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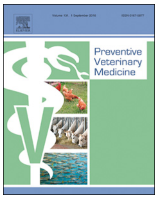

Seroprevalence of canine *Leishmania infantum* infection in the Mediterranean region and identification of risk factors: The example of North-Eastern and Pyrenean areas of Spain

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ABSTRACT

The Mediterranean basin is an endemic region for canine leishmaniosis (CanL), where it represents a major veterinary problem and raises human health concerns. However, the distribution of the disease is heterogeneous and not all countries and locations have been equally studied and characterized. This work describes the situation of CanL in Girona province (Catalonia, Spain), for which no data has been previously reported, and presents a relevant study to exemplify other areas with similar characteristics across the region. Four cross-sectional seroprevalence surveys were performed from 2012 to 2016 throughout the province, including 36 sampling stations in 26 localities and a total of 593 dogs. For each animal, individual and location variables were also collected. Additionally, each dog owner answered a questionnaire about their knowledge of CanL and preventive methods used. Blood samples were analysed by an in-house ELISA and a mixed logistic regression model was used to assess the relationship between pre-determined variables and dog seropositivity. A Spearman's correlation was used to assess the association between dog owners' perceived risk of CanL and *Leishmania infantum* seropositivity in dogs at a given location. The overall true seroprevalence estimated for Girona province was 19.5% (95%CI: 15.5–23.5), of which only 6.8% (10/146) were considered symptomatic. Age of the dog [OR = 1.21 (95%CI: 1.11-1.31); $p < 0.001$] and altitude [OR = 0.02 (95%CI: 0.001-0.19); $p = 0.001$] were identified as risk factors for the infection. The results obtained in this study are expected to aid in the implementation of directed control programmes in CanL endemic areas throughout Europe, as well as to provide suitable data for the design of better risk assessment maps of the disease.

1. Introduction

Canine leishmaniosis (CanL) is a zoonotic parasitic disease caused by *Leishmania infantum*, widely distributed in the Mediterranean area (Dujardin et al., 2008). In this region, *L. infantum* transmission is mainly vectorial through the bite of phlebotomine sand flies of the genus *Phlebotomus*, subgenus *Larroussius*. The domestic dog is the main vertebrate reservoir of the parasite (Alvar et al., 2004). CanL is a multisystemic disease that can present variable, usually unspecific, clinical signs. However, in endemic regions, the high proportion of asymptomatic dogs favours the unnoticed spread of *L. infantum* infection in the dog population (Baneth et al., 2008). Asymptomatic seropositive dogs are at risk of developing the clinical disease throughout their lives (Baneth et al., 2008) and are infectious for sand flies, which makes them permanent and unnoticed reservoirs of the parasite for other dogs and humans (Molina et al., 1994). Likewise, the early detection of these asymptomatic carriers is crucial for the control of the disease both in endemic and in non-endemic areas, as it is known that the infection is

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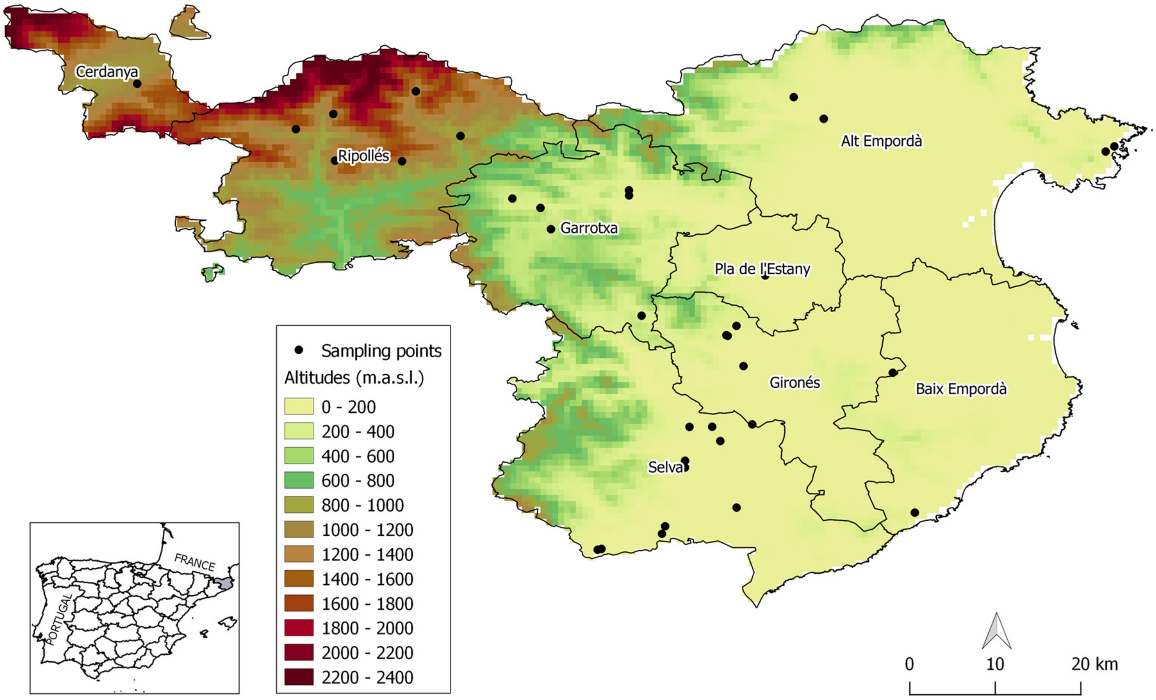

Fig. 1. Map of altitudinal distribution in Girona province. Study sampling locations are marked as black dots.

spreading to northern European regions through dog movement (Maia and Cardoso, 2015).

Spain is an endemic country for CanL and, as observed in other endemic areas, the distribution of the infection is highly heterogeneous throughout the territory (Miró and Molina, 2006). For this reason, Mediterranean endemic regions would benefit greatly from CanL directed control efforts, targeted at areas with higher levels of infection. CanL seroprevalence in owned dogs in Spain ranges from 1.6% in the northwest (Miró and Molina, 2006) to 34.6% in the south (Morillas-Márquez et al., 1996), with a range of intermediate values reported across the territory (Alcover et al., 2013; Ballart et al., 2013; Goyena et al., 2016; Martín-Sánchez et al., 2009; Miró et al., 2017; Solano-Gallego et al., 2001). Nevertheless, as in other Mediterranean countries, the map of CanL distribution in Spain is far from complete, with many regions still lacking documented information.

Catalonia, in the north-east of Spain, is considered one such endemic area for CanL. Here, like in other regions, identifying locations for the implementation of CanL directed control programmes is constrained by the heterogeneous distribution of the infection and the lack of published data on CanL prevalence. Historically, the south of Catalonia was known for the presence of well-established and important foci of CanL (Fisa et al., 1999; Portús et al., 2007) but recent studies in northern areas such as the Lleida region (Ballart et al., 2013) showed that the infection is more widespread than previously thought. Furthermore, results of a recently published questionnaire-based study suggest that Girona province, in the north-east of Catalonia, may be an endemic area of CanL (Lladró et al., 2017). In a survey of local veterinarians, the general opinion was that CanL is well established throughout the province and the number of autochthonous cases has risen in the last years. Additionally, Girona province shares a range of physical and climatic characteristics with other Mediterranean locations and is therefore an appropriate example for CanL epidemiological studies.

The objectives of this study were to provide the first data on CanL seroprevalence for Girona province (Catalonia, north-eastern Spain), which could also confirm the hypothesis of CanL endemicity in this region suggested by Lladró et al. (2017), and to identify possible individual and location risk factors associated with the infection in the study area which can be applied in the control of CanL in other Mediterranean endemic regions.

2. Materials and methods

2.1. Study area and population

Girona province (42°10′0″N, 2°40′0″E; area of 5,910 km^2) is located in the north-east of Catalonia (Spain). It is delimited by the Mediterranean Sea (to the east), France (to the north), and by Barcelona and Lleida Catalan provinces (to the south and west, respectively), all endemic for CanL. Girona is divided into nine counties with altitudes ranging from zero meters above sea level (m a.s.l.) to 2,910 m a.s.l. Habitats and climates vary from Mediterranean on the coast to alpine in the Pyrenees. Annual mean temperatures range from 16 °C in the southern counties to 5 °C in the north of the province, though maximum and minimum temperatures can reach 39 °C and-16 °C, respectively. Mean relative humidity varies from 61% to 81% and average annual rainfall ranges from 550 mm to 1350 mm (Servei Meteorològic de Catalunya, 2016).

Study individuals were recruited through local veterinarians registered in the regional veterinary association (Col.legi Oficial de Veterinaris de Girona – COVGi). After an informative talk about CanL, a number of professionals were willing to participate by being the link between their clients and the project researchers. Dog owners consisted mostly of wild boar hunters, who usually keep large packs of dogs, allowing the sampling of several animals in the same location. Four cross-sectional surveys were conducted between April 2012 and March 2016 in different locations of north-eastern and Pyrenean areas of Spain, in Girona province, including 36 sampling stations in 26 localities (Fig. 1).

2.2. Sample collection and serological techniques

Blood samples from all animals were collected by cephalic or jugular venepuncture to 5 mL EDTA tubes. Plasma was obtained and preserved at −40 °C. Samples were analysed by an in-house enzyme-linked immunosorbent assay (ELISA) for the presence of anti-*L.*

Table 1
True seroprevalence for canine *Leishmania infantum* infection observed in each locality and overall true seroprelavence calculated per county and for Girona province.

County	Locality	No. sampling points	No. sampled dogs (No. positive dogs)	Seropositive dogs (%)	True seroprevalence % (95% CI)
Alt Empordà	Cadaqués	2	29 (12)	41.4	41.8
	Darnius	2	18 (6)	33.3	31.1
	Ordis	1	31 (9)	29.0	25.4
	Total	**5**	**78 (27)**	**34.6**	**32.8 (20.6-45.0)**
Baix Cerdanya	Urtx	**1**	**30 (1)**	**3.3**	**0**
Baix Empordà	Sant Feliu de Guíxols	**1**	**19 (10)**	**52.6**	**56.8 (30.9-82.8)**
Garrotxa	Hostalnou de Bianya	2	21 (3)	14.3	5.7
	Montagut	2	55 (16)	29.1	25.5
	Olot	1	12 (5)	41.7	42.2
	Sant Esteve de Llémena	1	21 (2)	9.5	0
	Total	**6**	**109 (26)**	**23.9**	**18.5 (9.2-27.7)**
Gironès	Aiguaviva	1	30 (4)	13.3	4.4
	Canet d'Adri	3	44 (35)	79.6	92.7
	Madremanya	1	20 (3)	15.0	6.7
	Sant Gregori	1	24 (13)	54.2	58.9
	Total	**6**	**118 (55)**	**46.6**	**48.8 (38.4-59.2)**
Plà de l'Estany	Banyoles	**1**	**15 (7)**	**46.7**	**48.9 (19.7 -78.0)**
Ripollés	Bruguera	1	9 (0)	0	0
	Camprodon	1	8 (0)	0	0
	Ogassa	1	32 (0)	0	0
	Serrat	1	6 (0)	0	0
	Setcases	1	3 (0)	0	0
	Ventola	1	13 (0)	0	0
	Total	**6**	**71 (0)**	**0**	**0**
Selva	Brunyola	1	10 (0)	0	0
	Maçanet de la Selva	1	17 (3)	17.7	10.2
	Massanes	2	29 (3)	10.3	0.5
	Riells i Viabrea	2	31 (7)	22.6	16.8
	Sta Coloma de Farners	2	32 (0)	0	0
	Vilobí d'Onyar	2	34 (7)	20.6	14.1
	Total	**10**	**153 (20)**	**13.1**	**4.1 (0-10.3)**
Total for Girona province		**36**	**593 (146)**	**24.6**	**19.5 (15.5-23.5)**

infantum antibodies, using a technique previously described (Ballart et al., 2013; Riera et al., 1999). Briefly, dog plasma samples diluted at 1:400 were incubated in titration plates (Costar®) previously coated with sonicated whole promastigotes at a protein concentration of 20 μg/ml in 0.05 M carbonate buffer at pH 9.6. Protein A peroxidase ((1:30,000, Sigma®) was used as conjugate and reactions were stopped with H_2SO_4 3 M when a pre-determined positive control serum reached an optical density of 450 read at 450 nm. Sample optical densities were then read at 492 nm. All samples were run in duplicate and calibrator, positive and negative serums were included in all plates. Results were expressed in standard units (U) compared to a calibrator control sample set arbitrarily at 100U. The cut-off was established at 24U.

2.3. Data collection

In addition to sample collection, information was gathered from each sampling location (geographical coordinates, altitude, county, nearest locality, type of habitat, and presence of other domestic and farm animals) and each animal's individual characteristics (sex, age, breed, given use, type of night shelter, and presence of visible CanL clinical signs). Clinical exams were performed by veterinarians and the criteria for classifying dogs as "symptomatic" were the detection of the following clinical signs: weight loss, lymphadenomegaly, periocular or diffuse alopecia, onychogryphosis, ocular lesions, and/or pale mucous membranes. Dog owners were asked about their previous knowledge of CanL, as well as control measures regularly taken to prevent the disease. This data was then used to determine possible risk factors associated with CanL seroprevalence in the population studied.

2.4. Statistical analysis

True seroprevalence was calculated following the method described in Cortes et al. (2012). The formula used was: true prevalence (TP) = [apparent prevalence (AP) – 1 + test specificity (Sp)] / [test sensitivity (Se) – 1 + Sp]. Confidence intervals for true prevalence were also calculated with the following formula: TP 95%CI = 1.96 x √ [AP x (1-AP) / sample size (n) x (Se + Sp-1)].

The relationship between CanL seropositivity and a series of individual and location variables was assessed through a mixed logistic regression model. The choice of variables to analyse, as well as the categories defined, were based on those used in previous publications (Ballart et al., 2013; Gálvez et al., 2010a; Martín-Sánchez et al., 2009) and adapted to the characteristics of the present study. In summary, the covariates considered in the analysis were: altitude (< 800/ > 800 m.a.s.l.), type of habitat (rural or between villages/periurban or at the edge of villages/urban or inside villages), presence of other animal species (yes/no), sex (male/female), age (< 1 to 13 years, introduced as a continuous variable), breed (purebred/crossbred), use given (hunting/breeding/others, which includes racing and pet dogs), night shelter (indoors/outdoors), dog owner knowledge of preventive methods against CanL (yes/no), dog owner use of preventive methods against CanL (yes/no) and dog owner use of prophylactic methods against other arthropods (yes/no).

A bivariate logistic regression analysis was performed, in which the relationship between the outcome variable ("dog seropositivity") and each explanatory variable listed above was assessed individually. Statistical significance was set at $p < 0.05$. This was followed by a multivariable mixed logistic regression analysis, in which non-significant covariates ($p > 0.05$) were sequentially deleted through a backward stepwise selection method until a final model was obtained. In this model, "Locality" was introduced as a random-effects variable to account for geographic clustering of the data (Alonso et al., 2010; Ballart et al., 2013) and the year of the survey was included as a fixed-effects variable.

The association between CanL seroprevalence calculated per dog shelter and owner's perception of risk of infection (graded in percentage categories from 0 to 90–100%) was assessed through a Spearman's coefficient correlation.

All statistical analyses were performed using Stata 15 software (StataCorp LP, College Station, TX, USA). Maps were produced in QGIS Desktop version 2.18.11.

3. Results

3.1. Descriptive analysis of the study population

A total of 593 blood samples were obtained from dogs distributed throughout the north-east and Pyrenean areas of Spain, in Girona province, with sampling points ranging from 1 to 10 per county (Table 1).

Sampling sites were mainly rural (corresponding to 50.1% of the dog sample) and periurban (41.8% of sampled dogs), with dog density per site ranging from 3 to 34. Altitudes ranged from 50 to 1,300 m a.s.l., with the majority of dogs living below 800 m a.s.l. (83%). Most dogs were hunting animals (78.9%), but breeding (16%), shelter (2.5%), racing (2.2%) and pet dogs (0.3%) were also represented. A large number of sampled dogs were born in Girona province (60.4%) and were not reported to have left the region. All animals included in the study were kept with other dogs in open kennels during the day time, and the majority were also kept outdoors at night (87.9%). There were other animal species kept in close proximity to 49.6% of the sampled dogs. These included cats, horses, cows, goats and pigs. Observed age average was 3.6 years (SD = 2.9), 58.9% of the dogs were males and 55.4% were crossbred.

3.2. Dog owners' perception on CanL and use of preventive measures

The majority of dog owners showed previous knowledge of CanL (93.9%) and approximately half of them knew preventive methods against CanL (57.6%), although only 27.3% had ever used them (Table 2).

Only a small number of dog owners believed that their dogs were not at risk of contracting CanL during their lifetime (12.1%), with the majority of them believing that the risk of CanL ranged from 5 to 20% (63.7%). The result of the Spearman's correlation showed a positive association between dog owners' perceived risk of CanL infection and CanL seroprevalence ($r_s = 0.5046$; $p = 0.0027$).

Prophylactic methods against CanL, when used, included dog collars (44.5%), spot-on (33.3%) and combined insecticide treatments (22.2%). Vaccination against CanL or immunomodulatory prophylactic treatments had not been used by any of the dog owners. The main reasons given for not using any preventive method against CanL were unawareness (58.3%) and not believing that prophylaxis worked (12.5%).

3.3. CanL study results

From the 593 dogs analysed, 146 were considered seropositive by ELISA. Apparent seroprevalence at the sampling point level ranged from 0% to 79.6%, with a total apparent seroprevalence calculated for Girona province of 24.6% (95% CI: 21.2–28.3). The L. *infantum* in-house ELISA has a specificity of 90% and a sensitivity of 85%, when the chosen cut-off is used. These values were calculated based on a population of 77 dogs (Fisa et al., 2001; Iniesta et al., 2002). Reference positivity status for L. *infantum* infection was determined by parasite detection (culture and/or direct exam and/or PCR) (provided as supplementary material). Considering these values, the estimated true CanL seroprevalence for Girona province was 19.5% (95%CI: 15.5–23.5). Estimated seroprevalence at the county level ranged from 0 to 56.8%. Results for all localities and counties are summarized in Table 1.

Only 10 out of 146 seropositive dogs were considered symptomatic (6.8%). Observed clinical signs included onychogryphosis (n = 9), weight loss (3), skin wounds (3), diffuse alopecia (2), popliteal lymphadenomegaly (2), periocular alopecia (2), and ocular lesions (2).

Table 2
Results of the questionnaire asked to dog owners regarding their knowledge of canine leishmaniosis and the methods used to prevent the infection (n = 33).

Question	No. replies (%)
Have you ever heard of CanL?	
Yes	31 (93.9)
No	2 (6.1)
In your opinion, how great is the risk of any of your dogs having CanL throughout their lives?	
0%	4 (12.1)
5%	9 (27.3)
10%	5 (15.2)
20%	7 (21.2)
50%	1 (3.0)
50-90%	4 (12.1)
90-100%	3 (9.1)
Do you know of any measures to protect your dogs against CanL?	
Yes	19 (57.6)
No	14 (42.4)
Do you use any measure to protect your dogs against CanL?	
Yes	9 (27.3)
No	24 (72.7)
If YES, which method do you use? (n = 9)	
Collar	4 (44.5)[b]
Spot-on	3 (33.3)[b]
Others[a]	2 (22.2)[b]
If NO, why not? (n = 24)	
Unawareness	14 (58.3)[b]
Do not believe it works	3 (12.5)[b]
Too expensive	2 (8.3)[b]
Do not believe there is CanL	1 (4.2)[b]
Others/no answer	4 (16.7)[b]
Do you use any measure to protect your dogs against other arthropods (e.g. ticks, fleas, etc.)	
Yes	25 (75.8)
No	8 (24.2)
If YES, which method do you use? (n = 25)	
Pour-on	12 (48.0)[b]
Sprays	2 (8.0)[b]
Spot-on	1 (4.0)[b]
Others[a]	10 (40.0)[b]

[a] Includes the use of others or multiple preventive measures.
[b] Percentage based on the total for the subgroup YES or NO of the previous answer.

3.4. Bivariate statistical analysis

One of the dog kennels included in the seroprevalence study (Banyoles, Plà de l'Estany) was excluded from the statistical analysis, following the criteria used in similar studies (Ballart et al., 2013). This is a shelter kennel that collects stray dogs, which means that some of the individual data, as well as owners' perception of CanL, could not be collected. Therefore, the statistical analysis included 578 individuals and 25 localities. Results of the bivariate analysis are summarized in Table 3. Dogs' age and location altitude ($p < 0.001$) showed a very strong relationship with dog seropositivity. In our population, a bimodal CanL seroprevalence distribution according to age was observed, with a first peak at 3–4 years and a second at 7–8 years old, with the risk of infection rising by each year of life [OR = 1.18 (95%CI: 1.09–1.27)] and decreasing at altitudes above 800 m a.s.l. [OR = 0.012 (95%CI: 0.002-0.07)]. Also, according to the results, being a crossbred dog raises the risk of infection [OR = 2.19 (95%CI: 1.18–4.06); $p = 0.013$] and the use of unspecific insecticides against arthropods has a protective effect [OR = 2.94 (95%CI: 1.58–5.45); $p = 0.001$]. All the other variables (sex of the dog, type of habitat, dog purpose, type of nocturnal refuge, presence of other animal species, owner's knowledge

Table 3
Number of dogs analysed and *Leishmania infantum* seropositivity observed for each category of the explanatory variables, followed by the results of the bivariate analysis expressed in odds ratios (OR). Statistically significant variables ($p < 0.05$) are marked with (*).

Explanatory variables and categories	No. dogs analysed	No. seropositive dogs (% seropositive dogs)	Bivariate analysis	
			OR (95% CI)	*p*-value
Altitude (m a.s.l.)				
< 800	492	144 (29)	Ref	
> 800	109	6 (6)	0.012 (0.002-0.07)	< 0.001*
Type of habitat				
Rural	297	70 (24)	Ref	
Periurban	228	51 (22)	1.64 (0.93-2.88)	0.082
Urban	53	19 (36)	0.97 (0.42-2.22)	0.934
Presence of other animals (other than dogs)				
Yes	302	73 (24)	Ref	
No	299	78 (26)	1.10 (0.58-2.08)	0.763
Sex				
Male	338	87 (26)	Ref	
Female	240	53 (22)	0.88 (0.56-1.38)	0.581
Age (years)			1.18 (1.09-1.27)	< 0.001*
< 1	53	6 (11)		
1	83	12 (14)		
2	91	13 (14)		
3	79	20 (25)		
4	51	16 (31)		
5	42	10 (24)		
6	42	12 (29)		
7	33	15 (45)		
8	33	14 (42)		
9	15	3 (20)		
10	18	5 (28)		
11	10	4 (40)		
12	3	0 (0)		
13	1	1 (100)		
Breed				
Purebred	258	51 (20)	Ref	
Crossbred	320	89 (28)	2.19 (1.18-4.06)	0.013*
Use given				
Hunting	468	118 (25)	Ref	
Breeding	95	21 (22)	2.28 (0.78-6.63)	0.130
Others[a]	15	1 (7)	0.20 (0.02-1.73)	0.145
Night shelter				
Outdoors	506	126(25)	Ref	
Indoors	72	14 (19)	0.50 (0.20-1.23)	0.131
Owner knows preventive measures against CanL				
Yes	362	103 (28)	Ref	
No	216	37 (17)	0.58 (0.29-1.19)	0.138
Owner has used preventive measures against CanL				
Yes	140	46 (33)	Ref	
No	438	112 (26)	1.24 (0.63-2.43)	0.539
Owner has used prevention methods against other arthropods				
Yes	472	112 (24)	Ref	
No	106	28 (26)	2.94 (1.58-5.45)	0.001*

[a] Includes racing (n = 13) and pet dogs (n = 2).

of prophylactic measures against CanL and the regular application of these methods) showed no statistically significant relationship with dog seropositivity.

3.5. Multivariable mixed model

The final multivariable mixed logistic regression model identified age of the dog and altitude of the location as the explanatory variables that affect dog seropositivity. According to this model, the odds of being infected rise in 1.21 per each year of life [(95%CI: 1.11–1.31); $p < 0.001$] and decrease at locations above 800 m a.s.l. [OR = 0.02 (95%CI: 0.001-0.19); $p = 0.001$]. The final model explains 53.7% of the total variance of the outcome variable, of which 42% is explained by the fixed effects terms and 11.7% by the random effects variable.

4. Discussion

Until now, data on CanL in north-eastern and Pyrenean areas of Spain is scarce and fragmented. The only published study regarding CanL in Girona province is a questionnaire-based survey of veterinary practitioners working in the region (Lladró et al., 2017). This work provided the first data from a previously recognized, but non-documented CanL endemic area in north-east Spain and highlights gaps in the epidemiological picture in Mediterranean regions considered to be endemic for CanL (Ready, 2017). The veterinary survey showed that new cases of CanL in autochthonous animals were diagnosed annually, including some asymptomatic cases detected by CanL pre-vaccination screening (Lladró et al., 2017). The present study confirms the suspected endemicity of CanL in the region, providing results for canine seroprevalence, as well as an overview of the infection distribution throughout Girona province. Preliminary exploratory surveys showed the presence of phlebotomine vectors in the surroundings of many of the sampling points (authors' unpublished data), confirming that all conditions are present for a complete *L. infantum* biologic cycle to be maintained in this region. In addition, the characterisation of all individuals and locations included in the study allowed for the identification of risk factors associated with CanL distribution.

As previously mentioned, there was an active search for individuals to be enrolled in the study, assisted by local veterinarians. There was therefore a constraint in the distribution and type of animals recruited, depending on the availability of veterinary practitioners' clients willing to participate. As a result, the dog population was mainly composed of hunting dogs. These animals have inherent characteristics, such as the fact that they are usually kept with other dogs in open kennels, in rural or periurban settings, and generally do not have the same type of veterinarian monitoring as pet dogs. Therefore, this type of population is usually considered a good sentinel for CanL (Ballart et al., 2013; Cabezón et al., 2010). As similar hunting activities take place throughout Mediterranean areas in Europe, it can be expected that comparable dog populations are widespread. An overestimation of the overall infection prevalence can however occur due to an expected lower incidence in urban centres, mostly explained by a decreased probability of contact between dogs and sand fly vectors (Ballart et al., 2013). Additionally, there was an increased difficulty in recruiting dogs from higher altitude regions, mainly because these areas are more inhospitable and less populated. Consequently, dogs living at locations above 800 m a.s.l. are less represented.

Some degree of spatial clustering may have been introduced by sampling several dogs in the same kennel or locality. This could also have had a clustering effect on the positive results, as higher dog densities tend to favour the transmission of the parasite, especially if some of the dogs are already infected (Alonso et al., 2010). Nevertheless, in the present study we have used similar dog populations in the different sampling points, allowing comparison between them. Additionally, this methodology has also been used in similar studies describing other regions of Spain (Alcover et al., 2013; Ballart et al., 2013). In the statistical analysis, the potential clustering effect was dealt with by introducing "Locality" as a random-effects term in the final multivariable mixed logistic regression model.

The serological technique used to measure antibody levels to *L. infantum* was an in-house ELISA. ELISA is one of the methods recommended by the World Organization for Animal Health for performing CanL surveillance studies and to determine prevalence of infection (OIE, 2014), the other one being the indirect immunofluorescent antibody test (IFAT). Unlike IFAT, ELISA is easy to perform and interpret, being particularly useful in field study settings, where a large number of samples must be analysed (Maia and Campino, 2008). In addition, this ELISA has been widely used for CanL diagnosis, as well as in other CanL epidemiological studies (Alcover et al., 2013; Ballart et al., 2013; Fernández-Bellon et al., 2008; Fisa et al., 2001; Iniesta et al., 2002; Riera et al., 1999; Rodríguez-Cortés et al., 2010; Solano-Gallego et al., 2005).

The overall estimated seroprevalence for Girona province was 19.5%, ranging from 0 to 56.8% across the different counties. These results are in accordance with previous reports for other regions of Spain, as well as the Mediterranean basin, (Ballart et al., 2013; Ntais et al., 2013; Cortes et al., 2012; Maroli et al., 2008). A series of CanL seroprevalence surveys undertaken in France, Italy, Spain and Portugal between 1971 and 2006 showed an overall seroprevalence of 23.2%, with point prevalences of 0% and higher than 80% in some locations (Franco et al., 2011). These values are comparable to the ones obtained in the present study and correspond to the previous claims of the heterogeneous distribution of the disease. However, as pointed out by Franco et al. (2011), caution must be taken when comparing studies with different experimental designs and different criteria used in the selection of the target dog population, as this can introduce significant variations in seroprevalence results. A common European strategy for leishmaniosis surveillance and control would aid the implementation of standardized methodology. However, although leishmaniosis is currently listed as a notifiable disease by the World Organization for Animal Health (OIE, 2018), this is not clearly reflected in the European or Spanish legislation (BOE, 2014; Official Journal of the European Union, 2012).

From the 146 seropositive dogs, only 10 (6.8%) showed clinical signs compatible with CanL and more than 50% presented low standard ELISA units (inferior to 50U). This can be explained by the cryptic nature of the infection and the wide clinical spectrum it can present, ranging from asymptomatic or mild symptomatic cases to very severe clinical stages (Solano-Gallego et al., 2009). There is also the possibility that some of the dogs are in an early stage of infection (Fisa et al., 2001; Miró et al., 2012) or are immunologically resistant and only transiently seropositive, eventually showing spontaneous clearance of the parasite (Fisa et al., 1999). In such populations, serological techniques could have a lower sensitivity (Otranto et al., 2009). It is also known that, in endemic areas, only a small proportion of dogs display symptoms of CanL, while the majority of infected dogs do not show any clinical evidence of the disease (Baneth et al., 2008). It is believed that the high prevalence of asymptomatic infected dogs, comparable to that observed in Lleida province (other north-eastern and Pyrenean region studied in Spain), is strong evidence for a well-established CanL focus in Girona province (Ballart et al., 2013). In the present study, clinical signs compatible with CanL were also identified in 10 out of 447 seronegative dogs (2%), illustrating the lack of specificity of the disease's clinical presentation and the added difficulty in detecting affected dogs. As mentioned before, the ability of serological tests to detect infected animals is limited, especially in endemic settings, and a small number of seronegative asymptomatic infected dogs should be expected, as previously reported in other studies (Iniesta et al., 2002;. Otranto et al., 2009; Solano-Gallego et al., 2001). These animals can harbour parasites in the skin, detectable by PCR (Otranto et al., 2009), and could also be infectious to sand flies, as has been demonstrated for asymptomatic seropositive dogs (Molina et al., 1994; Quinnell and Courtenay, 2009). Considering this, any control programme for CanL should be based on multiple diagnostic methods, as serology alone can prove to be insufficient in detecting all infected and infectious dogs.

In the present study, the risk of infection increased with dogs' age. This is an individual factor commonly reported as being positively related with *L. infantum* infection (Alonso et al., 2010; Ballart et al., 2013; Cortes et al., 2012; Gálvez et al., 2010a; Maresca et al., 2009; Martín-Sánchez et al., 2009; Miró et al., 2012), and which can be explained by an incremental risk of exposure to infected sand flies. The bimodal CanL seroprevalence distribution observed has been previously described by other authors (Gálvez et al., 2010a; Miró et al., 2012). This pattern suggests that *L. infantum* may be able to infect immunologically vulnerable animals at an earlier age, followed by a later infection of resistant animals either by cumulative exposure or due to concomitant diseases that weaken the dogs' immune system (Miranda et al., 2008).

According to the results, altitude shows a negative correlation with *L. infantum* infection. This is mainly related to the bioclimatic needs of the phlebotomine vector species present in Spain, *Phlebotomus perniciosus* and *P. ariasi* (Rioux et al., 1986). Altitude is known to be closely linked to temperature, precipitation and land cover (Baron et al., 2011; Rivas-Martínez, 1983). In temperate regions, as atmospheric temperature rises, a higher biting rate is expected (Hartemink et al., 2011), therefore increasing the risk of sand fly bites to vertebrate hosts. Simultaneously, a shorter extrinsic incubation period (Hartemink et al., 2011) and a more effective development of the parasite inside the vector (Rioux et al., 1985) are observed, raising the risk of *L. infantum* infection. Also, an increased altitude may provide a more hostile environment for sand fly survival (Gálvez et al., 2010b), not only because of the more extreme bioclimatic conditions, but also due to a possible scarcity of vertebrate hosts. However, a relationship between altitude and risk of CanL infection was not observed in the neighbouring province of Lleida (Ballart et al., 2014, 2013) or in France (Chamaillé et al., 2010), where both vector species are present and show different altitudinal preferences. In these areas, *P. perniciosus* is known to occupy ecological niches commonly below 800 m a.s.l., while *P. ariasi* shows a higher abundance above this altitude. Therefore, it would be of particular interest to perform entomological studies and risk factor analysis associated with the vector populations present in the study area. This could also help to improve the ability of the present model to predict the outcome variable. One of the possible reasons for the moderate performance of the final statistical model presented (which explains 53.7% of variance of the outcome variable) is the absence of data on the abiotic factors mentioned above, which are known to have an important impact on sand fly populations, and indirectly on *L. infantum* infections (Dantas-Torres et al., 2014; Gálvez et al., 2010b).

The present study failed to detect an effect of type of habitat (rural/urban) or access to night shelter, which several other authors identified as significantly related to *L. infantum* infection (Ballart et al., 2013; Cortes et al., 2012; de Almeida et al., 2012d; Gálvez et al., 2010a; Martín-Sánchez et al., 2009; Oliveira et al., 2016). According to these studies, dogs that live in rural habitats and are left outdoors at night show an increased risk of infection. In this study, the high percentage of dogs living in rural/periurban areas and kept permanently outdoors may not have allowed detection of such an effect. Also, periurban areas are increasingly described as the most suitable ecosystems for sand flies, due to the ideal microclimate offered by house gardens associated with the abundance of vertebrate hosts (Alvar et al., 2004; Ballart et al., 2013).

Results from the bivariate statistical analysis identified dog breed and the use of general insecticide treatment against arthropods as variables associated with dog seropositivity. In the first case, crossbred dogs would be at higher risk of infection [OR = 2.19 (95%CI: 1.18–4.06); p = 0.013]. However, previous studies have shown that this should not be the case, as crossbred, autochthonous dogs tend to be more resilient to *L. infantum* infection (Alvar et al., 2004; Solano-Gallego et al., 2000). This is even more noticeable when the purebred dogs belong to exotic breeds like boxers and beagles (both represented in this study), known for their higher sensitivity to CanL (Solano-Gallego et al., 2009). The effect of dog breed was absent in the mixed model, showing that the previous results were most probably induced by confounding factors related to the kennel locations (e.g. altitude) or dog owners' attitudes (e.g. use of prophylactic measures against CanL). The non-use of generalist insecticide preventive methods against arthropods was also identified as a risk factor for *L. infantum* infection [OR = 2.94 (95%CI: 1.58–5.45); p = 0.001], while the use of specific prophylaxis against CanL failed to show a protective effect (p = 0.539). Again, this may be related to confounding factors, such as a possible partial effect of some insecticides against phlebotomine vectors, even though they may not be licensed for sand fly prevention. Additionally, the improper use of specific sand fly prevention treatment, such as failure to apply it to all dogs or to maintain it during the whole transmission season, may impair the protective effect of these products (Courtenay et al., 2009). Once again, the effect of this variable lost significance in the multivariable analysis and was not included in the final statistical model.

The majority of dog owners showed previous knowledge of CanL and to be aware of preventive methods for the infection. Although a positive correlation was observed between owners' perceived risk of infection and CanL seropositivity at the dog kennel level, only 27.3% of dog owners stated that they regularly used CanL prophylactic measures. This result is in accordance with those reported by Lladró et al. (2017), in which all veterinary practitioners working in Girona province recommended at least one preventive measure against CanL, though the majority did not believe that dog owners protected their dogs properly. When used, the most frequent prophylactic methods applied against CanL were dog collars and spot-on insecticides, as recommended by veterinarians. However, most owners did not keep their dogs indoors at night and did not report the use of vaccination against CanL or immunomodulatory agents, as also suggested by veterinarians (Lladró et al., 2017). Our study, being an example for other Mediterranean endemic areas, shows that the implementation of prophylactic measures by dog owners should be reinforced in order to reduce *L. infantum* transmission between dogs, as well as to reduce the public health risk (Miró and López-Vélez, 2018).

5. Conclusions

According to the results presented, Girona province shows characteristics of a stable, endemic focus of CanL: a high *L. infantum* seroprevalence observed in dogs, together with a large number of asymptomatic cases and the presence of the sand fly vector. The majority of these dogs are autochthonous and have never left the province. Dogs' age and altitude were identified as risk factors for the disease, providing additional information to complement the design of risk assessment maps for *L. infantum* infection as well as for the implementation of CanL control measures in endemic areas across the Mediterranean basin.

Ethics approval

The research protocol was submitted to the Ethics Committee on Animal Experimentation (CEEA) of University of Barcelona, which considered that an ethical approval was not required for this study. The project was also submitted to and approved by ISGlobal Internal Scientific Committee (ISC). All dog owners were informed about the research protocol and signed an informed consent allowing for sample and data collection.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2018.10.015.

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