field and even proved the presence of favorable cross-reactions with other sand fly vectors of canine leishmaniasis. Hence, this improved rS03B Sero-Strip represents a valuable tool in monitoring dog exposure to vectors of CanL and may contribute significantly to future control programmes.
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Figure 2

Seroprevalence of bitten dogs per month

Results rSP03B sero-strip: longitudinal sampling
Figure 3

- P. perniciosus
- P. papatasii
- P. sergenti
- P. tobbi
- I. ricinus
- C. felis
- Cu. molestus
Figure 4

- P. perniciosus
- P. papatasii
- P. sergenti
- P. tobbi
- I. ricinus
- C. felis
- Cu. molestus
5 SUMMARY AND CONCLUSIONS

This Ph.D. thesis was focused on improving the evaluation of vector control methods by using salivary proteins of phlebotomine sand flies. The thesis can be divided into three parts, starting with the validation of an exposure marker for *P. perniciosus* bites and concluding with the proposition of a new vector exposure tool – a rapid test unique in the field of vector exposure which is validated for fast evaluation of dog exposure to *P. perniciosus* in the field.

The first part of this Ph.D. was focused on the validation of the 43 kDa yellow-related recombinant protein (rSP03B) as an exposure marker to *P. perniciosus*, previously proposed as a highly antigenic salivary protein in studies on both laboratory- and naturally-exposed animals (dogs, mice, hares, and rabbits) [172,175,176]. These studies showed that the rSP03B protein from *P. perniciosus* is a valuable replacement for the use of whole saliva, indicated by high correlations between the Ab responses against both salivary Ags. Furthermore, a longitudinal study performed in Campania, Italy demonstrated that (a) the anti-SGH and anti-rSP03B IgG showed a comparable pattern of Ab kinetics and (b) that these kinetics were clearly seasonal, rising during summer and decreasing during the winter months. The results observed in this study were in accordance with the sand fly season in central and southern Italy, generally starting in late May and lasting until late October [229].

During this Ph.D. the rSP03B protein was further used to test canine sera samples collected cross-sectionally in CanL endemic settings in Italy and Portugal in order to validate the use of the protein across southwestern Europe. A substantial antigenic cross-reactivity between populations of *P. perniciosus* was observed. Furthermore, we also demonstrated that our recombinantly expressed SP03B possesses the same antigenic epitopes as its native form present in whole saliva, as was shown by performing an inhibition immunoblot with canine sera positive to *P. perniciosus*’ SGH. These data were summarized in Kostalova *et al.* (2017) published in *Medical and Veterinary Entomology*.

During a subsequent study, we evaluated the anti-*P. perniciosus* SGH and anti-rSP03B Ab response in a naturally exposed heterogenous dog population. Dogs living in 12 different locations of a *L. infantum* endemic area in north-eastern Spain were sampled for 14 months. This study showed that Ab responses against both salivary proteins followed the expected trend of *P. perniciosus* activity in the region with significantly lower IgG levels during the non-transmission season. Altogether, these results support the use of the Ab response against the rSP03B protein as a tool to investigate exposure to *P. perniciosus*, even in natural heterogeneous dog populations living in CanL endemic areas. The data of this study were summarized in Velez *et al.* (2018) published in *Parasites & Vectors*. 
The second part of my Ph.D. thesis was aimed at using this rSP03B yellow-related salivary protein from *P. perniciosus* to prepare a lateral flow immunochromatographic assay which would allow rapid evaluation of canine exposure to *P. perniciosus*. In order to do so, pre-immune sera from non-bitten laboratory bred Beagles and sera from laboratory-bred Beagles experimentally exposed to *P. perniciosus* bites were used as negative and positive controls, respectively. The immunochromatographic test (ICT) was prepared using the bacterially expressed recombinant protein rSP03B as Ag and optimized according to the control sera. The principle of the resulting rSP03B sero-strip is comparable to an indirect ELISA in the sense that canine Abs present in the sample bind to the *P. perniciosus* salivary Ag immobilized on the NC membrane of the ICT. The reaction is then visualized by an anti-dog IgG Ab gold-conjugate.

Final optimization of the ICT led to sensitivity and specificity values of 100% and 86.79%, respectively. Furthermore, when comparing three serological methods (SGH-ELISA, rSP03B-ELISA and the rSP03B sero-strip), the SGH-ELISA was highlighted as being the most sensitive (100%) and the most specific (98.11%) amongst all. Hence, the SGH-ELISA was appointed the golden standard for determining canine exposure to *P. perniciosus*. When performance values of the rSP03B-ELISA and the rSP03B sero-strip were compared the highest sensitivity was assigned to the rSP03B sero-strip (100% compared to 69% for the rSP03B-ELISA), whereas the highest specificity was obtained by the rSP03B-ELISA (98.11% compared to 86.79% for the rSP03B sero-strip). Overall these predictive values suggested that the rSP03B sero-strip performs better than the rSP03B-ELISA. This was confirmed when the ICT was compared to the SGH-ELISA, resulting in an almost perfect agreement between the two. Taken together, a simple, rapid, and easily operated colloidal gold ICT possessing high predictive values was developed to screen for dog exposure against *P. perniciosus* bites. Even more so, these results confirm the use of the rSP03B sero-strip as a replacement tool for the standard SGH-ELISA used in numerous previous studies [158,172,175,176]. These data were summarized in Willen et al. (2018) published in *PLoS Neglected Tropical Diseases*. However, the question remained if this ICT would also function in a similar fashion with dogs naturally exposed to various frequencies of sand fly bites.

Therefore, during the third part of this Ph.D. thesis I focused on confirming the field detection accuracy of the test, by not only screening canine samples collected from naturally exposed field dogs, but also evaluating potential cross-reactions with other bloodfeeding arthropods. Even more so, to improve the ICT for better field application efforts were made to improve the specificity of the ICT and to allow the utilization of whole canine blood. More specifically, the ICT was optimized using the rSP03B recombinant protein expressed in a human cell line for which the previously optimized protocol for high-density transfection of the suspension adapted HEK293S GnTI- cell line was used.
This resulted in an increased specificity (94.92%) compared to the first proposed ICT (86.79%), plausibly caused by proper protein folding and post-translational modifications introduced by the HEK293 cells expression system [231,232]. Furthermore, in order to ascertain that test functionality is not influenced by different individuals reading or performing the ICT, we evaluated the inter-rater/performer reliability and reproducibility of the ICT, resulting in an excellent agreement between individuals (raters or performers) and a good agreement between measurements obtained on different days. Similar results were obtained after validation of the test with whole canine blood, by spiking one canine blood sample with sera samples from different CanL endemic regions, revealing an excellent agreement between the use of serum and whole canine blood.

The most important part of this study was the evaluation of the field detectability of the ICT. This was achieved by (a) screening serum samples from canine populations exposed to various frequencies of sand fly bites and (b) testing possible cross-reactions with other hematophagous arthropods present across the Mediterranean area using sera of experimentally bitten hosts. The ICT-results were transformed into a proportion of bitten dogs per area (calculated as the amount of positive dogs divided by the total amount of dogs per area) and compared to the seroprevalence per region, calculated by the SGH-ELISA results. It is generally accepted that the prevalence in an area affects the functionality of a diagnostic test. Areas with a lower prevalence often do not support a good functionality of rapid tests as indicated by a low PPV of the test in these areas. This also reflects in the results of this ICT. That is to say, samples classified in category (1) represent a grey zone, comprising both true positive dogs (in areas with a high prevalence) as well as negative dogs (in areas with a low prevalence). Excluding these samples from the analysis gave optimal results, as is indicated by accordance between the ICT and the SGH-ELISA for all areas (McNemar χ²) and for six out of eight areas when the Cohen’s kappa was calculated. When screening longitudinally collected canine sera samples, higher signal intensities (and hence a higher proportion of bitten dogs) corresponded to the peaks in antibody response observed in the SGH-ELISA. Altogether, these results support the use of the ICT in naturally exposed field dogs since (1) it is able to distinguish recent from past exposure to *P. perniciosus* and (2) the ICT-results from most areas were in concordance with the standard SGH-ELISA. It has to be borne in mind, however, that for areas with a low seroprevalence of bitten dogs re-evaluation of the results by another method or the use of an automated strip reader is recommended (reviewed in [184]).

A last part to validate the field applicability of the test was the evaluation of potential cross-reactions between other hematophagous arthropods and salivary proteins of *P. perniciosus*. Results demonstrated the presence of cross-reactions between salivary proteins across members of the subgenus *Larroussius* and the absence of unspecific interactions between *P. perniciosus* and other
hematophagous arthropods, including two sand fly species – *P. papatasi* and *P. sergenti*, refractory to *L. infantum* [233,234]. These results support the idea of expanding the use of this ICT to a universal vector exposure test of CanL in the Mediterranean basin, which can be employed during large-scale epidemiological studies of CanL in the Mediterranean area to evaluate the efficacy of vector control programs. The data obtained during the third part of this Ph.D. thesis were summarized in a final manuscript (*Willen et al*).

In conclusion, we showed that the antibody response against the rSP03B protein is a valid marker of exposure to *P. perniciosus* in regions endemic for CanL and have exploited these results in the preparation of a rapid vector exposure test, the first in the field of vector biology. The resulting rSP03B sero-strip showed high sensitivity and specificity values, was proven to be compatible with whole canine blood, showed favorable cross-reactions with other vector species, and was validated for use on naturally exposed field dogs, especially in areas with a higher seroprevalence of bitten dogs. The test is now available to be used during dog exposure studies throughout the western part of the Mediterranean. It is evident that evaluating more canine samples from different CanL endemic areas in the future, perhaps together with the introduction of a strip reader, will enable proper implementation of this rapid test in the field. Furthermore, future studies will focus on the development of similar tests for human exposure, especially in areas where anthropoontic transmission prevails such as foci of VL in the Indian subcontinent caused by *L. donovani* or certain regions in East Africa where parasites of the *L. donovani* complex are transmitted by *P. orientalis*. Interestingly, suitable salivary Ags of *P. orientalis* have already been characterized in our laboratory, encouraging the preparation of such a rapid human vector exposure test.
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