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Serological survey of *Leishmania infantum* in apparently healthy dogs in different areas of Spain

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Abstract

Background: Canine leishmaniosis caused by *Leishmania infantum* is an endemic disease in Spain. The dog is considered the main reservoir, and the detection of specific serum antibodies against *L. infantum* antigen is the most used technique for diagnosing this infection. The LEISCAN LEISHMANIA ELISA test is a commercialized enzyme-linked immunosorbent assay for the detection and measurement of canine anti-*Leishmania* serum antibodies.

Objectives: The aim of this study was to assess seroprevalence results of apparently healthy dogs in different areas of Spain using LEISCAN.

Methods: Collection of sera from 5451 apparently healthy dogs was performed between 2020 and 2021 in different areas of Spain. Dogs were of adult age (≥12 months), were not previously diagnosed with clinical leishmaniosis or vaccinated against *Leishmania* and did not present clinical signs compatible with *L. infantum* infection. LEISCAN was performed following the manufacturer's protocol.

Results: The overall seroprevalence was 5.5%. The highest seroprevalences were found in the Southeast of Spain: Comunidad Valenciana (14%) and Región de Murcia (14%), whereas the lowest seroprevalences were found in Northern Spain: Galicia (1%), Navarra (2%) and Castilla y León (2%) (p-value <0.001).

Conclusions: In conclusion, the seroprevalence for *L. infantum* in apparently healthy dogs in Spain varied from almost no infection to being over 10%.

KEYWORDS
canine, diagnosis, ELISA, leishmaniosis, seroprevalence

1 | INTRODUCTION

Canine leishmaniosis caused by the protozoan *Leishmania infantum* is a zoonotic and endemic disease in Spain (Díaz-Regañón et al., 2020; Gálvez et al., 2020; Montoya-Alonso et al., 2020). *Leishmania infantum* is usually transmitted by the bite of a female phlebotomine sand fly following a digenetic cycle that alternates between two differentiated phases: (a) an extracellular and motile promastigote that colonizes the digestive tract of the vector sand fly and (b) an intracellular and non-motile amastigote that colonizes the monocyte-macrophage system of the vertebrate hosts (Dostálová & Volf, 2012). The dog is considered the main domestic and peridomestic reservoir for *L. infantum* infection in Spain (Dantas-Torres, 2007; Solano-Gallego et al., 2011), whereas other mammals, such as wild canids (Oleaga et al., 2018), rodents (Alcover et al., 2020) and lagomorphs (Molina et al., 2012), may be able to maintain a wild cycle.

Moreover, when a dog is infected, the development of clinical disease depends on the dog's immune response. Classically, two polarized outcomes have been described: (a) a protective T cell-mediated immune response (the dog is infected but does not develop clinical disease) and (b) a non-protective marked humoral immune response with a reduced or absent T cell mediated immunity (the dog develops clinical leishmaniosis) (Toepp & Petersen, 2020). Thus, a wide spectrum of clinical manifestations is documented in dogs with leishmaniosis (Solano-Gallego et al., 2011). Furthermore, in endemic regions, such as Spain, there is a high prevalence of *L. infantum* infection in apparently healthy dogs (Baxarias, Homedes, et al., 2022; Müller et al., 2022), although the prevalence of clinical illness is frequently lower than 10% (Solano-Gallego et al., 2009).

Detection of serum specific antibodies against *Leishmania* is the most frequently used technique for detecting infected dogs (Baxarias, Homedes, et al., 2022; Maurelli et al., 2020; Paltrinieri et al., 2016). Furthermore, as an anti-*Leishmania* vaccine is available in Europe, serological screening is mandatory prior to vaccination in dogs (Solano-Gallego et al., 2017). Several commercial serological techniques are available, such as immunochromatographic tests, enzyme-linked immunosorbent assays (ELISA) and immunofluorescent antibody tests (IFI) (Baxarias, Homedes, et al., 2022; Maurelli et al., 2020; Miró et al., 2017; Paltrinieri et al., 2016; Solano-Gallego et al., 2014). However, the interpretation of these techniques differs as ELISA and IFI allow the quantification of antibody levels, whereas immunochromatographic tests are only qualitative techniques and, thus, only give a positive or negative result (Maurelli et al., 2020; Solano-Gallego et al., 2014). Regarding serological quantitative techniques, the difference lies in their interpretation as ELISA presents objective results depending on optical density (OD), whereas IFI presents subjective results depending on the operator's experience (Maurelli et al., 2020; Solano-Gallego et al., 2014).

The LEISCAN LEISHMANIA ELISA test is an enzyme immunoassay for the detection and measurement of canine serum anti-*Leishmania* antibodies (Rodríguez-Cortés et al., 2013; Solano-Gallego et al., 2014). Previous studies have evaluated LEISCAN in dogs and obtained good diagnostic sensitivity and specificity (Rodríguez-Cortés et al., 2013; Solano-Gallego et al., 2014).

The aim of this study was to assess seroprevalence results of apparently healthy dogs in different areas of Spain using LEISCAN.

2 | MATERIALS AND METHODS

2.1 | Dogs

Collection of sera from 5451 apparently healthy dogs was performed between June of 2020 and June of 2021 by veterinarians from 68 veterinary practices and 12 dog shelters in different areas of Spain (Table 1). The inclusion criteria of dogs enrolled were adult age (≥12 months), not have been previously diagnosed with clinical leishmaniosis nor vaccinated against *Leishmania*, and the absence of clinical signs based on clinical history and a full clinical examination that includes general appearance (physical body condition, mentation, posture), record of vital signs (temperature, pulse, heart and respiratory rates and capillary refill time) and to check all body from head to tail.

2.2 | Detection of anti-*Leishmania* antibodies using LEISCAN

LEISCAN (Ecuphar Veterinaria SLU, Spain) was performed to detect anti-*L. infantum* antibodies in serum following the manufacturer's protocol. Briefly, samples were diluted using the dilution solution included in the kit and incubated for 10 min at room temperature in 96-well plates. Then, washes were performed five times with the diluted washing solution, and afterwards, 100 μL of conjugate were added in each well. After incubating the plate for another 5 min at room temperature, washes were repeated, and 100 μL of substrate were added to each well. Finally, after an incubation of 10 min at room temperature in the dark, stop reaction solution was added to the plate, and the results were read at 450 nm in a spectrophotometer (MB-580 HEALES; Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China).

LEISCAN results were calculated using the following formula: ratio sample = OD sample/OD low control positive. Samples were classified following the protocol as positive (when the ratio sample was ≥1.1), dubious (when the ratio sample was ≥0.9 and <1.1) and negative (when the ratio sample was <0.9). Furthermore, when samples were positive, samples were further classified between low positive (when the ratio sample was ≥1.1 and <2.0) and high positive (when the ratio sample was ≥2.0).

2.3 | Statistical analysis

The statistical analysis was performed using the package Stats for the software R i386 3.6.1 for Windows, using *t* test to compare the altitudes of the centres between the LEISCAN results (positive or negative) and using chi-square tests to compare seroprevalence and antibody levels between autonomous communities, the different areas of Spain (North, South, East and West) and the type of centre that collected the samples (veterinary practice or dog shelter). A *p*-value of <0.05 was considered statistically significant. Maps were created using the Free and Open Source QGIS 3.10.4 for Windows. Information about altitudes of the centres were collected from Google Earth Web (https://earth.google.com/web/).

3 | RESULTS

The overall seroprevalence and dubious results of the 5451 dogs and their geographical distribution are shown in Table 1. The highest seroprevalences were found in the Southeast of Spain: Comunidad Valenciana and Región de Murcia, whereas the lowest seroprevalences were found in Northern Spain: Galicia, Navarra and Castilla-León (Table 1) (Figure 1) (chi-square: $\chi^2 = 88.96$, $df = 1$, $p < 0.001$). Furthermore, 170

TABLE 1 Seroprevalence and dubious rates of *L. infantum* infection in apparently healthy dogs classified by Spanish autonomous community.

Autonomous community (number of dogs)	Bioclimate (Gálvez et al., 2020)	Number of veterinary practices and dog shelters (total)	Mean of the metres of altitude (±SD)	Percentage of seroprevalence (95%CI)	Percentage of dubious results (95%CI)
Andalucía (1234)[a]	TM, MM, SM, OM and CM	11 and 2 (13)	468.8 (±287.4)	4.5 (3.4–5.9)	2 (1.3–2.9)
Aragón (516)	MM, SM, ST and OT	7 and 1 (8)	625.3 (±356)	6.7 (4.7–9.2)	1.6 (0.7–3)
Islas Baleares (189)	TM and MM	3 and 0 (3)	64.9 (±40.6)	7.4 (4.1–12.1)	3.7 (1.5–7.5)
Castilla-La Mancha (18)[b]	MM, SM, OM and CM	2 and 0 (2)	552.2 (±10)	41.2 (18.4–67.1)	5.6 (0.1–27.3)
Castilla y León (216)	MM, SM, OM and CM	3 and 0 (3)	873.2 (±259.4)	1.9 (0.5–4.8)	2.3 (0.8–5.3)
Cataluña (851)[a]	TM, MM, SM, ST and OT	12 and 1 (13)	240.2 (±221.7)	3 (1.9–4.3)	0.8 (0.3–1.7)
Comunidad de Madrid (358)	MM, SM, OM and CM	6 and 1 (7)	599.4 (±45.9)	5.7 (3.5–8.6)	1.1 (0.3–2.8)
Comunidad Valenciana (325)	TM, MM, SM, OM and CM	5 and 1 (6)	65.6 (163.9)	13.9 (10.3–18.2)	2.5 (1.1–4.8)
Extremadura (472)	MM	4 and 0 (4)	298 (±89.8)	3.8 (2.3–6)	0.8 (0.2–2.2)
Galicia (235)	SM, MT and ST	2 and 1 (3)	38.8 (±16.3)	0.9 (0.1–3.1)	0.4 (0–2.4)
Navarra (414)	SM, MT and ST	3 and 3 (6)	362 (± 67.9)	2.4 (1.2–4.4)	0.2 (0–1.3)
País Vasco (35)[b]	SM, MT and ST	1 and 0 (1)	500 (±0)	5.9 (0.7–19.7)	2.9 (0.1–14.9)
Región de Murcia (468)	TM, MM, SM, OM and CM	7 and 1 (8)	254.7 (±176.9)	13.7 (10.7–17.3)	3.6 (2.1–5.8)
La Rioja (120)	SM, MT and ST	2 and 1 (3)	383.3 (±66.7)	2.5 (0.5–7.1)	0 (0–3)
Total (5451)		68 and 12 (80)	373.3 (±294)	5.5 (4.9–6.1)	1.6 (1.3–2)

Abbreviations: CI; confidence interval; CM, crioro-Mediterranean; MM, meso-Mediterranean; MT, mesotemperate; OM, oro-Mediterranean; OT, orotemperate; SD, standard deviation; SM, supra-Mediterranean; ST, supratemperate; TM, thermo-Mediterranean.

[a]The Spanish autonomous communities from which high numbers of dogs were included were Andalucía and Cataluña.

[b]The significance of results obtained in País Vasco and Castilla-La Mancha remains to be further studied due to the limited number of dogs collected in these regions.

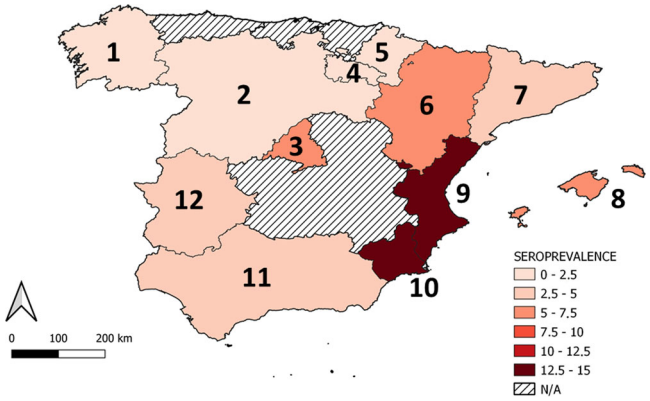

FIGURE 1 Geographical distribution of *L. infantum* seroprevalence in apparently healthy dogs in Spain: (1) Galicia, (2) Castilla y León, (3) Comunidad de Madrid, (4) La Rioja, (5) Navarra, (6) Aragón, (7) Cataluña, (8) Islas Baleares, (9) Comunidad Valenciana, (10) Región de Murcia, (11) Andalucía, (12) Extremadura. N/A, not applicable.

(56.7%) of the seropositive dogs were considered low positive, whereas 130 (43.3%) were high positive. No differences in antibody levels were observed among dogs from different Spanish regions ($p > 0.05$).

The majority of centres that collected samples were veterinary practices (68/80, 85%), whereas only a few were dog shelters (12/80; 15%). The geographical distribution of centres is shown in Table 1. The number of veterinary practices and dog shelters was similar in all regions studied ($p > 0.05$). Practitioners collected samples from 4733 apparently healthy dogs (86.8% of the total), whereas dog shelters collected 718 samples (13.2%). The majority of dog shelter samples were collected in Andalucía (147, 20.5%) and Navarra (137, 19.1%). Furthermore, there was a higher proportion of dogs sampled in dog shelters in Northern Spain (243, 28.1%) when compared to the Southeast of Spain (111, 13.7%) (chi-square: $\chi^2 = 52.14$, $df = 1$, $p < 0.001$). No differences in seroprevalence were detected between dogs from veterinary practices and dogs from shelters ($p > 0.05$).

The mean altitude depending on the geographical distribution of the veterinary practices is also shown in Table 1. The highest altitudes were found in Castilla-León (over 800 m) and Aragón (over 600 m), whereas the lowest altitudes were found in Galicia (under 40 m), Islas Baleares (under 70 m) and Comunidad Valenciana (a under 70 m) (Table 1). No differences in altitude were detected when comparing between seropositive and seronegative dogs ($p > 0.05$).

4 | DISCUSSION

The seroprevalence of *L. infantum* infection in dogs in Spain has been previously investigated, and seroprevalences of around 10% between 2011 and 2020 have been described (Table 2) (Díaz-Regañón et al., 2020; Gálvez et al., 2020; Miró et al., 2022; Montoya-Alonso et al., 2020). The seroprevalence in the present study (5.5%) is lower than expected. However, the reason could be explained by the inclusion criteria as only apparently healthy dogs with no clinical signs were included in the study as it is well known that sick dogs presented higher seroprevalence when compared with apparently healthy dogs (Solano-Gallego et al., 2006). Besides, some regions (País Vasco and Castilla-La Mancha) sampled a limited number of dogs, and thus, the results obtained were not representative of the specific region. Furthermore, dubious results were not considered positive results and; therefore, the seroprevalence was lower in all Spanish regions. In addition, it is likely that the differences observed on seroprevalences might also be due to the variable diagnostic performance of previous studies performed (Maurelli et al., 2020; Rodríguez-Cortés et al., 2013; Solano-Gallego et al., 2014).

Previous serosurveys performed in dogs from other countries have documented different results (Table 2). In a study performed in Northwestern Iran (Barati et al., 2015), a higher seroprevalence in asymptomatic dogs (23%) was observed when compared to previous studies (17%) performed in the dog population in the same area (Moshfe et al., 2008). On the other hand, in a serosurvey performed in asymptomatic dogs in Kosovo, an overall seroprevalence of 4% was observed (Xhekaj et al., 2023), which was lower than the seroprevalence detected in both healthy and sick dogs in previous years (18%) (Xhekaj et al., 2020). In the present study, a similar result to Kosovo (Xhekaj et al., 2020, 2023) was observed as the overall seroprevalence (5.5%) was lower than the previous seroprevalences (around 10%) found in the Spanish dog population (Díaz-Regañón et al., 2020; Gálvez et al., 2020; Miró et al., 2022; Montoya-Alonso et al., 2020).

In terms of specific investigated regions, previous serological surveys in Spain have documented similar results, detecting lower seroprevalences in the North of Spain and higher in the Southeast (Díaz-Regañón et al., 2020; Gálvez et al., 2020; Montoya-Alonso et al., 2020) which is also the nearest region to the Mediterranean. However, the results found in Islas Baleares are lower than expected (7.4%) when compared to previous studies that found seroprevalences of around 20% (Baxarias, Viñals, et al., 2022; Gálvez et al., 2020; Montoya-Alonso et al., 2020; Solano-Gallego et al., 2001, 2006). This could be explained with the same reasons as the lower overall seroprevalence: only sampling apparently healthy dogs, not considering dubious results as positive and the test performed. Furthermore, the samples were collected all year around in the present study, whereas in previous studies, all the samples were collected in a specific time of the year (Baxarias, Viñals, et al., 2022; Solano-Gallego et al., 2001) or, conversely, were collected for various years (Gálvez et al., 2020; Montoya-Alonso et al., 2020). These results highlight the need to use preventive measures against *L. infantum* in any region of Spain (Baxarias, Homedes, et al., 2022; Miró et al., 2017) as well as to perform an annual health check-up and serology for the detection of anti-*Leishmania* antibodies in dogs living in *L. infantum*-endemic countries (Miró et al., 2017; Solano-Gallego et al., 2017).

Regarding the antibody levels of the seropositive dogs, surprisingly, there were similar percentages of low (57%) and high (43%) positive dogs. Apparently healthy dogs usually present low anti-*Leishmania* antibody levels, whereas dogs with clinical leishmaniosis present high antibody levels (Solano-Gallego et al., 2006, 2017). However, these percentages could be also affected by not considering dubious results

TABLE 2 Seroprevalence rates of *L. infantum* infection in dogs in different countries with similar macroclimates.

Country	Macroclimate (European Environment Agency, 2017)	Number of dogs	Type of dogs	Percentage of seroprevalence (serological test) (%)	References
Albania	Mediterranean and alpine	602	Dogs with and without clinical signs	5.1 (IFI)	Hamel et al. (2016)
		308	Dogs with and without clinical signs	3.2 (ELISA)	Myrseli et al. (2016)
Algeria	Mediterranean and desertic	4812	Symptomatic and asymptomatic dogs	21.2 (ELISA, IFI, DAT, rapid test)	Touhami et al. (2023)
		227	Dogs with and without clinical signs	35.7 (IFI, rapid test)	Medkour et al. (2020)
Bosnia and Herzegovina	Mediterranean, continental and alpine	180	No data	16.7 (IFI)	Colella et al. (2019)
		172	No data	11 (ELISA)	Miró et al. (2022)
Croatia	Mediterranean and alpine	200	Asymptomatic dogs	8 (IFI)	Zivicnjak et al. (2007)
		1761	No data	7 (ELISA)	Miró et al. (2022)
		435	Apparently healthy dogs	1.4 (rapid test)	Mrljak et al. (2017)
Cyprus	Mediterranean	278	Symptomatic and asymptomatic dogs	1.1 (IFI)	Beyhan et al. (2016)
		281	Symptomatic and asymptomatic dogs	3.6 (IFI)	Çanakçı et al. (2016)
Egypt	Mediterranean and desertic	450	Asymptomatic dogs	21.3 (ELISA)	Selim et al. (2021)
France	Mediterranean, Atlantic, continental and alpine	80	Symptomatic and asymptomatic dogs	6.2 (IFI)	Davoust et al. (2013)
		5307	No data	8.7 (ELISA)	Miró et al. (2022)
Greece	Mediterranean	2620	Asymptomatic dogs	20 (ELISA, IFI)	Athanasiou et al. (2012)
		9568	No data	18.5 (ELISA)	Miró et al. (2022)
Iran	Alpine, anatolian and steppic	508	Asymptomatic dogs	23.4 (DAT)	Barati et al. (2015)
		384	Symptomatic and asymptomatic dogs	17.4 (DAT)	Moshfe et al. (2008)
Israel	Mediterranean and desertic	60	Dogs with and without clinical signs	37 (ELISA)	Baneth et al. (2020)
Italy	Mediterranean and continental	90,532	No data	11.9 (ELISA)	Miró et al. (2022)
		13,292	Dogs with and without clinical signs	6.7 (IFI)	Rombolà et al. (2021)
Kosovo	Mediterranean, continental and alpine	125	Dogs with and without clinical signs	18.4 (ELISA)	Xhekaj et al. (2020)
		285	Asymptomatic dogs	4.2 (ELISA)	Xhekaj et al. (2023)
Malta	Mediterranean	289	No data	15.9 (ELISA)	Miró et al. (2022)
Montenegro	Mediterranean and alpine	1500	Dogs with suspected leishmaniosis	83 (IFI)	Andric et al. (2013)
Morocco	Mediterranean and desertic	1514	No data	15.8 (rapid test)	El Berbri et al. (2020)
		3900	Dogs with and without clinical signs	14.9 (ELISA, IFI, rapid test	El-Mouhdi et al. (2022)
Portugal	Mediterranean and Atlantic	1860	Dogs with and without clinical signs	12.5 (DAT)	Almeida et al. (2022)
		1329	No data	13.8 (ELISA)	Miró et al. (2022)

(Continues)

TABLE 2 (Continued)

Country	Macroclimate (European Environment Agency, 2017)	Number of dogs	Type of dogs	Percentage of seroprevalence (serological test) (%)	References
Slovenia	Mediterranean, continental and alpine	80	Asymptomatic dogs	3.7 (ELISA)	Dumitrache et al. (2016)
		465	Asymptomatic dogs	1.9 (ELISA)	Kotnik et al. (2021)
Spain	Mediterranean and Atlantic	556	Dogs with and without clinical signs suggestive of vector-borne diseases	9 (rapid test)	Díaz-Regañón et al. (2020)
		1076	Dogs with and without clinical signs	8.1 (IFI)	Gálvez et al. (2010)
		1739	Dogs with and without clinical signs	10.1 (IFI)	Gálvez et al. (2020)
		439	Dogs with and without clinical signs	13 (IFI)	Martín-Sánchez et al. (2009)
		1100	Dogs with and without clinical signs	15.7 (rapid test)	Miró et al. (2013)
		98,737	No data	10.7 (ELISA)	Miró et al. (2022)
		4643	No data	10.4 (rapid test)	Montoya-Alonso et al. (2020)
		466	Dogs with and without clinical signs	30 (ELISA)	Solano-Gallego et al. (2006)
Tunisia	Mediterranean and desertic	317	Dogs with and without clinical signs	58.3 (IFI)	Bouattour et al. (2021)
Turkey	Mediterranean, black sea and anatolian	490	Dogs with and without clinical signs	5.3 (IFI, DAT)	Ozbel et al. (2000)

Abbreviations: DAT, direct agglutination test; ELISA, enzyme-linked immunosorbent assay; IFI, indirect immunofluorescence assay.

as positive dogs and the test performed. Other tests with higher sensitivities (Solano-Gallego et al., 2014) could detect a proportion of these dubious results as very low seropositive dogs increasing the number of low positive dogs of the study.

Interestingly, it has been described that owned dogs usually have a lower risk of infection than dogs living in dog shelters or kennels (Rombolà et al., 2021), which could be associated to environmental factors such as living outdoors, although it has also been described the opposite, being dogs living in kennels less likely to present *L. infantum* infection (Tamponi et al., 2021). In the present study, no differences in seroprevalence were detected between owned dogs from veterinary practices and dogs from shelters. However, a higher percentage of samples from dogs living in dog shelters was observed in Northern Spain, which was also the region with the lowest seroprevalences and could be affecting the overall seroprevalence of dog shelters. Another important factor could be that owned dogs are more frequently tested in the clinical setting (for clinical suspicion or annual health check-up) than dogs living in dog shelters (usually only sampled at kennel admittance) (Rombolà et al., 2021). In endemic areas, it is appropriate to screen dogs for *L. infantum* antibodies at least every 6–12 months (Solano-Gallego et al., 2009, 2017).

Regarding the vector importance over seroprevalences, the distribution and density of sand flies in a region affect directly the transmission of *L. infantum* and the rate of infected dogs, and thus, changes in the vector distribution can determine changes in the seroprevalence of a region (Ballart et al., 2014; Díaz-Sáez et al., 2021). For example, in recent studies, phlebotomine sand flies have been described to be able to maintain *L. infantum* infection in regions above 1300 m (Díaz-Sáez et al., 2021), which are higher altitudes than previously reported and, therefore, could be able to infect animal populations that were not previously affected by *L. infantum* (Alten et al., 2016; Hartemink et al., 2011). However, in the present study, all sampled areas were under 1000 m of altitude, and no differences were detected among different altitudes. Another factor that could also impact vector density and species is the bioclimate of the region and its climate changes (Ballart et al., 2014; Semenza & Suk, 2018). Spain presents several bioclimates such as supratemperate and mesotemperate in the North and meso-Mediterranean and thermo-Mediterranean in the Southeast (Ballart et al., 2014; Gálvez et al., 2020). For example, Ballart et al. (2014) reported that *Phlebotomus perniciosus* is mainly present in meso-Mediterranean and supra-Mediterranean bioclimates, whereas *Phlebotomus ariasi* preferred coline, subalpine and montane bioclimates. In the present study, higher seroprevalences were detected in the Southeast of Spain (meso-Mediterranean bioclimate) and could be related to higher densities of sand flies in this region as previously reported (Ballart et al., 2014).

Dog populations of countries that have a Mediterranean macroclimate in some regions present a similar seropositivity to *L. infantum* with a range from 4% to 20% (Table 2). For example, Spain, Portugal, France and Italy have similar seroprevalence rates of around 10%. Interestingly, Greece, which is the country that only presents a Mediterranean macroclimate, reports higher seroprevalence rates of 20% (Table 2).

This study presents some limitations. First, there were some Spanish regions with a limited number of sampled dogs which could not be truly representative of that region. Second, there was access to limited information about the sampled dogs, and therefore, statistical analysis of dog characteristics such as age or breed was not possible to perform. Finally, LEISCAN protocol classified some samples as dubious and recommended to repeat the test in 6 months which was not possible in this study. Even when repeating the test with the same sample, the majority of samples presented a second dubious result which could not be further classified as positive and negative.

In conclusion, the seroprevalence for *L. infantum* as detected by the ELISA technique used in apparently healthy dogs in Spain varied from almost no infection in the Northern areas of Spain to being over 10% in the Southeast close to the Mediterranean basin. These results highlight the need to use preventive measures against *L. infantum* in any region of Spain and to perform an annual check-up that includes a quantitative serological test.

AUTHOR CONTRIBUTIONS

Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing – original draft; writing – review and editing: Marta Baxarias. *Funding acquisition; methodology; project administration; resources; supervision*: Cristina Mateu. *Investigation; resources; writing – review and editing*: Guadalupe Miró. *Conceptualization; funding acquisition; project administration; resources; supervision; validation; writing – review and editing*: Laia Solano Gallego.

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CONFLICT OF INTEREST STATEMENT

CM has financial competing interests as they receive a salary from Ecuphar Veterinaria SLU which markets and LEISCAN.

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Ecuphar veterinaria SLU

DATA AVAILABILITY STATEMENT

The data is available from the corresponding author on reasonable request.

ETHICS STATEMENT

Study authorization was obtained from the Spanish authority, Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) with the authorization number 008/EPA-2383ESP.

CONSENT TO PARTICIPATE

Consent was obtained from the owner or the tutor of the dog(s) to collect the sample and perform LEISCAN.

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