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ANTI-*LEISHMANIA* ANTIBODIES SURVEY IN DOGS FROM PORTUGAL

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DISSERTATION TO OBTAIN THE MASTER'S DEGREE IN BIOMEDICAL SCIENCE, WITH A SPECIALIZATION IN MOLECULAR BIOLOGY IN TROPICAL AND INTERNATIONAL MEDICINE

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Resumo

Portugal é um país endémico para a leishmaniose canina (CanL), zoonose causada pelo parasita *Leishmania infantum*, em que o cão é o principal hospedeiro reservatório doméstico. Sendo uma zoonose, o controlo e monitorização da CanL é de grande relevância para a Saúde Pública humana e animal. Nas últimas décadas, têm sido realizados diversos estudos de prevalências da CanL, incidindo em diferentes regiões de Portugal. O último estudo de prevalência a nível nacional conta com mais de uma década, e com a introdução no mercado de novas medidas profiláticas, nomeadamente vacinas contra a CanL. O presente estudo teve como objectivos fazer uma atualização da seroprevalência de CanL em Portugal Continental face ao estudo anterior de 2009, bem como a determinação de fatores de risco associados à infecção por *L. infantum*.

Para o presente estudo, 98 Centros de Atendimento Medico-Veterinário (CAMV) de todo o país efetuaram colheitas de amostras de sangue em papel de filtro num total de 1860 amostras e de questionários sobre características dos cães e hábitos de vida. Foi efetuada a técnica serológica de Aglutinação Direta (DAT) para a pesquisa de anticorpos anti-*Leishmania* e efetuada a análise estatística das variáveis qualitativas e quantitativas face aos resultados da técnica serológica utilizando o programa IBM® SPSS® Statistics.

Observou-se uma seroprevalência global de 12.5% (CI 10.3 – 13.2%), que se traduz num aumento comparativamente ao estudo de 2009, no qual foi determinada uma seroprevalência de 6.31%. Os distritos com maior seroprevalência são Portalegre (30.5%, CI 19.9 – 43.8), Castelo Branco (29.9%, CI 20.1 – 42.0) e Guarda (19.3%, CI 9.6 – 35.1). A menor seroprevalência registou-se em Viana do Castelo (0.0%, CI 0.0 – 7.5).

Os fatores de risco identificados associados à infecção por *L. infantum* em cães foram estes terem idade superior a 2 anos (aOR = 2.14, CI 1.45 - 3.14) e residirem no interior de Portugal (aOR = 1.63, CI 0.91 – 1.72). Para cães que não tinham sido vacinados, o não uso de repelentes ou insecticidas foi também identificado como factor de risco (aOR = 1.74, CI 1.20 – 2.53). Uma vez que a técnica serológica utilizada – DAT - não permite a discriminação entre anticorpos vacinais e anticorpos resultantes de exposição ou infecção ao parasita *Leishmania*, uma especial atenção foi prestada à

vacinação, pois esta aparentava ser um fator de risco.

Quando comparados cães vacinados e não vacinados com resultado positivo e com sinais clínicos compatíveis com CanL, verificou-se que apenas 7% de cães vacinados positivos apresentavam sinais clínicos, comparativamente com 26% de cães não vacinados positivos, demonstrando a eficácia da vacinação na prevenção da progressão da doença.

O desenvolvimento de testes serológicos capazes de diferenciar entre anticorpos vacinais e de infeção será determinante para o melhoramento de estudos seroepidemiológicos de CanL. Igualmente o uso adequado de repelentes/inseticidas e vacinação devem ser privilegiados para reduzir a CanL. Globalmente, o presente estudo proporcionou informação atualizada referente à seroprevalência da infeção por *L. infantum* em Portugal.

Palavras-Chave: Leishmaniose Canina; Seroprevalência; Fatores de Risco; DAT; Portugal

Abstract

Portugal is endemic for canine leishmaniosis (CanL), a zoonosis caused by the *Leishmania infantum* parasite, for which dog is the main domestic host. The control and monitoring of CanL, as a zoonosis, is of great relevance for public and animal health. In the last few decades, several studies have been performed to determine CanL seroprevalence in various regions in Portugal. The last nationwide seroprevalence study was conducted over a decade ago (2009), and since then new prophylactic measures have been introduced, namely vaccines against CanL. The present study aimed to update seroprevalence of CanL in Mainland Portugal, compared to the 2009 study, as well as determine risk factors associated with *L. infantum* infection.

For the present study, 98 veterinary clinics throughout the country collected blood samples on filter paper, in a total of 1860 samples, and questionnaires regarding dog's characteristics and living habits. Direct Agglutination Test (DAT) was the chosen method for the anti-*Leishmania* antibody survey and a statistical analysis of quantitative and qualitative variables and serological results was performed using IBM® SPSS® Statistics.

A national seroprevalence of 12.5% (CI 10.3 – 13.2%) was determined, which translates into an increase in comparison with the 2009 study, in which a seroprevalence of 6.31% was determined. The districts with higher seroprevalence were Portalegre (30.5%, CI 19.9 – 43.8), Castelo Branco (29.9%, CI 20.1 – 42.0) and Guarda (19.3%, CI 9.6 – 35.1). The lowest seroprevalence was recorded in Viana do Castelo (0.0%, CI 0.0 – 7.5).

The risk factors associated with *L. infantum* infection in dogs were being 2 years old or older (aOR = 2.14, CI 1.45 - 3.14) and residing in the interior of Portugal (aOR = 1.63, CI 0.91 – 1.72). When vaccinated dogs were excluded from the analysis, the non-use of repellents/insecticides was also identified as a risk factor (aOR = 1.74, CI 1.20 – 2.53). Since the serological technique used – DAT – does not discriminate between vaccinal antibodies and antibodies resulting from infection or exposure to *Leishmania* parasite, this variable was further analyzed, as it was an apparent risk factor. When

comparing vaccinated and unvaccinated dogs that had a positive result and presented with compatible clinical signs for the disease, only 7% of vaccinated positive dogs presented clinical signs, compared to 26% of unvaccinated positive dogs, demonstrating the efficiency of vaccination in disease progression prevention.

The development of serological tests capable of differentiating between vaccinal and infection antibodies is determinant for the improvement of CanL seroepidemiological studies. Moreover, the correct use of effective repellents/insecticides and vaccination should be a strategy to reduce CanL. Overall, the present study provided updated information regarding the seroprevalence of *L. infantum* infection in Portugal.

Keywords: Canine Leishmaniosis; Seroprevalence; Risk Factors; DAT; Portugal.

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List of Abbreviations

aOR – Adjusted Odds Ratio

CanL – Canine Leishmaniosis

CI – Confidence Interval

CKD – Chronic Kidney Disease

CL – Cutaneous Leishmaniasis

DAT – Direct Agglutination Test

DGAV – Direção-Geral de Alimentação e Veterinária

DNA – Deoxyribonucleic Acid

ELISA – Enzyme-Linked Immunosorbent Assay

FelL – Feline Leishmaniosis

HIV – Human Immunodeficiency Virus

IFA – Indirect Fluorescent Assay

IFAT – Immunoflourescence Antibody Test

IRIS – International Renal Interest Society

LST – Leishmanin Skin Test

ML – Mucocutaneous Leishmaniasis

NNN – Novy-MacNeal-Nicolle

OIE – World Organization for Animal Health

OR – Odds Ratio

ORBEA-IHMT – Orgão Responsável pelo Bem Estar Animal – Instituto de Higiene e Medicina Tropical

PCR – Polymerase Chain Reaction

PKDL – Post Kala-Azar Dermal Leishmaniasis

qPCR – Quantitative Polimerase Chain Reaction

SIAC – Sistema de Informação de Animais de Companhia

VL – Visceral Leishmaniasis

WHO – World Health Organization

Introduction

1. Origin of Leishmaniasis

Leishmaniasis is a vector-borne parasitic disease, caused by the protozoa parasite of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae). Currently, there are over 20 species capable of causing disease in humans. It is transmitted by the bite of phlebotomine sand flies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World to a variety of mammal hosts (WHO, 2021). Majority of leishmaniases are zoonoses and various animals are responsible for *Leishmania*'s long-term survival in nature.

Two fossil ambers from prehistoric times have shown the presence of *Leishmania*-like organisms within a blood-filled female of the extinct sand fly *Palaeomyia burmitis* on a 100 million-year-old Cretaceous Burmese (Steverding, 2017). Therefore, it is believed that the *Leishmania* genus likely evolved in the Mesozoic era, however there are different hypotheses regarding the geographical origin of *Leishmania*.

The Palaearctic hypothesis suggests that the genus originated in the Palaearctic region, consisting of Europe, part of Asia, Northern Africa and Arabia; the Neotropical hypothesis indicates that due to the larger diversity of New World *Leishmania* compared to Old World, it should have been originated in the Neotropical region. Lastly there is the Supercontinent hypothesis, in which it is suggested that due to the breakup of the supercontinent Gondwana, different subgenera evolved in Africa and in South America (Steverding, 2017).

Although considered an ancient disease, it is unknown when leishmaniasis in humans was first identified. Several descriptions of sores from as early as the 16th century in the Middle East have been identified as possible Leishmaniasis. William Leishman and Charles Donovan first identified the parasite that causes visceral Leishmaniasis or kala-azar in the early 20th century, independently, through the identification of parasites in splenic pulp of sick soldiers and native Indians, respectively. After much discussion regarding the newly found parasites, initially thought to be trypanosomes by Leishman, in 1904 the term *Leishmania donovani* was adopted for the species in honor to these to doctors (Gibson, 1983; Steverding, 2017).

2. Epidemiology of Leishmaniasis

Leishmaniasis is a tropical neglected disease, endemic in the Americas, Southeast Asia, East Africa and the Mediterranean Region, although some sporadic cases of human Leishmaniasis are found in other areas all around the globe. A total of 98 countries, spanning 5 continents, have reported cases of Human Leishmaniasis, the majority of which are Cutaneous Leishmaniasis cases (Alvar et al., 2012). According to the World Health Organization (WHO), between 700 000 and 1 million new human cases occur every year. Human leishmaniasis is more prevalent in developing countries, where living conditions are not ideal, and these often promote the growth of the transmission vector and more compromised immune systems due to poor sanitary living conditions. Although it is a widespread disease, just 6 countries account for over 90% of all cases of human leishmaniasis: Brazil, India, Bangladesh, Ethiopia, Sudan and South Sudan (WHO, 2021).

In developed countries, cases are less common and often associated with a compromised immune system. According to Cipriano et al., (2017), visceral leishmaniasis (VL), is the third most common opportunistic parasitic infection in European HIV patients, which poses treatment difficulties for these individuals. In Portugal, the most common species is *L. infantum* (Campino & Maia, 2010), with very rare cases of infections with other species of *Leishmania* from imported cases. Moreover, in Portugal, VL is a compulsory notification disease, with 30 confirmed cases between 2013 and 2016, according to Direção-Geral da Saúde (DGS).

2.1. Visceral Leishmaniasis

Visceral leishmaniasis (VL), which can be fatal, if untreated, is also known as Kala-Azar disease, and etiological agents are *L. donovani* and *L. infantum*. An array of symptoms characterizes the disease, such as fevers, enlarged organs, due to their inflammation, such as the liver, spleen and lymph nodes, mostly found in Brazil, India and the African continent (Figure 1). There are several risk factors for disease progression such as other concomitant infections (HIV), malnutrition and genetic factors. Post Kala-Azar Dermal Leishmaniasis (PKDL) is a complication of VL, in which the patient develops a macular or papulonodular rash months or years after

having VL. This condition is not fatal and can often resolve itself without medication. (WHO, 2012, 2021; Boelaert & Sundar, 2014;).

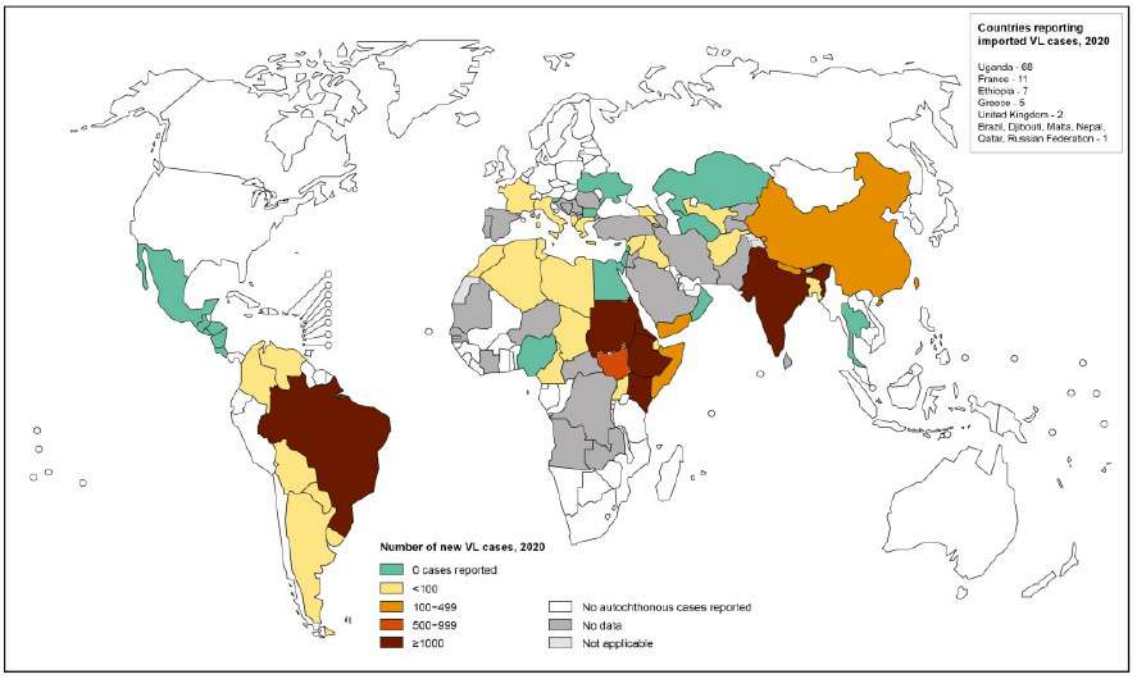

Figure 1 – Status of endemicity of visceral leishmaniasis worldwide (Source: WHO)

2.2. Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form of the disease in humans (WHO, 2021), and mostly found in Asia, South America and the Middle East (Figure 2). It is caused by several species, including *L. major* and *L. tropica* in the Old World and well as *L. braziliensis*, *L. guyanensis, L. peruviana, and L. mexicana* in the New World (Boelaert & Sundar, 2014). Symptoms include skin ulcers and/or nodules that often leave scars. In most cases it heals spontaneously. Not so frequently, *L. infantum* is also responsible for cutaneous leishmaniasis in the Mediterranean region.

2.3. Mucocutaneous Leishmaniasis

Mucocutaneous Leishmaniasis (ML) is caused mostly by *L. brasiliensis* and less frequently *L. panamensis*, and causes the partial or total destruction of mucous membranes in the mouth, nose and throat. Due to the aggressive nature of the lesions, this form of Leishmaniasis can be very disfiguring and lead to social stigma and/or permanent disabilities, and is most found in Bolivia, Brazil, Ethiopia and Peru. (WHO,

2021; Boelaert & Sundar, 2014).

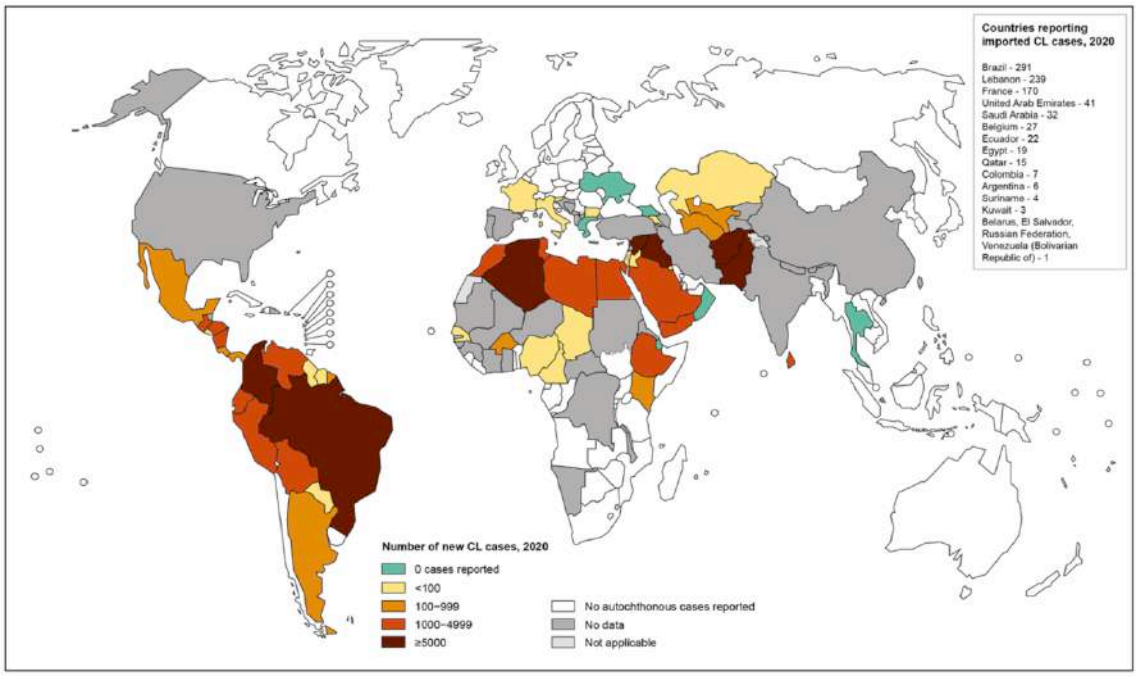

Figure 2 – Status of endemicity of cutaneous leishmaniasis worldwide (Source: WHO, 2020)

3. The Parasite *Leishmania* sp. and it's Life Cycle

3.1 The Parasite

Leishmania is a protozoan parasite that has two morphological forms during its life cycle:

i) An intracellular non-flagellated form, the amastigote, which develops inside vertebrate of the mononuclear phagocytic host cells.

ii) An extracellular flagellated form, the promastigote, which develops inside the digestive tube of the invertebrate host, the phlebotomine vector.

The promastigote has a slender shape with 15-26 µm in length and 2-3 µm in width (Figure 3). The protozoan cell has a single nucleus and a free flagellum, which allows it to move. It multiplies through longitudinal binary fission inside the sand fly gut at a temperature of 22 – 26 ºC, it also has a unique subcellular structure with mitochondrial DNA, known as the kinetoplast, a characteristic of all tripanosomatids (Pearson & Sousa, 1996).

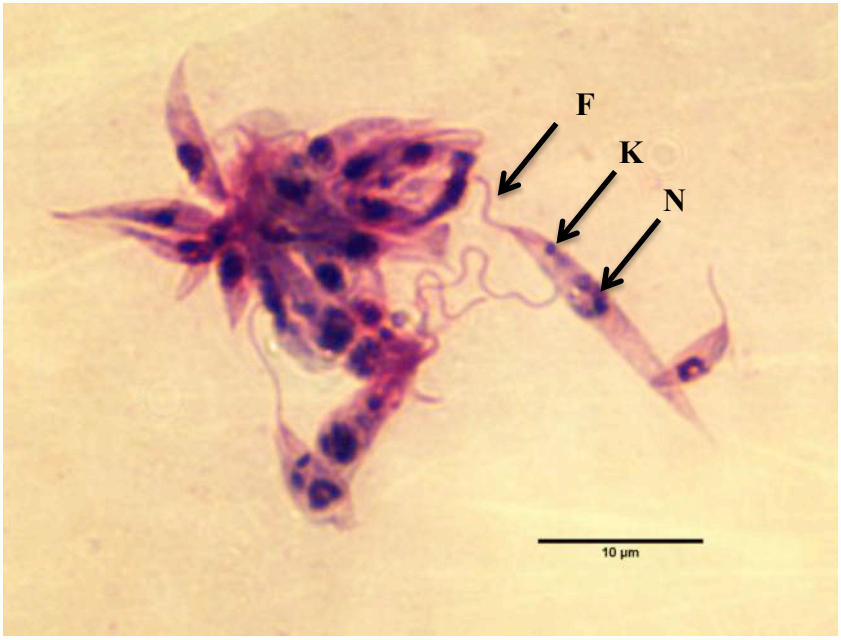

Figure 3 – *Leishmania infantum* promastigotes. N - nucleus; K - kinetoplast; F - flagellum (Amplification: 1000x). Maria Almeida, 06-10-2021.

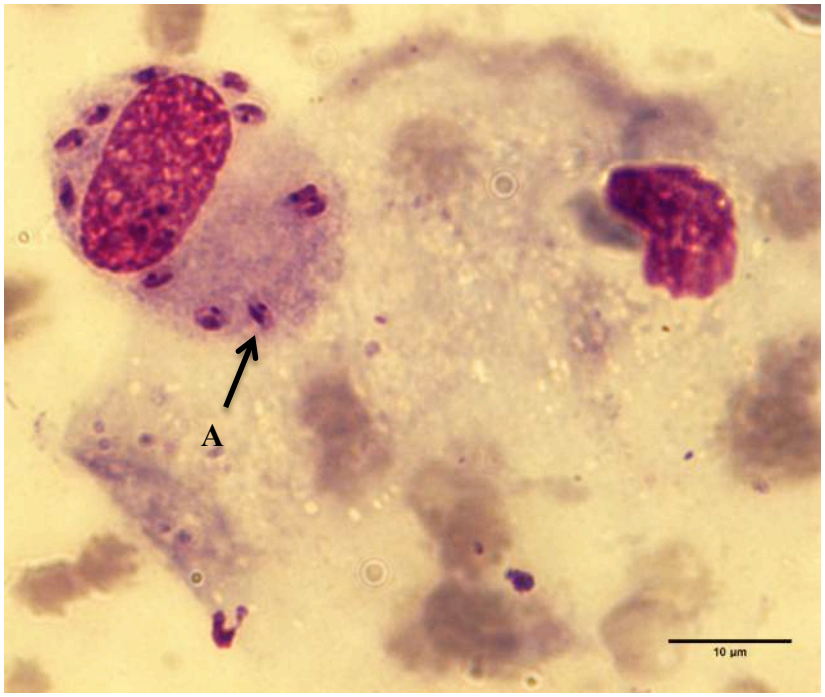

Figure 4 – *Leishmania infantum* amastigotes inside the macrophage. A – amastigote. (Amplification: 1000x). Maria Almeida, 06-10-2021.

Amastigotes are smaller, with a round, oval shape, 2-3 µm in diameter, and are obligate intracellular forms with tropism to macrophages of the vertebrate host where

they multiply by binary division (Figure 4). They have a central nucleus and kinetoplast with an internal flagellum. Amastigotes are adapted to the host body temperature, at around 36º C, as well as the acidic environment inside the macrophage they normally infect (Pearson & Sousa, 1996).

3.2. Life Cycle

The *Leishmania* parasite enters the vertebrate host through the skin, after a blood meal from an infected sand fly, which lacerates blood vessels and regurgitates infective promastigotes (metacyclics). Promastigotes enter the blood stream, where an immune response is triggered and macrophages and other cells, such as neutrophils and dendritic cells, are called to site. The promastigotes are then phagocyted and inside the macrophages, they migrate to the phagolysosome, where they differentiate into non-flagellated amastigotes. These cells then rupture, allowing for the infection of other nearby cells (Liu & Uzonna, 2012). Amastigotes released into the mammalian host will infect other macrophages, increasing the number of infected cells and expanding the infection. The infected macrophages circulating in the peripheral blood may be then ingested by another sand fly, allowing the life cycle to continue (Figure 5).

Once inside the macrophage, the *Leishmania* parasite is able to regulate the phagolysosome, through several more or less studied mechanisms, such as the class I nucleases (Freitas-Mesquita & Meyer-Fernandes, 2021), therefore compromising macrophage defense mechanisms, such as apoptosis, oxidative damage and antigen presentation, prolonging its' life inside the macrophage (Podinovskaia & Descoteux, 2015). When the intracellular development of amastigotes remains localized to the inoculation site, several cytokines are released and cellular reactions are generated, resulting in the development of a localized CL lesion. In other cases, the parasites spread to the organs of the mononuclear phagocytic system, giving rise to VL.

Although the *Leishmania* parasite has a simple life cycle, the relationships between the parasite and the host/reservoir or the vector are complex. Several different factors may affect transmission and infection rates, such as the possible effects of climate change, and genetic and immune factors may affect the parasites proliferation inside the host/reservoir.

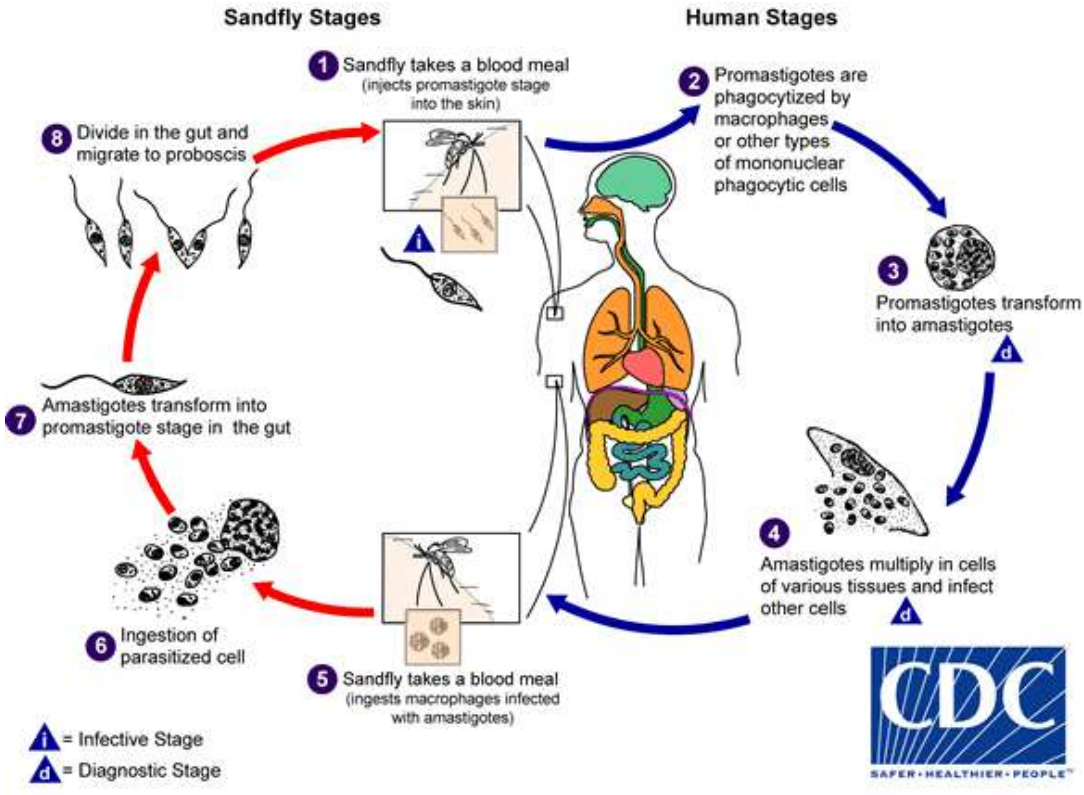

Figure 5 – *Leishmania* sp. life cycle (Source: CDC, 2020).

3.2.1. Vector

Sand flies are small insects, with blade-shaped hairy wings. In order for the female sandflies' eggs to have enough nutrients mature, a blood meal is required. Sugars are used as food for males and female sand flies. Sugars are also required for *Leishmania* to complete development in sandflies and produce the infectious metacyclic promastigote form (Almeida et al., 2003). Only female sand flies take a blood meal from an available vertebrate in order to complete egg maturation (Figure 6). When feeding, sand flies deposit a salivary mix in the vertebrate host, that among acts an anti-hemostatic (Sumova et al., 2020).

Sand flies prefer to rest during the day in dark places like cracks, henneries, rabbit hutches, wall holes, and become active at twilight and at night with some humidity.

Phlebotomus sp. is the proven vector in the Old World and *Lutzomyia* sp. is the vector in the New World (Maia & Campino, 2018).

Figure 6 – Female phlebotomine specimen – Maria Almeida, 02-12-2019

In Portugal, the main circulating phlebotomine species are *P. perniciosus* and *P. ariasi*, and proven vector of *L. infantum* and less frequently *P. papatasi* and *P. sargenti* (Campino et al., 2006; Branco et al., 2012). *P. perniciosus* and *P. ariasi* not only are the most common species of sand fly in Western Europe, but they are also the main transmission vectors of *Leishmania* in the Old World (Maia & Campino, 2018; Alten et al., 2016)

In a study performed in the Mediterranean region, it has been found that in this region, including in Portugal, *P. perniciosus,* is active from May until October, with some minimal activity recorded in November (Alten et al. 2016).

This dynamic may be changing however, as global warming is slowly changing climate around the world. In Kholoud et al. (2018), Morocco observed an increase in CL from 2028 annual cases in 2001 to 3414 annual cases in 2008. Moreover, it was observed that more territories were at ideal (warmer) conditions due to the increase in the average temperature for longer annual periods, leading to an increase in the phlebotomine population. Fischer et al., (2011) showed that the European climate may become increasingly more adequate for sand flies in northern regions, with possible implications in leishmaniasis epidemiology. However, a large spread of sand flies is unlikely due to their limited natural dispersal ability.

3.2.2 Vertebrate Host

The *Leishmania* parasite is known to infect several animal species, among which are humans, dogs, cats, rats and horses. These hosts have been studied and some, such as cats and hares, have been proven as sources of *L. infantum* for phlebotomine species in the Old World (Cardoso et al., 2021). In Alcover et al. (2020), wild animals in Catalonia, Spain, have been found to play a significant role as potential reservoirs of *L. infantum*, and surprisingly red squirrels presented with a significant prevalence rate.

In the case of zoonotic leishmaniasis caused by *L. infantum* in the Old World, the dog is considered the main reservoir host. A reservoir host is defined as an animal in which an infectious agent survives persistently and is therefore a main source of infection for the transmission vector. Additionally, a good reservoir host for leishmaniasis should be in close contact frequently with humans as well as the transmission vector whilst also be susceptible to infection but survive the chronic evolution of the disease until the next transmission season (Maia & Campino, 2018).

In case of infection, the susceptibility to the development of symptoms may be genetic, environmental or affected by other factors. In the last few decades, an increasing number of infected cats have also started to present clinical signs and disease, and therefore *Leishmania* infection in cats has deserved increased attention, as well as the role of cats as reservoirs.

Feline leishmaniosis (FelL) was first described in 1912 in Algeria. Different studies of *Leishmania* infection in cats have been published and the role of cats in the transmission of *Leishmania* parasites has been argued whether to consider cats primary hosts or secondary hosts for human infection caused by *L. infantum* in endemic regions (Maia & Campino, 2018; Fernandez-Gallego et al., 2020). Leishmaniosis may be an opportunistic infection in cats, since immunocompromised cats, or those who present with other health conditions, seem to be more prone to developing symptoms (Fernandez-Gallego et al., 2020). Regarding the prevalence of FelL worldwide a few studies have been done, varying between 0% and 60.7% in endemic regions in the Old World. This discrepancy is largely due to methodology differences as well as population and endemicity differences (Pennisi et al., 2015).

Regarding seroprevalence of FelL in Portugal, several studies have been done, with seroprevalences ranging from 0,3% to 9.9% (Maia & Campino, 2014). A few other

studies observed up to 30.4% of cats with *L.infantum* DNA in their peripheral blood (Maia et al. 2014; Vilhena et al., 2013).

4. Leishmaniasis Diagnosis

Diagnostic methods are similar for Human Leishmaniasis and Canine Leishmaniosis (CanL). This consists in the evaluation of clinical signs as well as sample analysis with parasitological, serological and molecular techniques.

4.1. Parasitological Methods

The classic method for the parasitological diagnosis of *Leishmania* consists in detection of parasite in cells from the mononuclear phagocytic system and in skin lesions with high specificity. Although the spleen and liver are the most affected organs in VL, bone marrow biopsy is most often used because it is of easier access and less invasive for the patient. In dogs, this search is often carried out on lymph node aspirates. Direct examination is carried out through observation of slides with Giemsa stained smears under an optical microscope (Kassi et al., 2004).

This allows for the direct identification of the parasite, but requires some expertise, as amastigotes may be difficult to identify or the number of amastigotes be too small for detecting the presence of the parasite, even with a long and careful observation, compromising its sensitivity (Solano-Gallego et al., 2011).

Culture of biological samples in Novy-MacNeal-Nicolle (NNN) medium in an incubator at 22-26ºC allows for detection of promastigotes under the microscope. NNN cultures present higher sensitivity than direct observation of stained slides. However, the definitive diagnosis may take until five weeks (Maia & Campino, 2008).

4.2. Serological Methods

Serological methods determine the presence of antibodies and are performed using blood samples. Although indicative of contact with *Leishmania* parasite, these methods do not confirm the presence of the parasite. They detect specific anti-*Leishmania* antibodies, therefore it is advisable to combine with other tests, such as molecular and/or cytology to confirm the presence of the parasite (Solano-Gallego et al., 2011).

Introduction

Immunofluorescence antibody test (IFAT) is considered the gold standard for the serodiagnosis of human leishmaniasis and CanL. IFAT is a semi-quantitative method and has shown a sensitivity and specificity up to 80.3% and 90.5%, respectively. Cross-reactions have been observed with *Trypanossoma cruzi*, the etiological agent for Chagas disease (revised in Thakur et al., 2020).

Enzyme-linked immunosorbent assay (ELISA), is a quantitative technique with high sensitivity and specificity, which may be as high as 100% each depending on the antigen used, to detect total or specific anti-*Leishmania* antibodies (Maia & Campino, 2008). In ELISA, the antigen may be whole amastigotes or promastigotes, whole extracts from amastigotes or promastigotes, purified proteins and recombinant proteins (Solano-Gallego et al., 2014). A few cross-reactions have been reported, namely with *Trypanossoma spp*. and *Babesia canis* (Krawczak et al., 2015).

Although an expensive test, Western Blotting has been shown to be one of the most sensitive serological techniques for the diagnosis of Leishmaniasis, with a sensitivity of up to 100% and specificity of 90.8% (Ashrafmansouri et al., 2015; Kalaurachchi et al., 2019)

Direct Agglutination Test (DAT) was developed in the 70's (Vattuone and Yanovsky, 1971) and further optimized for diagnosis of VL in the 80's (El Harith et al., 1988). Oskam et al. (1996), use for the first time DAT for the detection of anti-*Leishmania* antibodies in dogs, and proven to be a highly sensitive and specific technique.

The antigen used in DAT is mostly prepared as freeze-dried *L. donovani* promastigotes. Freeze-dried antigens are more stable than liquid antigens whilst remaining fully active (Meredith et al., 1995).

DAT requires no expensive/complex equipment and is an easy technique to perform. For CanL, DAT has been shown to have 100% sensitivity and 98.9% specificity (OIE, 2021). DAT also requires samples (blood) that are obtained with minimally invasive methods, such as venipuncture and even a few drops of blood can be spotted on filter paper and further eluted for DAT analysis (Cortes et al., 2012; Maia et al., 2016). Thus, DAT may be a good choice of technique for field studies (Mohebali et al., 2020).

Fast Agglutination Test (FAST) is based on DAT, and allows for the detection

of anti-*Leishmania* antibodies in samples in 3 hours, as opposed to 18-24 hours for a regular DAT. However when compared to DAT, FAST has a lower specificity and higher sensitivity, and a general high level of agreement of results (Schallig et al., 2002), allowing for faster screening of samples.

Rapid immunochromatographic tests may be performed as an indicative of infection or for monitoring therapeutic response, but not as a diagnostic test (Aronson et al., 2017). These however present much lower sensitivities, and therefore may produce more false-negatives. These typically come as dipsticks or strip-tests, using rK39 as an antigen. rK39 is a recombinant protein derived from *L. infantum*, with a repeating chain of 39 amino acids (Baneth et al., 2017).

4.3. Molecular Methods

"Polymerase Chain Reaction" (PCR) is the technique, which enables the detection and identification of the parasite's DNA in a wide variety of clinical samples, such as skin biopsies, bone marrow aspirates, lymph nodes and peripheral blood. Different *Leishmania* spp. molecular targets and protocols have been used with varying performances (Van der Auwera et al., 2016; Albuquerque et al., 2017). Although more sensitive than conventional PCR, nested-PCR is more time consuming and may be less specific due to the increased risk of contamination (Fisa et al., 2001).

Concerning dog samples, blood, buffy coat and urine samples are less sensitive samples to detect the parasite DNA (Solano-Gallego et al, 2017). Real Time PCR is the most sensitive technique, as it allows for a quantitative analysis (Solano-Gallego et al., 2011).

Some studies have showed that Loop-Mediated Isothermal Amplification (LAMP) has the potential to be used as a molecular technique in the diagnosis of Leishmaniasis, particularly in areas with fewer resources, as it requires less equipment, but still has a high sensitivity and specificity (Nzelu et al., 2019).

5. Canine Leishmaniosis

There are several species of *Leishmania* that can cause disease to dogs, but by far the most frequent is *L. infantum*. As a reservoir, their role in the transmission of

species other than *L. infantum* is unknown, and most likely negligible, although other species such as *L. arabica* and *L. donovani* have been isolated from dogs (Maia & Campino, 2018). In South America dogs were also found infected with *L. brazilliensis* and *L. panamensis* (Vélez et al., 2012; Peterson, 2020). Santos et al. (2020) reported the first case of a dog infected with *L. guyanensis*.

Dogs are the main domestic reservoirs of *L. infantum*. It has been documented that although up to 50% of infected dogs are asymptomatic (Ribeiro et al., 2018) they are still infectious to phlebotomine sand flies, enabling the parasite's life cycle to continue (Maia & Campino, 2018), and therefore allowing other animals, and humans, to be infected. This, as well as the fact that dogs and humans have a close relationship, often living in the same home, makes CanL a Public Health concern. In Ethiopia, a region endemic for both CanL and Human Leishmaniasis, it was observed dog owners had an increased risk of testing positive (Bejano et al., 2021). Although studies indicate a link between the prevalence of CanL and Human Leishmaniasis, this does not seem to apply in every endemic region.

Also endemic in the Mediterranean region, CanL has a much higher incidence than its human counterpart in this region, meaning Human Leishmaniasis is hypoendemic (Campino & Maia, 2010; Maia & Campino, 2018). Several studies have been performed in recent years for the determination of the prevalence of CanL in the Mediterranean Region. In the Lazio region of Italy, a true seroprevalence of 6.7% was determined (Rombolà et al., 2021), through IFAT, whereas in Spain, the seroprevalence of CanL was found to vary between 3.3% and 57.1% (Galvéz et al., 2020).

Canine Leishmaniosis is largely endemic in two major regions: South and Central America and the Mediterranean basin, spanning approximately 50 countries (Maia & Campino, 2018).

Occasionally CanL cases occur in non-endemic regions, but most are imported cases. Between 2005 and 2007, 257 cases of CanL were diagnosed in the UK, with 15% being imported cases and another 15% being rescue dogs (Shaw et al., 2009). CanL is considered by some authors to also be a neglected disease; largely due to the fact most cases occur in rural settings, where veterinary services are often lacking (Sasani et al., 2016).

CanL is endemic in Portugal and prevalence seems to vary throughout the years.

In the 80's, 3 endemic foci were identified Alto Douro, Lisbon and Algarve region (revised in Campino & Maia, 2010). Several studies performed in the end of the 90s and in the beginning of the 21st century showed that CanL it is not restricted to 3 main foci but occurs all over the country (Maia & Campino, 2014).

In 2007 a seroprevalence of 19.2% was found in the urban area of Lisbon among stray dogs (Cortes et al., 2007). Later, a national survey identified a seroprevalence for *Leishmania* infection, of 6.31%, using DAT (Cortes et al., 2012), using Direct Agglutination Test. In the same study, the seroprevalence was higher in the Interior of the country, with significant differences between different districts where the animals lived. The highest seroprevalences were found in Castelo Branco, Portalegre and Beja, with 17.40%, 12.54% and 12.12% true seroprevalence respectively, and more recent studies have found that this prevalence may reach as high as 56.0% in certain regions, such as Castelo Branco (Pires et al., 2019). The main circulating species in Portugal is *L. infantum* (Maia & Campino, 2014), with other species rarely being detected, such as *L. major* (Campino et al., 2013).

Studies determining the presence of *Leishmania* DNA in Portugal have also been performed. In recent studies, a prevalence of 69% was found, with qPCR, in South Portugal (Maia et al., 2016).

In CanL there is a wide range of clinical signs, and these include cutaneous alterations, such as alopecia, onychogryphosis, dermatitis, ulcers (Figure 7) and other skin lesions as well as ocular alterations, such as conjunctivitis. Other common manifestations include fever and vomiting, enlarged lymph nodes, weight loss, limping and epistaxis (Solano-Gallego et al., 2011).

Biochemical alterations are also detected in haemogram and protein analysis: anaemia, thrombocytopenia and proteinuria. Although uncommon, neurological alterations and arthritis can also occur (Ribeiro et al., 2018).

Figure 7 – Ulcer on a dog formed due to a *Leishmania* infection. (Source: Vélez et al. 2012)

Resistance to *Leishmania* infection in dogs is determined by several different factors, but not much extensive research has been done in determining genetic resistance factors, being that only two genes have been identified in the susceptibility/resistance to a *Leishmania* infection (Maia & Campino, 2018).

Susceptibility may depend on the breed. Some breeds, like the German Shepherd and Boxer, have been found to be more susceptible to developing CanL due to genetic factors related to immune response, and other breeds, like the Ibizan Hound, which is native to an endemic region (Spain), are more resistant (Ribeiro et al., 2018; Vasconcelos et al., 2019). This suggests that autochthonous breeds of endemic regions may be more resistant to infection.

5.1. Treatment of Canine Leishmaniosis

Miltefosine is a drug with oral administration that inhibits the production of a key receptor for *Leishmania* intracellular survival, and can also induce apoptosis. Miltefosine has been marketed in Europe as Milteforan since 2007 originally for treatment of VL and later for the treatment of CanL (dos Santos Nogueira et al., 2019).

Allupurinol is the major first line drug in the treatment of CanL. It is a purine analog that can be used alone or in combination with miltefosine or meglumine antimoniate to treat CanL. It impairs protein synthesis in parasites, but long-term use has side effects in mammals, such as xanthine urolithiasis, or renal stone formation.
Meglumine Antimoniate (Sykes & Papich, 2014).

Meglumine Antimoniate is a pentavalent antimonial drug that has parasiticidal activity, as is increases phagocytosis (Manna et al., 2015), but its precise mechanism of

action is unknown. In Europe, administering meglumine antimoniate in combination with allupurinol is considered the first line of treatment in CanL treatment (Santos et al., 2019). It may cause pain, inflammation and potential nephrotoxicity is a known side effect (Solano-Gallego et al., 2017)

Solano-Gallego et al. (2017) defined four stages of the disease, increasing in severity and based on clinical manifestations, biochemical alterations and serological tests. Treatment of CanL strongly depends on the stage of the disease. Domperidone is an immunostimulant which activates macrophages and cell immunity, therefore helping to prevent and control the multiplication of Leishmania parasites. The main potential side effect is galactorrhea (Maia & Campino, 2018; Travi & Miró, 2018).

In stage I the most common approach is to monitor the development of the disease, or administering an immunostimulant, such as domperidone (Solano-Gallego et al., 2011).

In stages II, III and IV treatment consists of a combination of allopurinol and meglumine antimoniate or miltefosine for different periods of time depending on the severity or improvement of the animal. Studies have shown that the combined use of meglumine antimoniate and allopurinol, in the long term, may be effective at preventing a relapse (Maia & Campino, 2018).

In addition to these, in stages III and IV, treatment of Chronic Kidney Disease (CKD) should be administered according to IRIS recommendations (Solano-Gallego et al., 2011, 2017; Elliot & Watson, 2015).

5.2. Preventative Measures

Several preventative measures have been developed for the prevention of Canine Leishmaniosis. These include repellent collars, spot-on formulations and vaccines, which were developed in more recent years and should be considered into a multimodal approach for Canine Leishmaniosis prevention (Solano-Gallego et al., 2017).

5.2.1. Impregnated Collars

Several studies have proved the efficiency of collars in the prevention of Canine Leishmaniosis, of which the most efficient can last up to 8 months (Solano-Gallego et al., 2017). There are two types of impregnated collars: deltamethrin collars, such as

Scalibor®, which has a repellent effect that peaks at 2 weeks and may last up to 8 months, and flumethrin and imidacloprid collars such as Seresto®, which last between 5-6 months, but information is lacking regarding contact repellency (Solano-Gallego et al., 2017; Miró et al., 2017).

5.2.2. Spot-on Formulations

These formulations have similar active compounds as collars, but shorter lasting effects, normally 3-4 weeks (Solano-Gallego et al., 2017). Many are formulated with combinations of active compounds. For instance, permethrin 50% and imidacloprid 10% have been shown to act synergistically to repel sandflies. Other combinations may include fipronil, indoxacarb, dinotefuran and pyriproxyfen (Miró et al., 2017).

5.2.3. Vaccination

In recent years four vaccines have been developed. In Brazil two vaccines are licensed for use: Leishmune® (Zoetis) and Leish-Tec® (Hertape Calier Saúde Animal)(Solano-Gallego et al., 2017). Leishmune® was launched in 2004, but due to non-compliance in phase III tests, it's commercialization was suspended in 2014. Leish-Tec® was launched in 2008 and shows a 71.8% efficacy (revised in Maia & Campino, 2018).

In Europe two other vaccines are licensed for use: Canileish® and Letifend®. Canileish® was launched in 2011. It consists of purified extracted-secreted proteins of *L.infantum* with a saponin coadjuvant. It has shown an efficacy of 68% (Maia & Campino, 2018). The Canileish® vaccine produces antibodies, which are detectable in ELISA and IFAT (Solano-Gallego et al., 2017). One month after vaccination, 74.1% of dogs would be classified positive in ELISA testing, and antibodies are detectable in 3.2% of dogs 1 year after vaccination (Veléz et al., 2020).

Letifend® was released in 2017. It consists of a recombinant protein Q from *L.infantum* with no coadjuvant (Miró et al., 2017). This vaccine induces the production of anti-QP IgG2 antibodies, however studies have not shown the detection of vaccine antibodies in commonly used serological tests, such as ELISA and IFAT (Solano-Gallego et al., 2017).

The development of vaccines has challenged the diagnostic methods for Canine Leishmaniosis. Since some vaccines produce antibodies, current serological methods,

such as IFAT, ELISA and DAT are unable to distinguish between infection antibodies and vaccine antibodies. This poses the need for the development of more specific diagnostic techniques, and a more comprehensive diagnosis procedure (Solano-Gallego et al., 2017).

5.2.4. Other Prophylactic Measures

Sprays are also used, but have shorter active time frames, of 1-2 weeks. Some natural compounds, such as lavender, are also known natural insect repellents, but have very short active time frames. Neem oil and lavander have shown a 7-hour protection period (Miró et al., 2017). Some studies have shown that use of these preventative measures may be part of the strategy for the control of CanL. In Brazil, prolonged use of deltamethrin-impregnated collars has proved to be an effective strategy in Canine Leishmaniosis prevention (Coura-Vital et al., 2018).

The key to control the disease in dogs, as well as reducing the threat to Public Health, lies therefore in preventative measures, by the use of prophylaxis and the vaccination of individuals, the early detection and monitoring of cases as well as monitoring the disease nation and worldwide.

6. Objectives of the present study

The objectives of the present study were to determine the presence of anti-*Leishmania* antibodies in dogs from the 18 Districts of Portugal, as well as determine the risk factors for seropositivity. Due to the implementation of vaccines in the last decade, another objective was to assess the impact of vaccines in dog's seroprevalence.

This study also aimed to assess the seroprevalence status of *Leishmania* infection compared to the 2009 national survey, and evaluate whether risk factors have changed.

Materials and Methods

The present study was performed at IHMT-NOVA in collaboration with veterinarian clinics ("Centros de Atendimento Médico-Veterinário") from the 18 Districts of Mainland Portugal, with the support of LetiPharma S.L.U. and MSD Animal Health. The present study was approved by Direção Geral de Alimentação e Veterinária (DGAV) (Ref. 0421/000/000/2021) and by the "Orgão Responsável pelo Bem Estar Animal" from IHMT (ORBEA-IHMT).

7. Survey Strategy

In an initial phase of the study, on March 2020, veterinarian clinics were invited to participate in the national survey. A total of 135 clinics agreed to participate through an online survey provided by "Ordem dos Médicos Veterinários". However, due to the beginning of the global COVID-19 pandemic and the beginning of the initial lockdown the study was postponed to 2021. These same clinics were contacted again on November 2020, regarding their previous interest in participating in the study.

At this stage only 98 clinics (Annex 1) maintained their interest in being enrolled in the study and agreed to collect canine blood samples. An explanatory letter was sent to each clinic along with a kit for each dog composed by filter paper in a small plastic bag (Figure 10) to collect a drop of dog's whole blood, a questionnaire (Annex 2) and informed consent for the dog owner (Annex 3) along with a prepaid envelope to send their samples to IHMT. It was solicited that the participating clinics randomized the collection of samples. The study was advertised to the dog's owners through posters and social media posts done by the clinics.

Although an initial period of one week was set for the samples' collection (18-24 January, 2021), this period was extended until March 2021 in order to increase the possibilities to obtain a considerable number of samples. During the time, Portugal went into the second nationwide strict confinement. Consequently, some clinics mentioned a lack of patients, due to the confinement or quarantine, others even closed doors temporarily due to small outbreaks, and hence were unable to collect and send as many

samples as it was initially foreseen.

7.1. Sample Size Determination

Portuguese mainland is divided into 18 districts distributed by five geographical regions: Alentejo, Algarve, Centre, Lisbon and Tagus Valley and North (Figure 8).

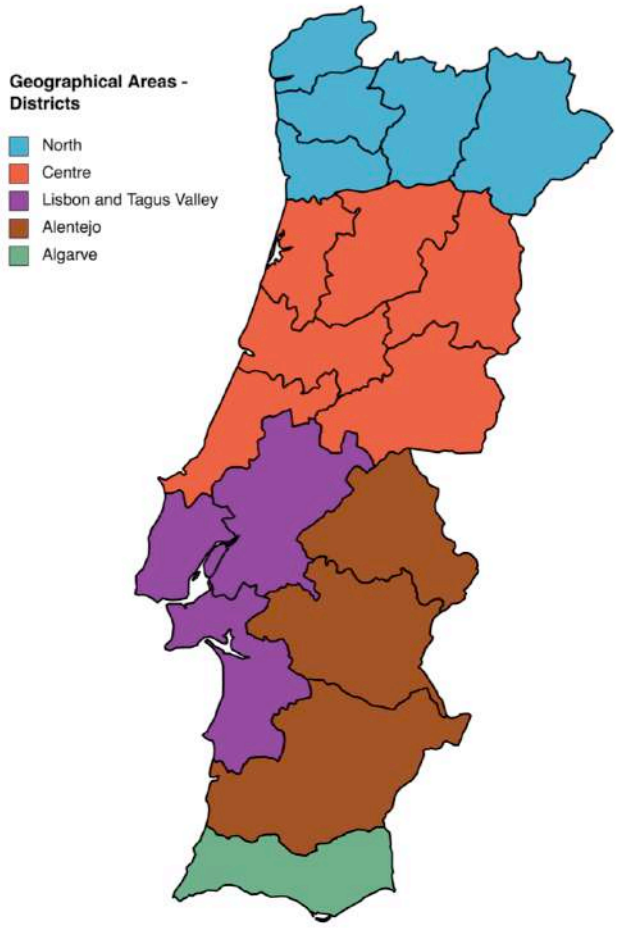

Figure 8 – Map of Mainland Portugal divided in districts and organized by geographical area. Made with mapchart.net

In order to estimate the Portuguese regions sample size the Sample Size calculator of Epitools© Epidemiological Calculators (Sargent, 2018; Humphry et al., 2004.) was used to estimate a proportion by a confidence interval, considering the performance of the used serological test. It was considered 95% confidence interval (CI), 100% test specificity and 93% sensibility for the DAT serological test (Ferreira et al., 2007). Additionally, the initial estimates for the true prevalence for each region were

based on the previous Portuguese national survey, namely: North: 3,8%, Centre: 7.8%, Lisbon and Tagus Valley: 6%, Alentejo: 10% and Algarve: 4,7% and established a 3% precision (Cortes et al., 2012). To estimate a minimum stratified proportional sampling by district, we used dogs' population provided by direct contact with Sistema de Informação de Animais de Companhia (SIAC) (Table 1).

Table 1 – Minimum stratified proportional sample sizes by Geographical region and District.

				Stratified Proportional Sampling	
Area / District	**Dog Population by Region***	**Dog population by District***	**Minimum Sample Size**	**Ratio per District**	**Proportional Sample**
NORTE	669 462		177		
Viana do Castelo		65 512		0,10	17
Braga		187 709		0,28	50
Vila Real		51 062		0,08	14
Bragança		47 778		0,07	13
Porto		317 401		0,47	84
CENTRO	590 478		340		
Aveiro		147 353		0,25	85
Viseu		94 621		0,16	54
Guarda		44 576		0,08	26
Coimbra		113 316		0,19	65
Castelo Branco		44 331		0,08	26
Leiria		146 281		0,25	84
LISBOA E VALE DO TEJO	788 090		261		
Lisboa		464 115		0,59	154
Santarém		134 678		0,17	45
Setúbal		189 297		0,24	63
ALENTEJO	158 270		417		
Portalegre		43 016		0,27	113
Beja		54 619		0,35	144
Évora		60 632		0,38	160
ALGARVE	160 671		219		
Faro		160 671		1	219
TOTAL	2 366 968	2 366 968	1414		1414

* SIAC, 2020

Although a minimum sample size was established (Table 1), considering the constraints and difficulties in the fieldwork, we decided to send a higher number of kits to guarantee a desired sample size. A total of 4220 samples kits were sent to the clinics.

Exclusion criteria were as follows: any animal 6 months or younger and animals vaccinated with Canileish® within 6 months before the sampling in order to exclude positive dogs due to vaccine antibodies. Moreover, samples that had repeated or no sample number on the questionnaire were also excluded.

7.2. Sample Collection and Serological Analysis.

The clinics were instructed to collect a coin sized (200µl-400µl) whole blood sample, onto the filter paper through venipuncture (Whatman® Grade 3, Cytiva). The samples were allowed to dry at room temperature, then sealed in their individual bags and shipped to the IHMT, where they remained stored at room temperature until use (Figure 9).

Figure 9 - Coin sized sample of whole blood collected onto filter paper.

Direct Agglutination Test (DAT) was used to detected total anti-*Leishmania* antibodies. The freeze-dried antigen used for DAT was derived from *L. donovani* promastigotes at a density of $5x10^7$ parasites per mL (KIT Biomedical Research, The Netherlands).

The first step was to elute blood from the filter paper. A 5.5mm diameter disk was collected with a paper punch and placed on the first column of a V-shape 96 micro-

well plate (BRAND plates, Pure grade S, BRAN, Germany)(Annex 6) and eluted with 120 µL of saline solution (NaCl 0.9%, w/v) overnight, at room temperature. According to manufacturer instructions, the blood eluted from the 5.5 mm disk diameter is equivalent to 5µL, which enables an initial dilution of 1:25.

A dilution solution was then prepared, using NaCl 0.9% and β-mercaptoethanol 0,2 M. The β-mercaptoethanol is added as an antigen-cleaving agent, significantly improving test sensitivity (Semião-Santos et al., 2014). The dilution solution was evenly distributed in the micro-well plate, 50µL per well, and two-fold serial dilutions of blood were made from the initial 1:25 to 1:800 or until 1:25600 when confirming positive samples. The samples were incubated one hour at 37. ºC. The antigen was reconstituted in 5mL of NaCl 0.9% (w/v) (Annex 7), according to manufacturer instructions. After incubation, 50µL of antigen/well was added with further 18h incubation at room temperature (Figure 10).

Semi-quantitative results provided by the DAT are expressed as an antibody titer, that is, the value reciprocal of the highest dilution for which agglutination is still visible. A cut-off (threshold for positivity) of 400 was set, according to Oskam et al. (1996). Any samples with a titer result equal or higher than 400 were repeated, with two-fold serial dilutions raging from 1:25 to 1:25600.

For agglutination assessment solid blue dots were considered negative results, while diffuse blue circles were considered positive results. Each plate included a negative and positive canine blood sample as controls.

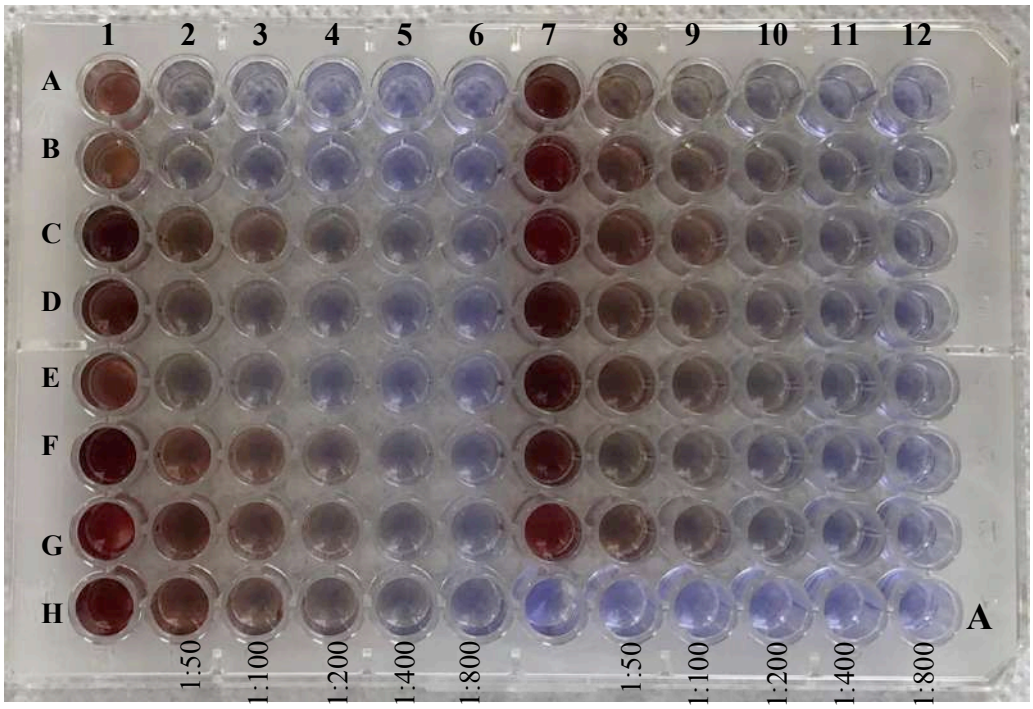

Figure 10 – 96-well microplate with samples dilution and antigen.
A: Antigen Control. Columns 1 and 7 are sample columns.

7.3. Statistical Analysis

A database was created using IBM® SPSS® Statistics Version 27. 2020, using the information collected from the questionnaire (Annex 2) and the serological test results.

The dogs' analyzed variables collected by questionnaire were: sex (male/ female), age groups (five classes: 0-2, 3-5, 6-8, 9-11 and 12-17 years), fur size (short, medium or long) and breed. Breeds were classified into four groups: pure autochthonous breeds, pure non-autochthonous breeds, cross breeds and mongrels. The dogs' habitual living place (geographic region, district, indoor or outdoors living and Littoral/Interior residence) was also analyzed. Prophylactic measures (use of repellents and insecticides and dogs' vaccination status regarding CanL) were analyzed to explore potential risk factors. The use of medication and presence of clinical signs compatible with CanL were also analyzed. Descriptive statistics were used to summarize each mentioned categorical variable, using absolute and relative frequencies and plots.

Materials and Methods

The Chi-Square test was used to compare proportions in two or more groups (e.g., prevalence by age group) or to test associations between categorical variables, checking the assumptions. Otherwise, Fisher exact test was used instead. In this last case of associations, the popular epidemiological measure Odds was used to quantify the association between an exposure and an outcome (Szumilas, 2010). However, this odds ratio is crude and may be affected by other third variables. Thus, a multivariate analysis, through multiple binary logistic regression models, was performed to analyze simultaneously several exposures and potential confounding variables, obtaining the corresponding adjusted odds ratio values (aOR) with 95% confidence interval, using IBM® SPSS® Statistics Version 2. 2020. Initial variables associated with outcome (Chi-Squared test result with a value <0.05) or other with relevant epidemiological interest were included in both analysis. Regarding any variables that presented as risk factors in Cortes et al. (2012), but no longer presented as risk factors in the present study, particular attention was given to those in the models. The Hosmer and Lemeshow Goodness-of-Fit test was obtained for each model of multivariate analysis. This test enables the user to verify if the model being applied to our dataset is adequate to describe it (Fagerland & Hosmer, 2012). When the significance is below 0.05, the test poorly describes the data.

True prevalence was estimated for each individual district and for each geographical area and for mainland Portugal as a whole. This was done using Epitools© Epidemiological Calculators (Sargent, 2018), considering 100% test specificity and 93% test sensitivity (Ferreira et al., 2007). All point estimates were accompanied by a 95% confidence interval. True prevalence was calculated based on the formula: True Prevalence (TP) = (test prevalence–1+specificity)/(sensitivity-1+specificity) (Rogen and Gladen, 1978). The confidence interval for true prevalence is calculated in Epitools© using the formulas described in Greiner and Gardner (2000).

Results

8. Sample characterization according with independent variables and their categories

A total of 1877 samples were sent to the laboratory from the 98 enrolled clinics, which included a blood spot on filter paper, questionnaire and owner's informed consent from each animal. A total of 1860 samples were analyzed as 17 were excluded due to exclusion factors for the study or were misidentified (Table 2).

Table 2 –Frequency of adherent clinics and samples per district and geographical region.

Geografic region/ District	No. adherent clinics	No. analyzed samples
NORTH	**TOTAL: 26**	**TOTAL: 458**
Viana do Castelo[a]	3	51
Braga[a]	6	93
Vila Real[b]	5	104
Bragança[b]	5	82
Porto[a]	7	128
CENTRE	**TOTAL: 31**	**TOTAL: 529**
Aveiro[a]	5	89
Viseu[b]	6	87
Guarda[b]	2	40
Coimbra[a]	8	131
Castelo Branco[b]	3	72
Leiria[a]	7	110
LISBON AND TAGUS VALLEY	**TOTAL: 25**	**TOTAL: 496**
Lisboa[a]	12	210
Santarém[b]	7	161
Setúbal[a]	6	125
ALENTEJO	**TOTAL: 11**	**TOTAL: 242**
Portalegre[b]	2	60
Beja[b]	4	60
Évora[b]	5	122
ALGARVE	**TOTAL: 5**	**TOTAL: 135**
Faro[a]	5	135
TOTAL	98	1860

[a]Littoral Districts; [b]Interior Districts

Concerning the total sampling set, as some questionnaires were incomplete, the analyzed variables retrieved different totals. The distribution of these samples per district and geographic regions is described in Table 2. The geographic region with the most samples analyzed was the Centre and the area with the least was the Algarve.

On average 18.94 samples were received from each clinic (raging from 2-39). A small number of clinics (n= 22) sent more than 30 samples (Figure 11).

Concerning previous knowledge about the study, 19.4% (357/1839) of dog owners knew about the initiative before attending to the veterinarian clinic.

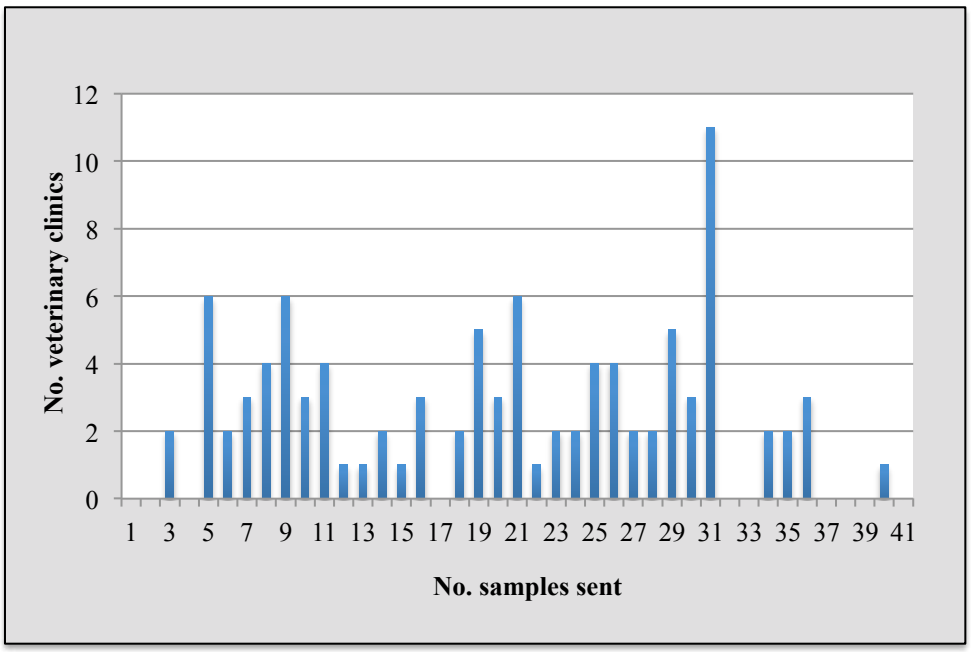

Figure 11 – Frequency of samples sent per clinic.

As presented in Table 3, the number of female and male dogs was similar with 51.5% female and 48.5% male dogs. Dog's age varied from six months to 17 years. With a mean age and standard deviation of 5.39 ± 3.763. It was observed a higher percentage of young dogs until eight years old, from 29.2% (age group 0.5-2) to 22.4% (age group 6-8).

Regarding breed, most of dogs were from non-autochthonous pure breed (43.9%) and mongrels (41.6%). The most frequent non-autochthonous breeds (includes pure and cross breeds) were: Labrador Retriever (n=175), German Shepherd (n=77) and French Bulldog (n=65). The most frequent autochthonous dog breeds are the Podengo

(n=46), Rafeiro Alentejano (n=27) and Cão da Serra da Estrela (n=23) (Annex 4). Most dogs had short fur (60.2%).

Table 3 – Frequency and percentage of dogs tested per sex, age, breed and fur size.

	No. tested dogs	**Percentage (%)**
Sex*		
Female	944	51.5
Male	890	48.5
Age group**		
[0.5-2]	506	29.2
[3-5]	465	26.9
[6-8]	387	22.4
[9-11]	243	14.0
[12-17]	130	7.5
Breed		
NAPB	817	43.9
APB	104	5.6
Cross breed	165	8.9
Mongrels	774	41.6
Fur size***		
Short	1109	60.2
Medium	505	27.4
Long	227	12.3

*In 26 samples there was no indication of the dog's sex; ** In 129 samples there was no indication of the dog's age; *** In 19 samples there was no indication of the dog's fur size. NAPB – Non-autochthonous pure breed; APB – Autochthonous pure breed

Concerning dog's living environment, majority of dogs lived mostly indoors (34.7%) followed by exclusively outdoors (21.6%), as presented in Table 4.

Table 4 – Frequency and percentage of dogs per living environment.

	No. tested dogs	Percentage (%)
Living environment*		
Exclusively Indoors	227	12.4
Mostly Indoors	637	34.7
Equally Indoors and Outdoors	323	17.6
Mostly Outdoors	252	13.7
Exclusively Outdoors	396	21.6

* In 25 samples information was missing.

Regarding prophylactic measures, and specifically vaccination status, most dogs were not vaccinated (85.1%) (Figure 12). Concerning the vaccinated dogs and the two commercialized vaccines in Portugal, Letifend® was administered more (7.8 %) than Canileish® (3.5%).

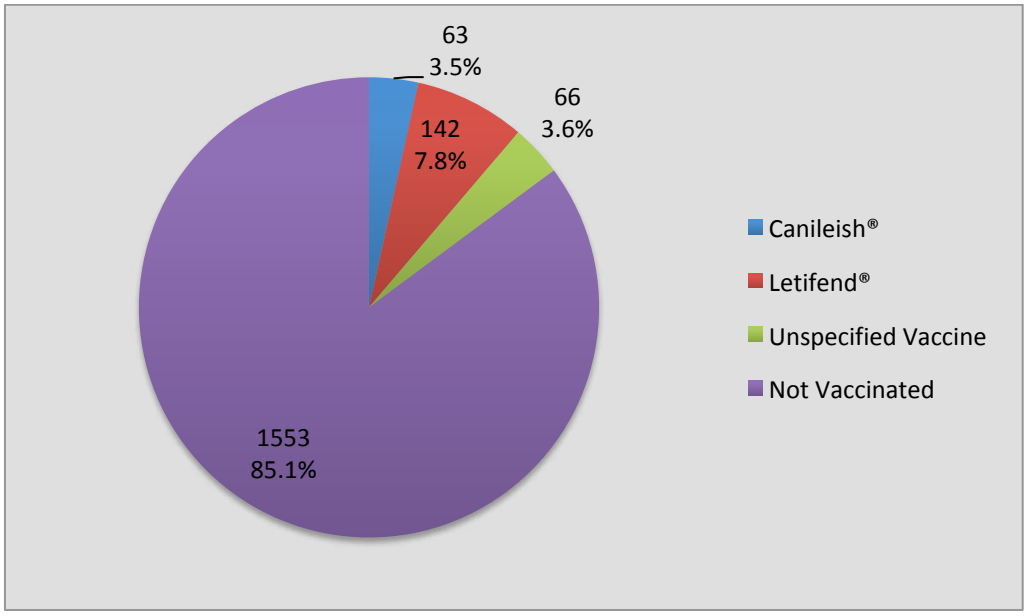

Figure 12 – Dog's vaccination status

Regarding the use of repellents/insecticides, each product was considered effective or not according to DGAV and EMA (Annex 5). 40.1% of dogs were using products with repellent effect against phlebotomine sand flies or in reducing *L. infantum* infection (as described in manufacturer instructions), and 30.5% were not using any product as repellent or insecticide (Figure 13). Moreover, it was observed that 81 dogs

use two forms of prophylactic products simultaneously, in most cases combining effective and non-effective repellents/insecticides. The most commonly used products were Seresto® collar (n=248) (imicloprid/flumethrin), spot-on Advantix® (n=206) (imicloprid/permethrin) and Bravecto® (n=181) (fluralaner), the latter being a systemic insecticide and acaricide that is administered orally, with no effect on sand flies.

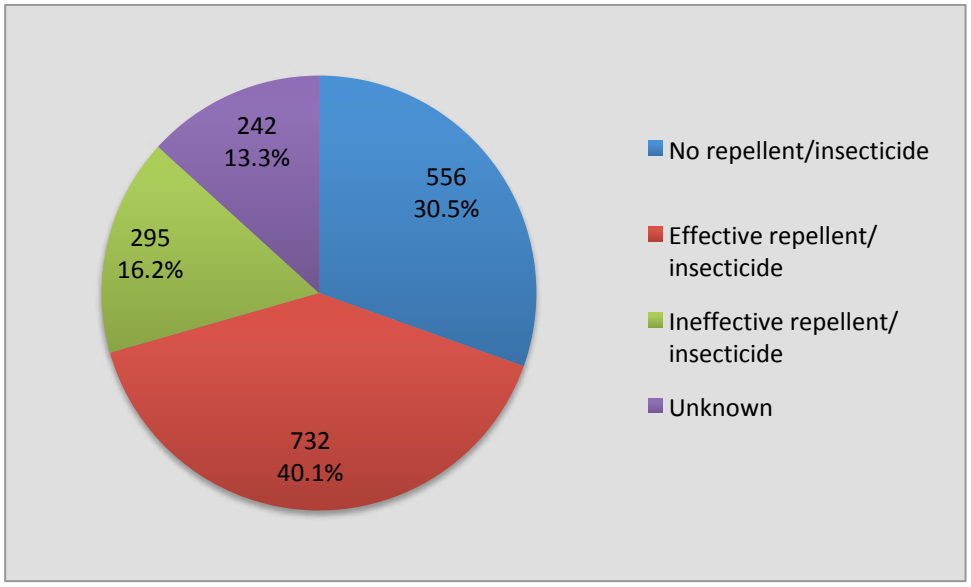

Figure 13 – Percentage of dogs according to use of repellents/insecticides.

Considering dogs that use any type of repellents/insecticides, most use these quarterly or monthly, as indicated in Figure 14.

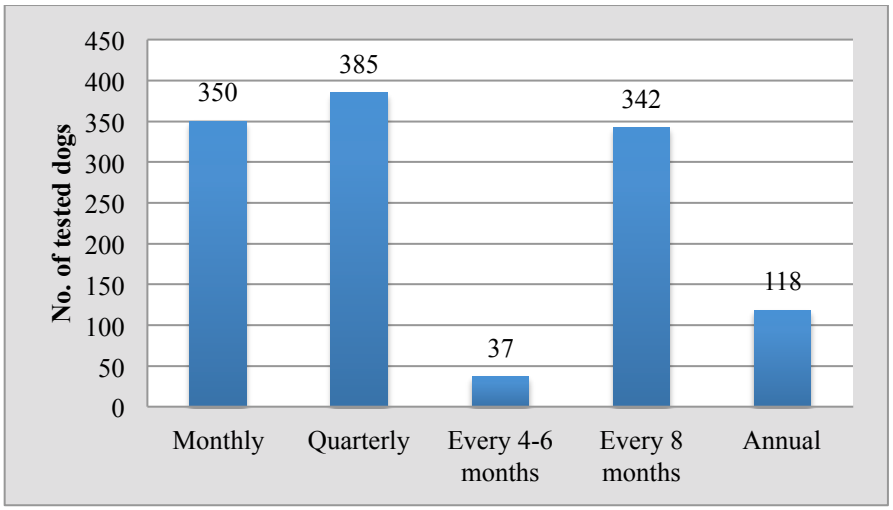

Figure 14 – Dog's periodicity of use of repellents/insecticides.

Results

In 6.2% of tested dogs (112/1804) the presence of clinical signs compatible CanL was reported (Table 5). The most common signs were appetite/weight loss, skin lesions and alopecia (Table 6).

Table 5 – Presence/absence of clinical signs

	No. tested dogs	Percentage (%)
Presence of Clinical Signs*		
Yes	112	6.2
No	1692	93.8

* In 56 samples there was no indication of the presence or absence of symptoms.

Table 6 – Frequency of clinical signs compatible with CanL compatible present in positive dogs.

Clinical signs	No. tested dogs
Loss of Appetite or Weight Loss	13
Ulcers, Wounds and other unspecified skin lesions	11
Alopecia	7
Onychogryphosis	6
Haemogram/Hepatic Alterations	6
Locomotion difficulties/articular pain	4
Ocular Lesions (such as eye discharge)	3
Pruriency	1
Dermatitis	1
Lymphadenomegaly	1

9. Seroprevalence for *Leishmania* infection according with the studied variables

A representative result of DAT is presented in figure 15 with controls and positive and negative samples. Positive samples presented a titer of ≥ 800.

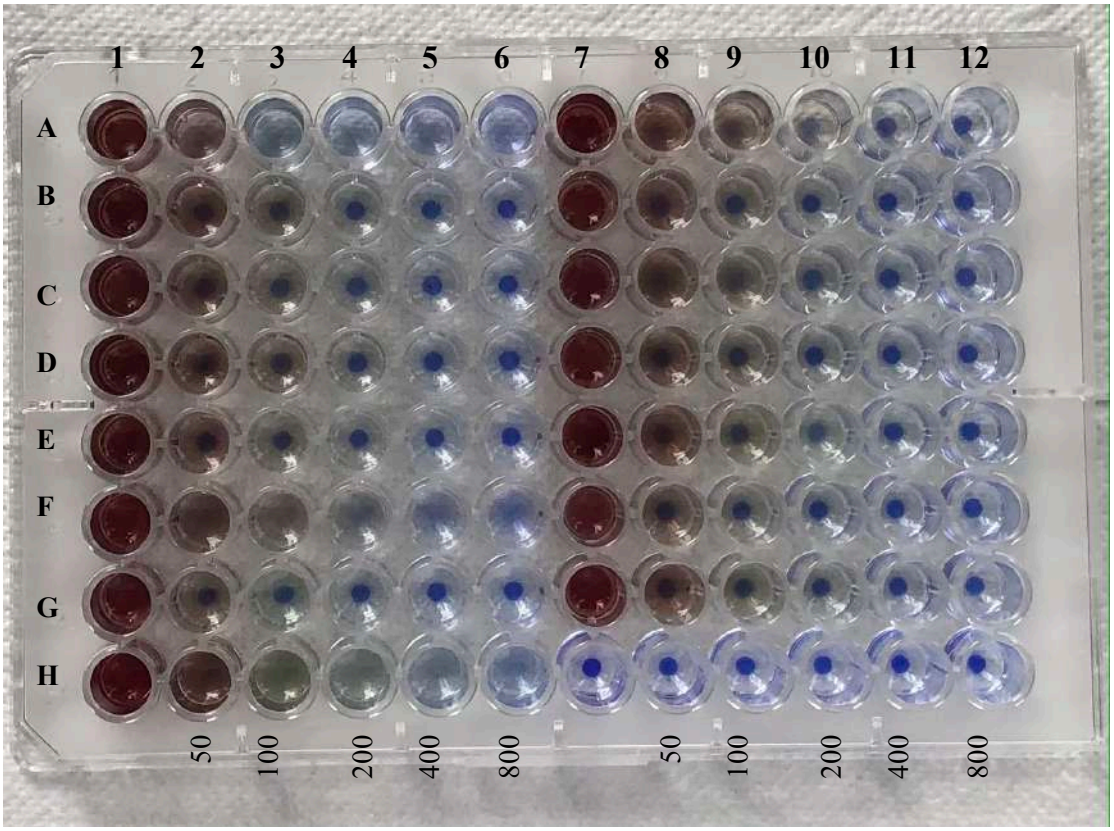

Figure 15 – 96-well microplate with representative DAT results from blood samples on filter paper (columns 1 and 7).

Line A1-6 – positive control; Line B1-6 – negative control; Line H7-12 – A+ antigen control; Lines F1-6 and H1-6 – positive samples with a titer ≥ 800; (with antigen agglutination) all other lines correspond to negative samples (with antigen precipitation)

Overall, 217 of the 1860 dogs examined tested positive for the presence of anti-*Leishmania* antibodies (11.7%) considering the cut-off titer of DAT equal or higher to 400 (Table 7). 61.8% (134 of 217) of positive samples presented a titer of 25600 (Table 9).

Excluding vaccinated dogs, seropositivity was 9.1%. Overall true seroprevalence considering the whole sample was 12.5%, and 9.8% excluding vaccinated dogs.

Results

Table 7 - True seroprevalence for *Leishmania* infection considering the whole sample and excluding vaccinated dogs.

	No. of positives (total)	Percentage (%)	True Prevalence (%)	95% CI
Whole Sample	217 (1860)	11.7	12.5	10.3-13.2
Excluding Vaccinated Dogs	142 (1553)	9.1	9.8	8.4-11.5

Due to the difference in seroprevalence between the whole sample and unvaccinated dogs, positives were analyzed between vaccines. Dogs vaccinated with Canileish® showed a 47.6% positivity, and dogs vaccinated with Letifend® showed a 18.3% positivity, as shown in Table 8.

Table 8 – Results of DAT titers for positive samples.

	No. tested dogs	Percentage (%)
DAT titer		(Total: 217)
400	38	17.5
800	28	12.9
1600	5	2.3
3200	7	3.2
6400	4	1.8
12800	1	0.5
25600	134	61.8

Table 9 – Results of DAT per vaccine.

Result of DAT test per vaccine	No. Positive tested dogs (total)	Percentage (%)
Not Vaccinated	142 (1553)	9.1
Vaccinated with Canileish®	30 (63)	47.6
Vaccinated with Letifend®	26 (142)	18.3

There are significant differences (*P*-value = 0,000) between groups.

Tables 10 and 11 present seroprevalences for *Leishmania* infection in the 18 Districts of Portugal considering the whole sample and excluding unvaccinated dogs. In Table 10 are presented the results for true seroprevalence per district for the whole sample, and in Table 11 are shown the results for true seroprevalence per district

excluding vaccinated dogs.

In the whole sample set, Portalegre, Castelo Branco and Guarda presented the highest true seroprevalences with 30.5%, 29.9% and 19.3% respectively.

True prevalence data from tables 10 and 11 are compiled onto maps presented in Figure 16. Differences in seroprevalence are observed, such as Coimbra, Faro and Guarda, which show a significant increase when vaccinated dogs are considered.

Table 10 – True prevalence of for *Leishmania* infection by District for the whole sample.

	Positives (of total)	**Positive dogs (%)**	**True Prevalence (%)**	**95% CI**
District				
Aveiro	1 (of 91)	1.1	1.2	0.00 – 6.4
Beja	8 (of 60)	13.3	14.3	7.4 – 26.0
Braga	6 (of 93)	6.5	6.9	3.2 – 14.4
Bragança	12 (of 82)	14.6	15.7	9.2 – 25.8
Castelo Branco	20 (of 72)	27.8	29.9	20.1 – 42.0
Coimbra	20 (of 131)	15.3	16.4	10.9 – 24.1
Évora	12 (of 122)	9.8%	10.6	6.2 – 17.6
Faro	22 (of 135)	16.3	17.5	11.8 – 25.2
Guarda	7 (of 39)	17.9	19.3	9.6 – 35.1
Leiria	4 (of 110)	3.6	3.9	1.5 – 9.6
Lisboa	18 (of 210)	8.6	9.2	5.9 – 14.1
Portalegre	17 (of 60)	28.3	30.5	19.9 – 43.8
Porto	11 (of 128)	8.6	9.2	5.2 – 15.8
Santarém	17 (of 161)	10.6	11.4	7.2 – 17.5
Setúbal	18 (of 125)	14.4	15.5	10.0 – 23.2
Viana do Castelo	0 (of 51)	0.0	0.0	0.00 – 7.5
Vila Real	13 (of 104)	12.5	13.4	8.0 – 21.7
Viseu	11 (of 86)	12.8	13.8	7.8 – 23.1

CI, confidence interval, Wilson Method

Results

Table 11 – True prevalence of for *Leishmania* infection in Mainland Portugal by district excluding vaccinated dogs.

	Positives (of total)	Positive dogs (%)	True Prevalence (%)	95% CI
District				
Aveiro	1 (of 82)	1.2	1.3	0.00 – 7.1
Beja	8 (of 55)	14.5	15.6	8.1 – 28.1
Braga	6 (of 88)	6.8	7.3	3.4 – 15.2
Bragança	10 (of 74)	13.5	14.5	8.1 – 24.9
Castelo Branco	13 (of 54)	24.1	25.9	15.8 – 39.7
Coimbra	7 (of 110)	6.4	6.8	3.4 – 13.5
Évora	9 (of 103)	8.7	9.4	5.0 – 17.0
Faro	12 (of 91)	13.2	14.2	8.3 – 23.3
Guarda	4 (of 31)	12.9	13.9	5.5 – 31.0
Leiria	1 (of 95)	1.1	1.1	0.00 – 6.2
Lisboa	10 (of 152)	6.6	7.1	3.9 – 12.6
Portalegre	13 (of 52)	25.0	26.9	16.4 – 41.1
Porto	10 (of 115)	8.7	9.4	5.2 – 16.4
Santarém	11 (of 133)	8.3	8.9	5.0 – 15.3
Setúbal	8 (of 90)	8.9	9.7	4.9 – 17.8
Viana do Castelo	0 (of 51)	0.0	0.0	0.00 – 7.5
Vila Real	11 (of 101)	10.9	11.7	6.7 – 19.8
Viseu	8 (of 76)	10.5	11.3	5.8 – 20.9

CI, confidence interval, Wilson Method

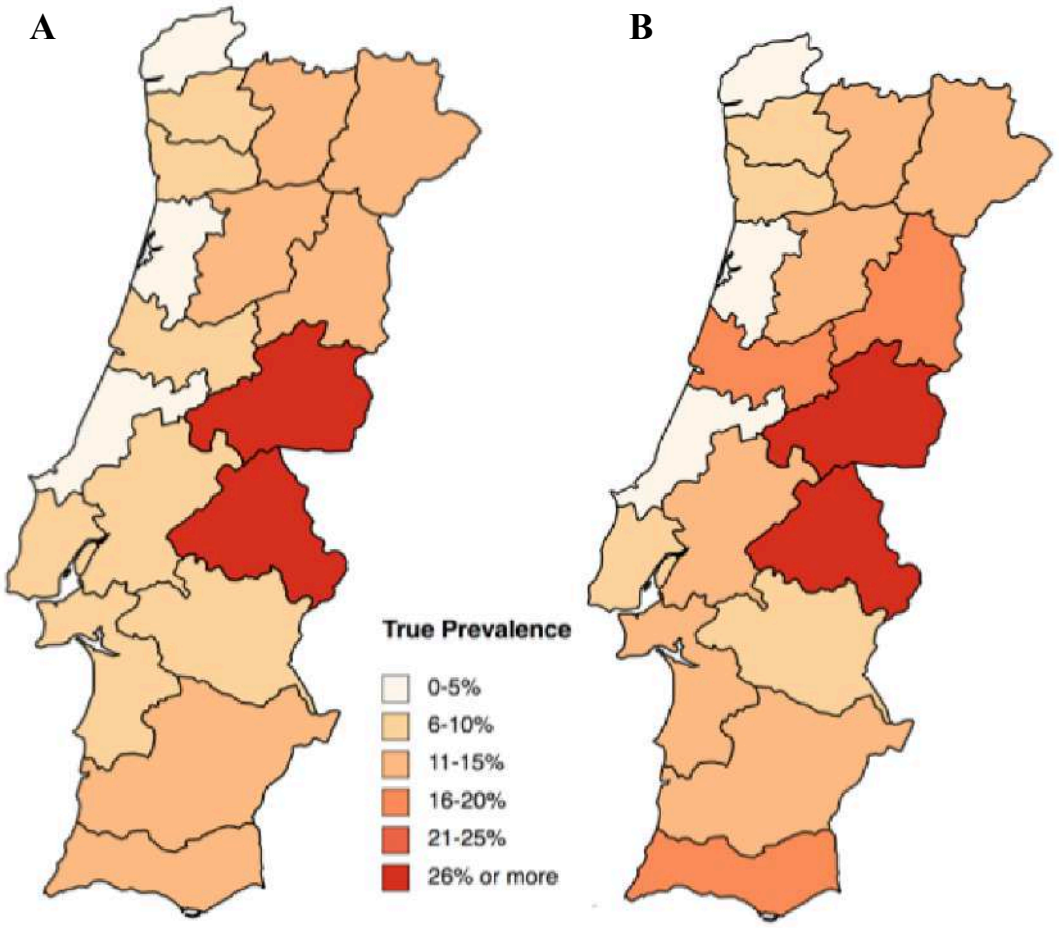

Figure 16 – Maps of Mainland Portugal with data for true seroprevalence. A: map with data excluding vaccinated dogs; B: map with data of the whole sample set.

Table 12 presents positivity results concerning dog's characteristics in the whole sample set and excluding vaccinated dogs. Female and male dogs equally tested positive, with no significant differences between sample sets.

Concerning age group distribution, age group [12-17] had the highest proportion of positives, and [0-2] age group had the lowest proportion, with significant statistical differences between all groups (p-value = 0.005).

In the whole sample set versus breed, the group with the highest proportion of positives was autochthonous pure breeds. Regarding fur size, positive dogs were presented mostly with short fur.

Table 12 – Percentage of positives per variable for sex, age, breed and fur size.

	Whole Sample			Excluding Vaccinated		
	No. of Positives (total)	**Percentage (%)**	**95% CI**	**No. of Positives (Total)**	**Percentage (%)**	**95% CI**
Sex						
Male	105 (944)	11.8	5.6-17.9	73 (757)	9.6	2.8-16.4
Female	111 (890)	11.8	5.7-17.8	68 (779)	8.7	2.0-15.4
Age Group						
[0-2]	36 (506)	7.1	0.0-15.4	29 (454)	6.4	0.0-15.3
[3-5]	64 (465)	13.8	5.3-22.2	41 (377)	10.9	1.4-20.4
[6-8]	51 (387)	13.2	3.9-22.5	28 (308)	9.1	0.0-19.7
[9-11]	27 (243)	11.1	0.0-22.9	17 (206)	8.3	0.0-21.4
[12-17]	20 (130)	15.4	0.0-31.2	13 (110)	11.8	0.0-29.3
Breed						
NAPB	104 (819)	12.7	6.3-19.1	57 (636)	9.0	1.6-16.4
APB	15 (104)	14.4	0.0-32.2	12 (92)	13.0	0.0-32.0
Cross breeds	12 (165)	7.3	0.0-22.0	8 (136)	5.9	0.0-22.2
Mongrels	86 (772)	11.1	4.4-17.7	65 (689)	9.4	2.3-16.5
Fur size						
Short	135 (1109)	12.2	6.7-17.7	93 (928)	10.0	3.9-16.1
Medium	57 (505)	11.3	3.1-19.5	34 (412)	8.3	0.0-17.6
Long	24 (227)	10.6	0.0-22.9	15 (200)	7.5	0.0-20.8

CI, confidence interval

NAPB – Non-autochthonous pure breed; APB – Autochthonous pure breed

Concerning preventive measures, namely use of repellents/insecticides, in table 13, a higher percentage of positive dogs was observed in the group not using any repellent/insecticide (13.1%). The same was found when vaccinated dogs were excluded

(12.0%), and in this group there are significant statistical differences (*p*-value = 0.008)

As shown in Table 14, most positive dogs reside in the Interior of Portugal. No significant statistical differences were found regarding the dog's living environment.

Table 13– Percentage of positives per variable for use of repellents/insecticides.

	Whole Sample			Excluding Vaccinated		
	No. of Positives (total)	**Percentage (%)**	**95% CI**	**No. of Positives (Total)**	**Percentage (%)**	**95% CI**
Repellents or Insecticides						
No use	73 (556)	13.1	5.3-20.8	61 (509)	12.0	3.8-12.2
Use Efficient	90 (732)	12.3	5.5-19.1	49 (569)	8.6	0.1-16.4
Use Inefficient	26 (295)	8.8	0.0-19.7	18 (258)	7.0	0.0-18.8
Unknown	25 (242)	10.3	0.0-22.2	13 (194)	6.7	0.0-20.3

CI, confidence interval

Table 14 – Percentage of positives per variable for Littoral/Interior and for living environment.

	Whole Sample		
	No. of Positives (total)	**Percentage (%)**	**95% CI**
Littoral/Interior			
Interior	118 (784)	15.1	8.6-21.6
Littoral	99 (1076)	9.2	3.5-15.0
Living environment			
Exclusively Indoors	22 (227)	9.7	0.0-22.1
Mostly Indoors	73 (637)	11.5	4.2-18.8
Equally Indoors and Outdoors	33 (323)	10.2	0.0-20.5
Mostly Outdoors	31 (252)	12.3	0.1-23.9
Exclusively Outdoors	58 (396)	14.6	5.5-23.7

There are significant statistical differences between the Littoral and Interior groups (*p*-value <0.001).
CI, confidence interval

Of the 112 dogs that presented clinical signs, 42 tested positive (37.5%).

Regarding positive dogs with clinical signs, a considerably lower number of vaccinated dogs presented clinical signs (7.2%), when compared to unvaccinated dogs (25.7%), as shown in figure 17. There are significant statistical differences between groups (*p*-value = 0.002), and being unvaccinated presents as a risk factor for the presence of clinical signs (OR = 4.4, CI 1.6 – 11.9)

The most frequent clinical signs compatible with CanL, as described by Solano-Gallego et al. (2011), in positive dogs of our sample set were: weight loss and/or loss of appetite, wounds and ulcers, alopecia, onychogryphosis and haemogram (Table 6). Although reported in two positive samples, urinary incontinence and pulmonary hypertension were not considered as clinical signs compatible with CanL.

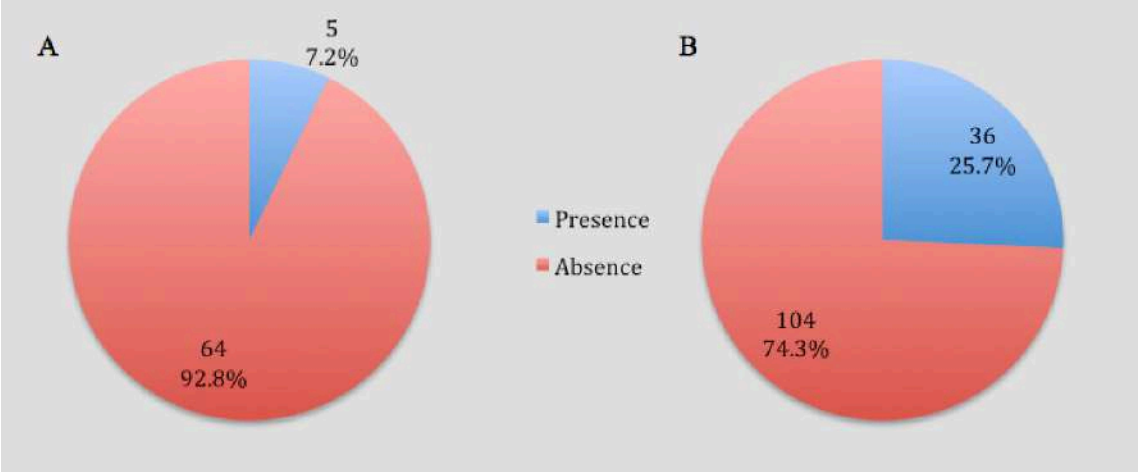

Figure 17 – Percentage of dogs with clinical signs compatible with CanL in vaccinated group (A) and the unvaccinated group (B).

10. Analysis of Risk Factors

Significant statistical differences were detected when comparing littoral and interior districts, age groups, geographical areas and use of repellents or insecticides. Concerning variables studied, in Table 16 are identified all the variables with significant statistical differences (*P*-value ≤ 0.050) in a univariate analysis, with their respective OR for the whole sample (n=1860) and excluding vaccinated dogs (n=1553).

Different variables have been found to be statistically different in the two groups and considered as risk factors;

i) whole sample: being 2 years or older and residing in the interior.

ii) excluding unvaccinated dogs: being 2 years or older, residing in the interior, residing in the Alentejo region and as not using any repellent/insecticide.

Other variables that presented statistical differences were considered as protective factor: residing in the North region, and using repellents/insecticides monthly or quarterly. Unexpectedly, being vaccinated presented as a "risk factor" (Table 15).

Table 15 – Risk factors identified in a univariate analysis.

Risk factor	**Univariate Analysis**					
	Whole Sample			**Excluding Vaccinated Dogs**		
	% in sample	Crude OR	95% Cl	% in sample	Crude OR	95% Cl
Older than 2 years	70.8	1.99	1.36-2.90	68.8	1.61	1.05-2.47
Residing in the Interior	42.1	1.72	1.29-2.28	43.5	2.21	1.55 – 3.14
Residing in Alentejo Region	--	--	--	12.2	2.01	1.29-3.12
Non use of Prophylaxis	--	--	--	33.3	1.60	1.13-2.28
Variables with statistically significant differences						
Residing in the North region	23.8	0.70	0.48-1.00	--	--	--
Using prophylaxis monthly (vs. non use)	--	--	--	17.8	0.58	0.34-0.98
Using Prophylaxis every 3 months (vs. non use)	21.1	0.58	0.37-0.90	22.2	0.53	0.32-0.88
Dogs who have clinical signs	6.2	5.37	3.55-8.13	6.5	7.32	4.64-11.56
With Vaccine	% in sample		Crude OR		95% Cl	
	11.9		3.60		2.61-4.95	

The identified risk factors were combined in a multivariate analysis in order to understand if these risk factors were maintained (Table 16). Being older than two years and residing in the Interior Districts remained as risk factors for seroprevalence of *Leishmania* infection. For unvaccinated dogs, the non-use of repellents/insecticides is also a risk factor.

Table 16 – Multivariate analysis results for the identified risk factors in the univariate analysis.

Risk factor	**Multivariate Analysis**					
	Whole Sample			**Excluding Vaccinated Dogs**		
	Adjusted OR	95% Cl	*P*-value	Adjusted OR	95% Cl	*P*-value
Older than 2 years	2.14	1.45-3.14	<0.001	1.68	1.09-2.60	0.020
Residing in the Interior	1.63	0.91-1.72	0.003	1.92	1.27-2.90	0.002
Residing in Alentejo region	--	--	--	--	--	--
Non use of repellents or insecticides	--	--	--	1.74	1.20-2.53	0.003
Constant	0.054	--	<0.001	0.037	--	<0.001
Hosmer and Lemeshow Test	Sig. = 0.835			Sig. = 0.759		

Since being vaccinated presented as a risk factor, further analysis was conducted to determine if the vaccination status is a confounding factor. Both vaccines used present a higher chance of positivity, being that Canileish® is significantly higher (OR = 9.03), as shown in Table 17.

Table 17 – Analysis of vaccination as a risk factor. Both vaccines were analyzed in comparison to unvaccinated dogs.

Vaccination	**Univariate Analysis**			
	% in sample	Crude OR	95% Cl	*P*-value
Vaccinated with Canileish®	3.5	9.03	5.35-15.25	<0.001
Vaccinated with Letifend®	7.8	2.23	1.41-3.52	0.001

When analyzing variables to identify risk factors excluding dogs vaccinated with Canileish®, which is known to show false positive results in common serological tests for CanL (Solano-Gallego et al., 2017), all risk factors previously identified remain the same, including vaccination, with the addition of another identified risk factor: residing outdoors, as shown in Table 18. With further multivariate analysis, residing outdoors no longer remained as risk factor.

Table 18 – Risk factors analysis excluding dogs vaccinated with Canileish®.

Risk factor	**Univariate Analysis**			**Multivariate Analysis**		
	% in sample	Crude OR	95% Cl	Adjusted OR	95% Cl	*P*-value
Older than 2 years	30.1	1.71	1.16-2.52	1.81	1.18-2.77	0.007
Interior regions	42.5	1.92	1.41-2.62	1.83	1.23-2.71	0.003
Residing in Alentejo	11.5	1.66	1.10-2.53	--	--	--
Living Outdoors	43.4	1.45	1.03-2.02	--	--	--
Non-use of prophylaxis	30.9	1.26	0.92-1.74	--	--	--
Constant	--	--	--	0.05	--	<0.001
Hosmer and Lemeshow Test				Sig.= 0.829		

4. Discussion

Although parasites of the genus *Leishmania* sp. can infect a variety of vertebrate animals, dogs are the principal reservoir for zoonotic Human Leishmaniasis caused by *L. infantum*. Canine Leishmaniosis is prevalent in Southern Europe, with infection rates up to 60% in exposed populations (using serological and molecular diagnosis) (ESCCAP, 2019).

Some authors suggest a positive correlation between human and canine *Leishmania* infection. The infection in dogs has much higher prevalence values compared to human infection (revised in Campino & Maia, 2010). Moreover, several studies have found that more than 50% of dogs can be asymptomatic (Solano-Gallego et al, 2001; Dantas-Torres et al, 2006). Thus, it is of upmost importance to perform epidemiological studies as infected dogs may represent a veterinary and public health concern.

In Portugal, many canine epidemiological studies have been performed since the eighties of the last century (revised in Campino & Maia, 2010; Cortes et al., 2012; Maia et al., 2015a; Pires et al., 2019), with different prevalences in dogs all over the country.

The present study aimed in collecting canine blood samples to assess seroprevalence in the 18 Districts of Portugal, which enables a representation of the country as a whole. The sample's collection phase started during the second nationwide lockdown in Portugal, due to the global ongoing COVID-19 pandemic. As result, clinics were unable to collect and send as many samples as desired, being some Districts underrepresented such as the ones from Alentejo.

The under representation in some districts implies careful consideration of the results. One of the Districts where this difference may be of importance is Portalegre, with a true prevalence of 30.5%. A larger sample number from this region would be required to confirm the obtained results and proportionately represent the Alentejo region. In the previous national seroepidemiological study (Cortes et al., 2012), Portalegre presented a true prevalence of 12.54% based on 249 dog blood samples.
Nevertheless, the total number of collected samples exceeded the previously calculated proportional stratified sampling for a precision of 3%.

For CanL diagnosis, various serological techniques, including qualitative and

quantitative tests, are used. These techniques present different sensitivity and specificity levels (Solano-Gallego et al., 2014). Serological assays utilizing entire parasites, soluble parasite extracts, or recombinant proteins generated from genes of interest have been employed in clinical and epidemiological research. ELISA is a method that allows for absolute antibody quantification and the use of numerous antigen combinations to increase the method's sensitivity and/or specificity, but it has a lot of stages and requires a spectrophotometer.

Like IFAT, which is the reference technique, DAT uses whole *L. donovani* complex promastigotes to detect anti-*Leishmania* antibodies. DAT has a high specificity, similar to IFAT and ELISA, and sensitivity higher that IFAT, reaching in some studies to 100% (Oskam et al., 1996; Ferreira et al., 2007).

However, some authors observed that sensitivity and specificity of IFAT and DAT can differ in areas where there are low versus high prevalence rates or the places where the disease is hypoendemic (Mendonça et al., 2017; Lockwood & Sundar, 2006). Overall, DAT is a simple technique, inexpensive, can be applied to a large amount of samples, does not require any equipment and has a track record of clinical accuracy for both canine and human diagnosis (Oskam et al., 1996; Sousa et al., 2011; Mohebali et al., 2020).

Molecular methods, such as PCR, are more sensitive than serological methods for the diagnosis of CanL (Solano-Gallego et al., 2017). Ideal samples for molecular methods, such as bone marrow and spleen, require more invasive procedures (Solano-Gallego et al., 2011). Although we are aware that molecular methods may give a more accurate picture of effective infection by the presence of *Leishmania* or its DNA, this study intended to compare the presence of anti-*Leishmania* antibodies to the national canine survey performed in 2009, in which the serological method used was DAT (Cortes et al., 2012).

The overall true seroprevalence found in this study was 12.5% (CI 10.3 – 13.2). The districts with the highest seroprevalences were Portalegre (30.5%, CI 19.9 – 43.8), Castelo Branco (29.9%, CI 20.1 – 42.0), Guarda (19.3%, CI 9.6 – 35.1) followed by Faro (17.5%, CI 11.8 – 25.2). If we exclude vaccinated dogs, in order to directly compare with the last national seroepidemiological study performed by Cortes et al. (2012), the highest seroprevalences are still Portalegre (26.9%, CI 16.4 – 41.1), Castelo

Branco (25.9%, CI 15.8 – 39.7) and also Beja (15.6%, CI 8.1 – 28.1), which are the previously considered with highest seroprevalence. Viana do Castelo had a seroprevalence of 0.0% (CI 0.0 – 7.5). This does not indicate that there are no dogs that have come into contact with the parasite, but rather that no cases were detected in the present study.

In a recent study on several municipalities of this District, it was found a maximum seroprevalence of 56.0%, confirming that Castelo Branco is still a region from the Interior with relevance for CanL (Pires et al., 2019). Overall, in our study it was observed that Interior Districts present higher seroprevalence for *Leishmania* infection than Littoral Districts, with statistical significance. This trend was also identified in Cortes et al. (2012). Faro District in Algarve presented a seroprevalence of 17.5% similar to the 18.2% found in a study performed between 2011 to May 2014 including owner and stray dogs using DAT (Maia et al., 2015a), but much higher than the last national seroepidemiological survey, in which Faro presented a seroprevalence of 4.71% (Cortes et al., 2012)

On the other hand, in this region substantial differences were observed in the presence of *Leishmania* DNA in dogs from 0.4% (Maia et al., 2015b) to 60.4%. These differences can be partially explained by the methodology used, in which molecular approaches can be more sensitive, but also sampling method. This region has been surveyed for entomological studies with a dynamic presence of *phlebotomine* sandflies (Maia et al., 2013), along the different counties of Faro District but also monthly densities, which can partially explain the relatively high seroprevalence. In a study performed in the Mediterranean sub region in which Portugal (Algarve) was also surveyed, it was observed that *Phlebotomine* season lasts from April to October in Portugal, some recorded activity up to November (Alten et al., 2016).

Concerning the other Portuguese Districts with the highest seroprevelances, these are known to have, on average, warm periods of time, which may favor *phlebotomine* activity and influence on the transmission season. Also, the dogs from these interior Districts, such as Portalegre, Castelo Branco and Guarda may live in a more rural environment, such as peridomestic biotopes, particularly sheep pens, cattle stable and henhouses, with possible accumulation of organic matter, which may provide favorable conditions for sand flies resting and breeding sites (Branco et al., 2013)

Nevertheless, as there is a lack of *phlebotomine* studies in the Districts with higher seroprevalences, it is difficult to assess the influence of the *phlebotomine* season and densities on the present results.

Regarding risk factors two were identified for the whole sample. Residing in Districts from the interior of Portugal (aOR = 1.63, CI 0.91 – 1.72) and being older that 2 years (aOR = 2.14, CI 1.45 – 3.14). With regards to age, being an older dog has been identified as a risk factor in several other studies in dogs from Italy, Egypt, and Spain (Rombolà et al., 2021; Selim et al., 2021; Galvéz et al., 2020; Diáz-Regañon et al., 2020). In a recent study performed in Spain, residing in the interior region, at higher altitudes, has been identified as a risk factor and the effects of an increasing average temperature in the Mediterranean Basin has been discussed as a factor for the increase of cases in the interior (Rombolà et al., 2021). In our study, residing in Interior Districts, in opposition to littoral Districts may be a risk factor also due to the average warmer temperatures in summer, when it is known to occur higher phlebotomine densities. Nevertheless, seroprevalence of Interior Districts has a whole is incremented by the values of the three Districts with higher seroprevalence.

When considering only unvaccinated dogs, no use of repellents/insecticides becomes a risk factor (aOR = 1.74, CI 1.20 – 2.53). This has also been reported in other studies, highlighting the importance of preventative measures (Miró et al., 2017; Bourdeau et al., 2014). When comparing risk factors identified in Cortes et al. (2012), the use of efficient repellents/insecticides as well as living outdoors, being purebred and having shorter fur are no longer present as risk factors in this study. Some of these variables are identified as risk factors in other countries of the Mediterranean Region such as Italy (Rombolà et al., 2021), Egypt (Selim et al., 2021) and Greece (Symeonidou et al., 2021). However the samples used in some of these studies are different from the present study. For example, in Rombolà et al. (2021), the sample does not include solely dog samples collected in Veterinarian Clinics, but also shelter dogs and hunting dogs.

The risk factors identified in the previous national seroepidemiological study concerning breed and dog's fur size, were no longer identified on the present study. In the survey from 2009 it was found that dogs from pure exotic breeds are at higher risk of acquiring CanL than mongrel or pure autochthonous breed Cortes et al. (2012). Also,

in that study, short-medium fur was considered a risk factor. We conclude that all dogs independently of breed and fur size are potentially susceptible to *Leishmania* infection.

In this study, all the samples were collected in Veterinary Clinics, therefore from animals with owners and access to veterinarian care. The sample may not be completely representative of Portugal's dog population, due to the lack of stray and sheltered dogs.

Using repellents/insecticides monthly and/or in 3-month intervals also presented significant differences. Nonetheless, these intervals may not be accurate since it is impossible to verify the correct use of the repellents/insecticides. Apart from the correct use of repellents/ insecticides, also in rural and peri-urban regions, measures may include raising awareness among animal owners to minimize conditions that favor the presence of sand fly breeding sites as accumulated garbage or organic material in corrals, chicken coops, rabbit hutches, where dogs may roam too.

The current vaccines approved for use in Europe for CanL - Canileish® and Letifend®- work to stimulate the dog's immune system in order to prevent infection in the animal and long-term disease progression. Unfortunately, they don't block parasite's life cycle (Solano-Gallego et al., 2017). In this study, significant differences in prevalence were found in vaccinated and unvaccinated dogs. Canileish® is known to produce vaccinal antibodies that are undistinguishable from infection antibodies. The common serological diagnostic techniques often detect total antibodies, but it is known that antibodies elicited by this vaccine may lead to false positives (Vélez et al., 2020). With Canileish®, vaccinal antibodies reach a peak one month post-vaccination but are detectable, in 3.2% of cases, up to a year after the first dose (Miró et al., 2018; Montoya et al., 2021). The results obtained in the present study are in agreement with these findings, as the unvaccinated group had a positivity of 9.1% and in the vaccinated group dogs with Canileish® had a positivity of 47.6% and with Letifend® 18.3 %.

Previous studies have shown that Letifend® vaccine containing the recombinant Protein Q made from the genetic fusion of 5 antigenic fragments, does do not interfere with results of serological tests, such as IFAT and ELISA, on the detection of total IgG antibodies (Cotrina et al., 2018). Letifend® is classified as a DIVA vaccine (**D**ifferentiating between **I**nfected and **V**accinated **A**nimals) which only elicit antibodies to protein Q and different from those of natural infection (Solano-Gallego et al., 2017). Nevertheless, further studies should be performed also using DAT and serological

comparative studies to assess if there is any interference associated to these vaccines.

After performing a multiple logistic regression, it was observed that being vaccinated can be a potential "risk factor" (OR = 3.60, CI 2.61 – 4.95). Upon further analysis, vaccination status appeared to be a confounding factor, due to DAT being a serological test and both vaccines having a higher positivity.

When the vaccines were analyzed separately, Canileish® had a significantly higher risk of positivity (OR = 9.03, CI 5.35 – 15.25) than Letifend® (OR = 2.23, CI 1.41 – 3.52). Being vaccinated is not a risk factor for CanL *per se*, but due to the antibodies produced, serological tests may result in false positives, as previously observed (Montoya et al., 2021), and as discussed above.

The effects of vaccination are reflected on the presence of clinical signs. For positive samples, 25.7% of unvaccinated dogs presented with clinical signs compatible with CanL, whereas it was only 7.2% of vaccinated dogs with presented with clinical signs. Being unvaccinated presents as a risk factor for symptom development in positive dogs (OR = 4.4, CI 1.6 – 11.9). As well as prevent infection, the goal of a vaccine for CanL should be to control the progression of the disease (Montoya et al., 2021). Thus, the results of this study show that despite dogs being infected or having contact with the parasite, the proportion of vaccinated animals that may develop clinical signs or eventually progress to disease is much smaller.

In the present study there was a possible case of cross-reaction. One positive sample in the Coimbra district had a titer of 25600 and after posterior confirmation, with tests performed outside the laboratory, the dog had been confirmed negative for leishmaniosis but positive for babesiosis, a tick-borne parasitic infection. The sample was analyzed a third time and the same positive titer was obtained. Although uncommon, cross-reactions using serological techniques, like ELISA, have been recorded when detecting anti-*Leishmania* and anti-*Babesia* antibodies (Krawczak et al., 2015). One drawback of serological tests is the possibility of a cross-reaction, such as with *T. cruzi* and *Ehrlichia canis* (Maia & Campino, 2018), which in turn may result in a false positive, or in an undetected concomitant infection. False positives may lead to unnecessary treatment of the dog, in some cases mandatory unnecessary euthanasia (Maia & Campino, 2018)

Concomitant infections with other parasites may also occur. Pathologically,

concomitant infections may severely worsen CanL symptoms and accelerate disease progression (Andrade et al., 2014; Maia et al., 2016).

In the present study, 19.3% (42 out of 218) of positive dogs presented with compatible CanL clinical signs. In Cardoso et al (2012), 25.2% of positive dogs presented compatible clinical signs. This study used ELISA. In another study, using molecular techniques, such as qPCR, 42.7% of positive dogs showed compatible signs (Maia et al., 2016). This disparity is likely due to the difference in sensitivity between ELISA and DAT and qPCR. With molecular techniques, sensitivity is much higher and allows for the detection of the parasite in seronegative dogs (Solano-Gallego et al., 2011). Moreover, positive dogs with no clinical signs may be asymptomatic dogs, or clinically healthy dogs, as studies have shown that 35-60% of positive dogs are asymptomatic (Idrissi et al., 2021). These results would need to be confirmed with more sensitive techniques, like PCR. Asymptomatic cases pose a risk, due to the fact that clinically healthy infected dogs are able to allow the parasite's life cycle to continue. Therefore proper assessment of these cases may contribute greatly to the control of the disease, particularly in endemic regions (Idrissi et al., 2021).

The most common clinical signs were cutaneous lesions, such as ulcers and other wounds, weight loss, onychogryphosis, lethargy and alopecia. These agree with other studies, where the most common signs were found to be similar: alopecia, weight loss, skin lesions and lymphadenomegaly (Bordeau et al., 2014; Díaz-Regañon et al., 2020; Miró & López-Vélez, 2018). Detectable antibody titers are associated with disease progression; therefore indicating these clinical signs may be indicators of late-phase infection (Maia & Campino, 2011). According to Cardoso et al. (2021), an increasing number of reports indicate that leishmaniosis is not limited to canines and affects a wide range of mammalian and avian species. As a result, the potential reservoir grows, posing a serious public and veterinary health risk.

In recent years the role of cats as potential reservoirs for *Leishmania* has been explored, and several studies indicate that cats may play a bigger role in the *Leishmania* life cycle than previously anticipated, as the number of FelL cases has increased in endemic regions including Portugal (Rombolà et al., 2021; Maia & Campino, 2011). FelL has shown a prevalence of up to 59% (Maia & Campino, 2011) in different regions, and on a recent study 68.8% of cases previously diagnosed with FelL, through

molecular, cytological and/or serological methods, presented compatible systemic signs (Fernandez-Gallego et al., 2020).

Overall, the seroprevalence of CanL in Portugal has increased, and the risk factors associated with the disease have remained similar, with a few alterations regarding efficiency of repellents and insecticides used. Although the main objective of the present study was to determine the seroprevalence and evolution of CanL in Portugal, as well as determine risk factors, vaccines have proven to not only be a key part of the management of CanL, but a topic to be further studied regarding serological diagnostic and survey methods.

This cross-sectional study has inherited limitations and as some data were obtained by questionnaire, some biases are expected, namely the social desirability bias. This is a type of response bias in which respondents tend to answer questions according to “a good answer viewed by others” instead of answering truthfully.

Final Considerations

This study was the second seroepidemiological assessment on anti-*Leishmania* antibodies in dogs from the 18 Portuguese districts, with the goal of determining risk factors and evaluating the effects of vaccination as a novel preventive measure.

Over a 12-year period, the seroprevalence of *Leishmania* infection increased from 6.31% to 12.5% with the non-use of repellents and insecticides representing a risk factor. Portalegre and Castelo Branco still remain the districts with higher seroprevalence. The use of effective repellents/insecticides and vaccination should be a strategy to reduce both CanL and the incidence in Human infection.

In this study, the lower number of vaccinated positive dogs with clinical manifestations showed the usefulness of CanL vaccines in preventing the development of clinical signs associated with Leishmaniasis.

Vaccines are proven efficient prophylactic measures to prevent disease progression. However, vaccinal antibodies pose difficulties for serological testing, due to the fact most serological tests, DAT included, do not distinguish between vaccinal antibodies and infection antibodies. Thus, an effort must be done in supporting research for the improvement or development of new diagnostic approaches to differentiate vaccinal antibodies from those due to natural infection.

More integrated studies should be implemented with ONE Health vision to tackle Leishmaniasis related to the human host, canine reservoir and the vector.

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Annexes

Annex 1 – Table of Adherent Veterinarian Clinics

Veterinary Clinic	District
Centro Veterinário de Oliveira do Bairro	Aveiro
Clínica Veterinária do Vouga	Aveiro
O MEU VET - Clínica Veterinária	Aveiro
Clínica Veterinária da Vagueira	Aveiro
Termasvet - Consultório Veterinário	Aveiro
Consultório Veterinário Dr. Nuno Costa Neves	Beja
Refúgio Animal	Beja
SingaVet- Centro Veterinário	Beja
Vetmoura - Centro Veterinário De Moura, Lda	Beja
Centro Veterinário de Merelim	Braga
Clínica Veterinária de Areia	Braga
Clínica Veterinária de Martim	Braga
Clínica Veterinária Saúde Animal	Braga
Vetbasto Serviços Médico-Veterinários, Lda	Braga
Centro Veterinário do Parque	Braga
Clínica Veterinária Dr. Duarte Diz Lopes	Bragança
Clínica Veterinária Terra Quente, Lda	Bragança
Consultório Veterinário Animal SOS	Bragança
Consultório Veterinário Dr.ª Isabel Lameira	Bragança
Dr4Patas	Bragança
Clínica Veterinária de Castelo Branco (Hupera)	Castelo Branco
Fundão Vet - Centro Veterinário	Castelo Branco
Clínica Veterinária Vetbeirão	Castelo Branco
Centro de Saúde Animal	Coimbra
Centro Veterinário Cantanhede- Dr. André Caldeira	Coimbra
Centro Veterinário Vale das Flores, Lda	Coimbra
Clínica veterinária das Nogueiras	Coimbra
Oficina dos Animais - Clínica Veterinária Lda.	Coimbra
VetConímbriga, Lda	Coimbra
Vetsoure - clínica veterinária, Lda.	Coimbra
VetSunpi	Coimbra
Clínica Veterinária 112 Animal	Évora
Hospital Veterinário Muralha de Évora	Évora
Optivet	Évora
Vetviana, Consultório Veterinário, Lda	Évora
Vetvila - Clínica Veterinária de Vila Viçosa	Évora
Algarvet- Centro Veterinário dos Olhos de Água	Faro
Clinica Veterinária de Loulé	Faro
Villapet	Faro

Clínica veterinária da Guia	Faro
Império do Animal	Faro
Animalvet Hospital Veterinário	Guarda
Clínica Veterinária Serra da Estrela	Guarda
Centro Veterinário de São Jorge	Leiria
Clínica Veterinária do Lis	Leiria
Clínica Veterinária Milagres	Leiria
Clínica Veterinária São Romão	Leiria
Farmanimal Centro Médico Veterinário	Leiria
Vetfigueiró	Leiria
Clínica Veterinária Pombalvet	Leiria
Animais & Cãopanhia Veterinários	Lisboa
Animalcare-centro Veterinário Póvoa da Galega	Lisboa
Clínica Veterinária do Lambert	Lisboa
Clínica Veterinária Mascotes Sortudas	Lisboa
Consultório veterinário da Galiza - Maria Gonçalves	Lisboa
Hospital Veterinário Vasco da Gama-Parque das Nações	Lisboa
Hospital Veterinário Vasco da Gama- Odivelas	Lisboa
Hospital Veterinário Vasco da Gama - Forte da Casa	Lisboa
Manuel Dargent Figueiredo	Lisboa
Pet´Spot/MVF-Veterinário de Família Lda	Lisboa
Vetzoo de Famões	Lisboa
Vip Pets Arruda dos Vinhos	Lisboa
Clínica Veterinária das Laranjeiras	Portalegre
Clínica Veterinária de Santo Onofre	Portalegre
Centro Veterinário de Amarante	Porto
Vallis Vet- Centro Veterinário	Porto
Clínica Veterinária da Areosa	Porto
LEÇAVET- Clínica veterinária	Porto
Consultório veterinário do Freixieiro	Porto
Clínica Veterinária de Oldrões	Porto
SOS Patinhas- Clinica medico veterinária Centralparl, Lda	Porto
Animabilis	Santarém
Bicos, Pêlos e Patas	Santarém
Clínica Veterinária Cão d'Amor	Santarém
Centro Veterinário de Alcanede	Santarém
Clínica Veterinária de Ourém	Santarém
Clinica Veterinaria Torres Pet	Santarém
Hospital Veterinário Tutivete - Centro Cirúrgico de Santarém	Santarém
Centro Veterinário da Costa Vicentina	Setúbal
Doutoras dos animais	Setúbal
Vet Santa Maria	Setúbal
VETSET - Hospital Veterinário	Setúbal

Vetzone - Consultório Veterinário de Grândola	Setúbal
Vetzone - Consultório Veterinário de Santiago do Cacém	Setúbal
Clínica médico-veterinária d'Areosa	Viana do Castelo
Clínica Veterinária de Viana	Viana do Castelo
Clivetviana- Praia de Âncora	Viana do Castelo
Clinica Veterinária de Chaves	Vila Real
Clínica Veterinária de Valpaços	Vila Real
Clinica Veterinária Marãovet	Vila Real
Consultório Veterinário Dra Marta Rebelo	Vila Real
Reguavet Clínica Veterinária	Vila Real
Caniféli - Clínica veterinária Lda - Tondela	Viseu
Caniféli - Clínica veterinária Lda - Santa Comba Dão	Viseu
Clínica Veterinária Douro Sul Lda-Lamego	Viseu
Clínica Veterinária Douro Sul Lda-Peso da Régua	Viseu
Clínica Veterinária Douro Sul Lda-Tarouca	Viseu
Rosela Clínica Veterinária	Viseu

Annex 2 – Questionnaire

Semana da Leishmaniose Canina
Dados do canídeo

CAMV:	Médico Veterinário:
Data colheita (dd/mm/aa):	Amostra nº (ref. papel filtro):
Nome do Canídeo:	Sexo: M ◯ / F ◯
Data nasc. (ou idade aprox.):	Raça:
Pelagem: curta ◯ / média ◯ / comprida ◯	
Localidade onde vive o animal:	
Freguesia onde vive o animal:	
Concelho onde vive o animal:	
O proprietário teve conhecimento do rastreio gratuito: antes de chegar ao CAMV ◯ / depois de chegar ao CAMV ◯	
O animal permanece: exclusivamente dentro de casa ◯ / a maior parte do tempo dentro de casa ◯ / igualmente dentro e fora de casa ◯ / a maior parte do tempo fora de casa ◯ / exclusivamente fora de casa ◯	
O animal é tratado com insecticidas/repelentes ? Não ◯ / Sim ◯ Qual (Quais)? Periodicidade: Mensal ◯ 3 em 3 meses ◯ 8 em 8 meses ◯ anual ◯	
O animal é vacinado? Não ◯ Sim ◯ CaniLeish® ◯ Letifend® ◯ Data da última dose:	
Aspectos clínicos: Animal assintomático ◯ / Clinicamente suspeito de Leishmaniose canina ◯	
O animal tomou medicação nos últimos 15 dias?	
Sinais físicos e laboratoriais eventualmente presentes (discriminar sff.):	
Referência do Proprietário:	

Critérios de exclusão: cães com idade inferior a 6 meses; cães vacinados há menos de 6 meses com CaniLeish.

Annex 3 – Informed Consent Form

Consentimento Informado de participação no projeto "Semana da Leishmaniose Canina"

A leishmaniose canina é uma doença causada por um parasita (*Leishmania*) transmitido por um inseto, o flebótomo, que pode causar, no animal, perda de apetite, perda de peso, feridas na pele, conjuntivite e, até mesmo, a morte.

O que pretendemos nós fazer?

Fazer um rastreio para avaliar cães expostos ao parasita responsável pela leishmaniose canina em Portugal.

O que tenho de fazer para o meu cão participar no estudo**?**

Dar o meu consentimento para que, uma pequena amostra de sangue do meu cão, que poderá ser colhida por outras razões (ex.: análises de rotina, despiste de outra doença, avaliação pré-cirurgica), seja alvo de uma análise prevista neste estudo.

Quais são os benefícios do estudo?

O estudo permitirá detetar cães expostos ao parasita em todo o país. No caso de o resultado ser positivo, este será comunicado ao médico veterinário no espaço de dois meses. Deste modo, o clínico pode avaliar o resultado e, caso ache necessário, sugerir procedimentos adicionais para o diagnóstico definitivo.

Consentimento informado

Ao aceitar que o meu cão participe neste estudo, declaro que me foram comunicadas, pelo médico veterinário assistente, informações suficientes relativas ao mesmo. A participação do(s) meu(s) cão (cães) de que sou detentor garante-me, ainda, o direito de colocar as perguntas que achar convenientes e de obter informação adicional relacionada com o estudo em questão. A análise para este rastreio é-me disponibilizada gratuitamente.

Nome do canídeo:_____

Assinatura do detentor do animal:_____

Local e Data: _____, ____ de _____ de 2021

Eu _____ declaro ter efetuado a colheita de sangue ao canídeo acima identificado e ter explicado ao seu detentor o âmbito e os passos do estudo Semana da Leishmaniose Canina.

Documento para o Médico Veterinário
Estudo realizado pelo IHMT, Universidade NOVA de Lisboa

Consentimento Informado de participação no projeto "Semana da Leishmaniose Canina"

A leishmaniose canina é uma doença causada por um parasita (*Leishmania*) transmitido por um inseto, o flebótomo, que pode causar, no animal, perda de apetite, perda de peso, feridas na pele, conjuntivite e, até mesmo, a morte.

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O estudo permitirá detetar cães expostos ao parasita em todo o país. No caso de o resultado ser positivo, este será comunicado ao médico veterinário no espaço de dois meses. Deste modo, o clínico pode avaliar o resultado e, caso ache necessário, sugerir procedimentos adicionais para o diagnóstico definitivo.

Consentimento informado

Ao aceitar que o meu cão participe neste estudo, declaro que me foram comunicadas, pelo médico veterinário assistente, informações suficientes relativas ao mesmo. A participação do(s) meu(s) cão (cães) de que sou detentor garante-me, ainda, o direito de colocar as perguntas que achar convenientes e de obter informação adicional relacionada com o estudo em questão. A análise para este rastreio é-me disponibilizada gratuitamente.

Nome do canídeo:_____

Assinatura do detentor do animal:_____

Local e Data: _____, ____ de _____ de 2021

Eu _____ declaro ter efetuado a colheita de sangue ao canídeo acima identificado e ter explicado ao seu detentor o âmbito e os passos do estudo Semana da Leishmaniose Canina.

Documento para o Detentor do Animal
Estudo realizado pelo IHMT, Universidade NOVA de Lisboa

Annex 4 – Table of Dogs by Breed

Breed	Frequency
Non-autochthonous breeds	
Akita	1
American Akita	1
American Bully	2
American Staffordshire Terrier	2
American Staffordshire	1
Argentine Dogo	5
Australian Shepherd	1
Basset Hound	4
Beagle	40
Belgian Shepard	12
Bichon (Frisé)	2
Bichon (Maltese)	8
Boerboel	1
Border Collie	22
Boston Terrier	2
Bouvier Bernois	7
Boxer	24
Bull Terrier	7
Bulldog (unspecified)	1
Bulldog (French)	65
Bulldog (English)	4
Cane Corso	8
Cavalier King Charles	1
Chihuahua	24
Cocker	2
Cocker Spaniel (Unspecified)	12
Cocker Spaniel (English)	3
Dalmatian	6
Doberman	4
Great Dane	9
Dogue de Bordeux	3
English Setter	9
Epagneul Breton	26
Fox Terrier	2
German Shepherd	77

Annexes

German Shorthaired Pointer	18
Golden Retriever	33
Golden Retriever (Flat)	1
Greyhound (Unspecified)	1
Greyhound (Galgo Español)	1
Greyhound (Irish)	3
Hungarian Shorthaired Pointer	4
Jack Russel Terrier	24
Labrador Retriever	175
Labrador Retriever (Flat Coated)	1
Pacho Nvarro	1
Pekingese	21
Pinscher	59
Pinscher (Miniature)	6
Pitbull	12
Pitbull Terrier	1
Pointer	11
Pointer (English)	1
Poodle	28
Poodle (Miniature)	1
Poodle (Standard)	1
Poodle (Medium)	1
Pug	12
Pyrenean Mountain Dog	1
Ratonero	1
Rodesian Ridgeback	3
Rottweiler	4
Rough Collie	1
Samoyed	1
Schnauzer	1
Schnauzer (Miniature)	5
Scottish Terrier	1
Siberian Husky	3
Shar Pei	8
Shih Tzu	6
Spitz	4
Spitz (German)	5
Staffordshire Terrier	1
Teckel (aka. Dachshund)	18

Teckel (Wire Haired)	1
Warren Hound (Andalve)	1
Warren Hound (Campanero)	1
Waren Hound (Pelo Cerdeño)	2
Weimaraner	3
West Highland White Terrier	5
Yorkshire Terrier	62
Autochthonous Breeds	
Cão de Fila dos Açores	3
Cão de Gado Transmontano	3
Cão do Barracal Algarvio	1
Castro Laboreiro Dog	6
Rafeiro Alentejano	27
Portuguese Sheep Dog	2
Portuguese Water Dog	2
Pointer (Portuguese)	7
Warren Hound*	40
Warren Hound (Portuguese)*	6
Serra da Estrela	23
Crosses Between Two Known Breeds	14
Mongrels	774

*Warren hounds are native to the Iberian Peninsula, and due to the three autochthonous breeds to Portugal, often are just referred as Podengo (Warren Hound).

Annex 5 – List of Repellents/Insecticides

Insecticide / Repelent	Type	Efficiency against *Phlemotomus*	Protection Duration	Use
Activyl	Pipette	No	--	Repellent
Actyvil Tick Plus	Pipette	Yes	3 weeks	Repellent
Advantix	Pipette	Yes	2-3 weeks	Repellent
Advocate	Pipette	No	--	Repellent
Beaphar/Canishield	Collar	Yes	5-6 months	Repellent
Bioband	Collar	Yes	4 months	Repellent
Bravecto	Chew Pill	No	--	De-wormer / Repellent
Credelio	Chew Pill	No	--	De-wormer
Cestal	Chew Pill	No	--	De-wormer
Dixie	Collar	No	--	Repellent
Dosalid	Chew Pill	No	--	De-wormer
Effipro	Spray	No	--	De-wormer / Repellent
Effitix	Pipette	Yes	4 weeks	Repellent
Frontline	Pipette	No	4 weeks	Repellent
Merlin	Collar	Yes	5-6 months	Repellent
Nextgard	Chew Pill	No	--	De-wormer
Pecusanol	Collar / Shampoo	No	4 months	De-wormer / Repellent
Permethrin	Active Ingredient	Yes	Depends on Dose	Repellent
Pyrethrin	Active Ingredient	Yes	Depends on Dose	Repellent
Prevendog	Collar	Yes	5 months	Repellent
Pulvex	Collar / Pipette	Yes	4 months /4 weeks	Repellent
Scalibor	Collar	Yes	12 months	Repellent
Seresto	Collar	No	8 months	Repellent
Simparica	Chew Pill	No	--	De-wormer / Repellent
Spot-On	Pipette	Unknown	Depends on Brand	Repellent
Vectra	Pipette	Yes	4 weeks	Repellent

Annexes

Notes:

Spot-On generally refers to monthly pipettes. Several are efficient, but some are not. In some cases, the Spot-On brand used was not referred, and therefore it is impossible to verify the efficiency.

To verify the efficiency of each repellent, insecticide or de-wormer in reducing *Leishmania* infection, manufacturer instructions were consulted in Medicine Information sheet through ema.europa.eu (European Medicines Agency) or medvet.dgav.pt (Direção Geral de Alimentação e Veterinária (DGAV)).

Annex 6 – 96-well microplate

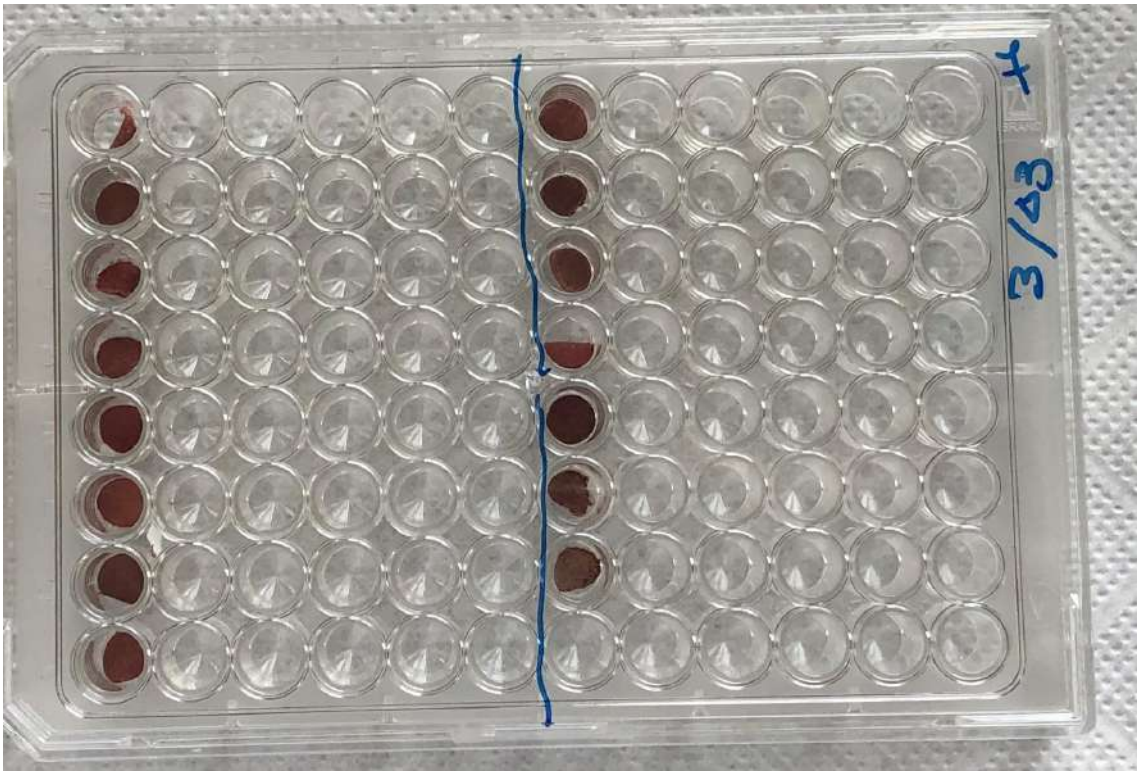

Figure 18 - 96-well microplate with 5.5 mm filter paper disks with blood corresponding to dog's samples.

Annex 7 - Flask with lyophilized DAT antigen

Figure 18 - Flask with lyophilized DAT antigen (KIT Biomedical Research)