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First survey on canine leishmaniasis in a non classical area of the disease in Spain (Lleida, Catalonia) based on a veterinary questionnaire and a cross-sectional study

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ABSTRACT

The Spanish distribution of canine leishmaniasis (CanL) is heterogeneous and very few data are available for the north of the country, including the province of Lleida (Catalonia, Spain). This work describes the results obtained from a questionnaire sent to veterinarians throughout the province of Lleida. The majority of veterinarians (25/32, 78.1%) believed CanL cases were increasing and that the dogs had been infected locally (30/32, 93.8%). Also, a cross-sectional study was performed on the seroprevalence of CanL in kennel dogs, with and without compatible clinical signs, in the county of Pallars Sobirà (Pyrenees of Lleida), where an autochthonous case of CanL had been previously detected. Four serological tests were used (IFAT, ELISA, Western blot, ICF) and dogs that tested positive with at least two immunological methods were considered seropositive and probably infected. 33.1% (48/145) of the dogs were seropositive. The results of a mixed logistic regression model showed that the risk of seropositivity increased with age (OR = 1.35, p-value = 0.002), among dogs living in the southern part of Pallars Sobirà (OR = 6.20, p-value = 0.025) and among dogs whose owners considered their animals to be at risk of leishmaniasis infection (OR = 1.26, p-value = 0.024) and who were unaware of anti-sand fly preventive methods (OR = 11.6, p-value = 0.009). The risk decreased when dogs lived in an urban-periurban habitat (OR = 0.17, p-value = 0.002). The information gathered in the veterinary questionnaires helped us to define the knowledge, perception and awareness of the disease in a naïve region, supporting the hypothesis of an existing CanL focus in Pallars Sobirà, which was confirmed by the seroepidemiological survey. The seroprevalence study carried out on kennel dogs of local origin proved useful for detecting an autochthonous focus of leishmaniasis through the analysis of a small number of animals.

1. Introduction

Changes in global climate, human activities and migration have resulted in the emergence or re-emergence of vector-borne diseases in some parts of the world, including leishmaniasis, the only tropical vector-borne disease that

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remains endemic throughout southern Europe (Dujardin et al., 2008).

Leishmaniasis in the Mediterranean region is due to *Leishmania infantum* and its transmission is mainly by the bite of permissive sand flies. Two proven vectors exist in the Iberian Peninsula, *Phlebotomus ariasi* and *P. perniciosus* (Guilvard et al., 1996; Portús et al., 2007), both present in the province of Lleida (Gállego et al., 1990) where *L. infantum* DNA has been found in *P. ariasi* (Alcover et al., 2012).

The first cases of human and canine leishmaniasis in Spain were published in 1912 and 1913, respectively (Pittaluga, 1912, 1913). The human incidence of the disease decreased at the end of the 1940s, which is attributed to the use of insecticides in the agricultural and domestic environment as well as an antipaludic campaign (Gil Collado, 1977; Botet Fregola and Portús Vinyeta, 1993; Portús et al., 2007). There has been a re-emergence in recent years, mainly in immunosuppressed adults (Alvar et al., 1997). From 1982 until 1996 human leishmaniasis (HL) was considered a notifiable disease in Spain, but its notification is currently mandatory in only 12 of the country's 17 autonomous communities, including Catalonia. From 1982 to 2010, 879 HL cases were officially recorded in Catalonia, 37 of them in Lleida province (updated from Ballart et al., 2012a). Nevertheless, as individual cases are not always officially recorded, the real incidence is not well defined (Gállego, 2004; Portús et al., 2007; Dujardin et al., 2008).

The distribution of canine leishmaniasis (CanL) in Spain is heterogeneous, with the lowest seroprevalence in the North (1.6%) and the greatest in the South (34.6%) (Alvar Ezquerra, 2001; Morillas et al., 1996). Nevertheless, data on CanL distribution is incomplete, as declaration of the disease is not compulsory, with very few data available for the north of the country, including the province of Lleida (Catalonia, NE of Spain) where the first case of CanL has been recently reported (Ballart et al., 2012a).

CanL is considered an important disease by the World Organization for Animal Health (OIE) from the socio-economical and sanitary points of view. Dogs constitute the main reservoir hosts of the parasite, which represents a risk for humans (Gállego, 2004; Ready, 2010), particularly considering the high number of asymptomatic animals (Alvar et al., 2004; Dujardin et al., 2008; Molina et al., 1994).

The true extent of CanL in many parts of southern Europe is unknown, due to a lack of survey data, its incidence only being inferred indirectly (de Ybáñez et al., 2009). For this reason, a questionnaire was designed by the EDEN project (Emerging Diseases in a changing European eNvironment) to rapidly obtain information from veterinarians about the presence, diagnosis and perception of the disease. This questionnaire was applied in the province of Lleida as has been done in other parts of Europe by EDEN team participants (Morosetti et al., 2009; Farkas et al., 2011; Gálvez et al., 2011).

Described here are the results obtained from the EDEN questionnaire as well as a cross-sectional study on the seroprevalence of CanL in Pallars Sobirà (Pyrenees) where the aforementioned CanL case was detected, with particular focus on the possible factors responsible for the CanL emergence in the area. This is the first survey carried out on dogs in the province of Lleida and was prompted by the possibility of the disease becoming wide-spread and established in the Pyrenees.

2. Materials and methods

2.1. Study area

A questionnaire-based survey on CanL was conducted among clinical veterinarians throughout the province of Lleida (Catalonia, NE of Spain). Lleida (41°36′N and 0°37′E; 12,173 km^2) shares its northern border with Andorra and France and is territorially divided in 13 counties, occupying a great variety of habitats that range from just over sea level to over 3000 m above sea level (m a.s.l.) (Fig. 1). The climate varies from Mediterranean high-mountain in the mountainous areas of the north to continental in the central depression. With a population of 436,402 inhabitants in 2009 (approximately 35.8 inhabitants/km^2) Lleida is the province with the lowest population density of Catalonia (IDESCAT Institut d'Estadística de Catalunya; http://www.idescat.cat). The only locality with more than 20,000 inhabitants is the capital, Lleida city, where 30% of the population lives.

Additionally, a cross-sectional serological study of CanL was carried out in Pallars Sobirà, a county of the province of Lleida located in the Spanish Pyrenees (42°25′N, 1°08′W), with a surface area of 1378 km^2. Its population density is very low (5.6 inhabitants/km^2 in 2009) and concentrated in small villages (IDESCAT Institut d'Estadística de Catalunya; http://www.idescat.cat). The altitude ranges from 600 to 3143 m a.s.l. and the climate is Mediterranean high-mountain, with cold winters and mild summers, except at over 1500 m a.s.l., where it could be considered alpine. The annual mean rainfall varies from 700 mm in the valleys to 1000 mm in the peaks, summer being the wettest season while winter is the driest. The orography of the region and its particular climate notably determine its vegetation, which is characterised by five different floors: basal with mixed oaks (from 600 to 1000 m a.s.l.), mountainous with oaks, beeches, firs and red pines (from 1000 to 1600 m a.s.l.), subalpine with black pine (from 1600 to 2200 m a.s.l.), alpine with pastures (from 2200 to 2800 m a.s.l.) and the mainly rocky snow floor (over 2800 m a.s.l.).

2.2. Veterinarian questionnaire on canine leishmaniasis

In 2009, a total of 41 questionnaires were sent to all the veterinarians with a pet veterinary clinic in the province of Lleida to survey their opinions on trends in CanL prevalence and control measures. The questionnaire was designed by members of the Leishmaniasis sub-project of the EDEN Project (http://www.eden-fp6project.net/emerging_diseases/leishmaniasis), and consisted of two pages of questions on different aspects of the disease in the area, including the approximate number of suspected and confirmed cases observed in the previous year, clinical signs and their frequency of appearance and utility in diagnosis, methods used to confirm CanL cases, etc. (Gálvez et al., 2011).

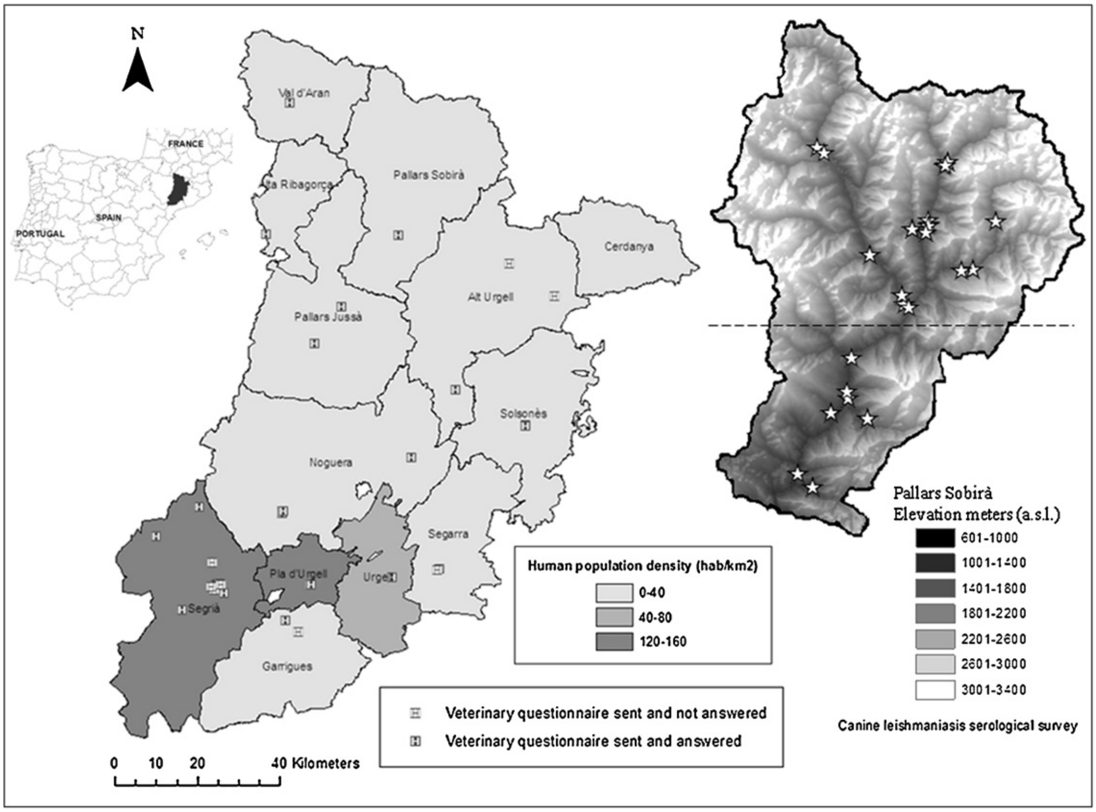

Fig. 1. Study area (Lleida province and Pallars Sobirà county), surveyed points for CanL and veterinarian questionnaires.

A conference was organized previously with the help of the *Col.legi Oficial de Veterinaris de Lleida* with the aim of involving the veterinarians in the study. Questionnaires were posted in a prepaid return envelope and also sent by e-mail. A second letter, following a telephone call, was sent when no answer to the questionnaire was received. The locations of veterinary clinics were geocoded using Google Earth (Fig. 1).

2.3. Serological study on canine leishmaniasis

2.3.1. Animals and samples

The cross-sectional study was performed in October 2009 in Lleida Pyrenees (Pallars Sobirà county). An active search for dog owners was necessary and was carried out with the help of the veterinarians of the area. The tested dogs belonged to 25 owners from 16 localities situated between 600 and 1300 m a.s.l. (Fig. 1). Blood samples of 145 dogs were obtained by cephalic vein puncture, with permission of the owners. Blood was collected in 5 ml tubes and on filter paper (Whatman 3). The tubs were centrifuged at room temperature and the serum was frozen and stored at −40 °C until use. Filter papers were also stored at −40 °C after air drying.

2.3.2. Data collection

Owner data and dog characteristics were collected in a PDA (Tungsten T5) using Pendragon Form v.5.0 software (PSC, Libertyville, IL, USA).

Clinical signs referable to canine leishmaniasis (Fig. 2) were also compiled during the clinical examination by local veterinarians, and dogs were classified as asymptomatic or symptomatic depending on the absence or presence of at least one of those clinical signs.

A GPS receiver (Tom Tom Wireless GPS MK II) was used to record geographical coordinates, and altitude data for each geocoded collection site was extracted from a 90 m resolution Digital Elevation Model (Jarvis et al., 2008) using ArcGIS 9.2 software (ESRI, Redlands, CA, USA).

2.3.3. Diagnostic techniques

Dog samples were analysed by four serological techniques: (1) an in-house immunofluorescent antibody test (IFAT), since this is considered the "gold standard" and reference test for CanL diagnosis (Alvar et al., 2004; OIE, 2008; WHO, 2010) and was chosen as the reference technique in the EDEN project; (2) an in-house enzyme-linked immunosorbent assay (ELISA), because it is habitually used by our team and has proved useful in epidemiological studies (Iniesta et al., 2002; Solano-Gallego et al., 2005; Fernández-Bellon et al., 2008); (3) an in-house Western Blot (WB) technique, because it is a more sensitive serological technique (Aisa et al., 1998, Iniesta et al., 2002), and (4) a commercial immunochromatographic test (ICF) for the detection of circulating anti-*Leishmania* kinesin antibodies, due to its rapidity and feasibility for use in the field.

The IFAT was performed according to standard procedures (Alvar et al., 2004). Sera from dogs were assayed in

DOG DEMOGRAPHIC DATA	VETERINARY DATA	DOG OWNER QUESTIONNAIRE
Name	Name and surname	Name and surname
Length of hair: Short ☐ Medium ☐ Long ☐	Address	Address
Colour of hair	Town	Longitude Latitude
Sex: M ☐ F☐ If female, is pregnant?	Province Post code	Town Province
Date of birth	Telephone nº	Post code
Age (years)	e-mail	Telephone nº
Weight (kg)		e-mail:

DOG DEMOGRAPHIC DATA (cont.)	CLINICAL SIGNS REFERABLE TO CANINE LEISHMANIASIS	SAMPLING DATE / TEST	DOG OWNER QUESTIONNAIRE (cont.)
Breed: Country of origin Region of origin Province of origin		SAMPLING DATE Form No.	Have you heard of visceral leishmaniasis? Yes ☐ No ☐ What do you think is the risk that your dog will get leishmaniasis during its lifetime? 0% ☐ 5% ☐ 10% ☐ 20% ☐ 50% ☐ 50-90% ☐ 90-100% ☐
DOG'S USUAL PLACE OF LIFE Address Locality Comarca Province Long Lat Resides in: In the home ☐ In the open ☐ In kennel ☐ Usual habitat: Urban ☐ Periurban ☐ Rural ☐ Plain ☐ Mountain ☐ Nocturnal shelter/home: Outdoors ☐ Indoors ☐	None ☐ Lymph node swelling ☐ Dermatitus furfuracea ☐ Alopecia ☐ Ulcers ☐ Onychogryphosis ☐ Epistaxis ☐ Pale mucosa ☐ Ocular lesions ☐ Splenomegaly ☐ Weight loss ☐ Other ☐ (specify)	Type of sample Serum ☐ Cutaneous sample ☐ Peripheral blood ☐ Lymph node ☐ Bone marrow ☐ DIAGNOSTIC TEST Microscopy ☐ ELISA ☐ IFAT ☐ WB ☐ In vitro culture ☐ PCR ☐ Other ☐ (specify)	Do you know of any measures to protect your dog against sandfly bites? Yes ☐ No☐ If YES, have you ever used any measures to protect your dog against VL? Yes ☐ No☐ If YES, which? Collar ☐ Scalibor ☐ Other collar (specify) ☐ Details collar: ☐ Spot-on ☐ Advantix ☐ Expot ☐ Other ☐ Details Spray ☐ Duowin ☐ Other (specify) ☐ Details spray: ☐ Shampoo ☐ Other (specify) ☐ Details: If NO, why not? Can't find them in the shop ☐ Too expensive ☐ Don't think they work ☐ VL risk not important ☐ Other (specify) ☐ Do you apply any insecticide on your dog to protect it from other insect or tick bites? Yes ☐ No☐ If YES, provide details
Occupation of dog: Pet ☐ Guard dog ☐ Sheep dog ☐ Hunting dog ☐ Dog in kennel ☐ Stray dog ☐ Other ☐ (specify): Lives with other dogs: Yes ☐ No ☐ If YES, How many: Lives with other animals Yes ☐ No ☐ If YES, which Movement/travel Yes ☐ No ☐ If YES, details of place If YES, period		Result Positive ☐ Negative ☐ Titre Observations	

Fig. 2. Questionnaire filled in by dog owners and veterinarians in the county of Pallars Sobirà.

serial two-fold dilutions from 1:40 to 1:1280, and antibody fixation was revealed with 1:100 FITC-conjugated rabbit anti-dog IgG (H + L) (ICN Plaza, Costa Mesa, California) in 0.01% Evans blue. The cut-off for positive sera was 1:80, in accordance with other authors (OIE, 2000; Alvar Ezquerra, 2001; Dereure et al., 2009; Gálvez et al., 2010).

The ELISA and WB tests were performed according to Riera et al. (1999). For the ELISA test, sonicated promastigotes coated to the plate were used as antigens. The sera were used at a dilution of 1:400 and Protein A peroxidase (Sigma) (1:30,000) was used instead of the second antibody. The reaction was stopped with H_2SO_4 3 M and the optical densities were measured at 492 nm using a Titertek Multiskan PlusMKII (Flow Laboratories International, SA, Lugano, Switzerland). The reaction was quantified in units (U) by reference to a positive control serum arbitrarily set at 100 U. The cut-off was established at 24 U (Riera et al., 1999).

For the WB, separation of different fractions in *L. infantum* antigen was performed with 0.1% SDS-12% polyacrylamide gel electrophoresis on a Mini-gel AE 6400 Dual Mini Slab Kit (Atto, Bunkyo-Ku, Japan) and transblotted onto nitrocellulose sheets (0.45 μm pore size, HAWP 304 FO; Millipore, Bedford, MA). Fractions were recognized by sera diluted to 1:50 and Protein A peroxidase (1:1000) was used instead of the second antibody. The reaction was considered positive when any of the polypeptide fractions considered most useful were revealed (14, 16, 18 or 24 kD) (Aisa et al., 1998; Iniesta et al., 2007).

The immunochromatographic dipstick test (Speed® Leish K, BVT Group, Virbac) was carried out on the serum following the manufacturer's instructions.

To avoid problems of nonspecificity of the techniques used, mainly present when results were near the cut-off, dogs that tested positive with at least two immunological methods were considered seropositive and probably infected (Iniesta et al., 2002).

2.3.4. Statistical analysis

A mixed logistic regression model was used to assess the relationship between seropositivity in dogs (outcome) and a series of individual and location variables (covariates). The villages where the dogs live were used as random effects to take into account the clustering of the data (Alonso et al., 2010), and maximum likelihood estimation was applied. The covariates considered in the analysis were: location of the village in Pallars Sobirà (North/South), altitude (600–800/801–1000/>1000 m), habitat (urban-periurban/rural), place of abode (outdoors/inside/in kennel), use given (pet dog/hunting dog/others), sleeping habits (outdoors/indoors), age in years (<1/1/2/3/4/5/6/>6), hair length (short/medium-long), sex (female/male), breed size in kg (≤25/>25), breed (pedigree dog/crossed dog), lives with other dogs (yes/no), lives with other animals (yes/no) and clinical signs (asymptomatic/symptomatic). Similarly, information on knowledge, attitude and practice was gathered from the owners: had they heard of leishmaniasis (yes/no), the risk of dogs having leishmaniasis (0%/5%/10%/20%/50%/50–90%/90–100%), did they use prevention methods (yes/no), if YES, what methods were known (collar/not proceed), if NO, why not (I don't think it works/leishmaniasis is not important/other/not proceed) and did they use topical insecticides (yes/no). The defined categories were based on those used in previous publications (Martín-Sánchez et al., 2009; Gálvez et al., 2010) and the characteristics of the data in the present study. Age and "perceived risk of dogs having leishmaniasis" were used as continuous variables in the model.

A two-step approach was used to determine the most parsimonious multivariate model. First, the association of all covariates and the outcome were assessed one by one in a bivariate analysis. Covariates with a p-value ≤ 0.2 were further tested in a multivariate mixed model (Gálvez et al., 2010). In this second step, a backward stepwise selection method was used to select the final model using a p-value of ≤ 0.05 as the threshold for removal.

All statistical analyses were performed using Stata 12 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Veterinary questionnaire survey

Thirty two of the 41 (78%) questionnaires sent to the veterinarians specialized in small animals were returned.

Table 1
Results obtained from the veterinary questionnaire surveying the entire province of Lleida.

Type of dogs population	N	(%)	Diagnostic methods	N	(%)
Clients			Epidemiology		
Mixed	23	(71.9)	No	30	(93.8)
Rural	7	(21.9)	Yes	2	(6.3)
Urban	2	(6.3)	Clinical signs only		
Dogs examined/week			No	32	(100)
4–10	3	(9.4)	Serology-IFAT		
11–20	11	(34.4)	No	17	(53.1)
>20	18	(56.3)	Yes	15	(46.9)
Dogs suspected (last 12 months)			Serology-ELISA		
1–5	5	(15.6)	No	20	(62.5)
6–10	8	(25)	Yes	12	(37.5)
11–20	6	(18.8)	Serology-rapid detection Kit (ICF)		
21–50	10	(31.3)	No	8	(25)
>50	3	(9.4)	Yes	24	(75)
CanL confirmed cases (last 12 months)			Microscopy-Lymph node punction		
1–5	10	(31.3)	No	30	(93.8)
6–10	8	(25)	Yes	2	(6.3)
11–20	8	(25)	Microscopy-Bone marrow punction		
21–50	3	(9.4)	No	26	(81.3)
>50	3	(9.4)	Yes	6	(18.8)
New cases of CanL			Microscopy-Cutaneous biopsy		
1–5	14	(43.8)	No	31	(96.9)
6–10	7	(21.9)	Yes	1	(3.1)
11–20	8	(25)	PCR		
21–50	1	(3.1)	No	29	(90.6)
>50	2	(6.3)	Yes	3	(9.4)
Dogs infected in the area			Place where diagnosis was confirmed		
No	2	(6.3)	Own laboratory		
Yes	30	(93.8)	No	11	(34.4)
Evolution (past 10 years)			Yes	21	(65.6)
Decrease	4	(12.5)	Private laboratory		
Increase	25	(78.1)	No	7	(21.9)
No evolution	3	(9.4)	Yes	25	(78.1)
Preventive measures recommended			Departmental veterinary laboratory		
Collar			No	27	(84.4)
No	0	(0)	Yes	5	(15.6)
Yes	32	(100)			
Spot-on					
No	13	(40.6)			
Yes	19	(59.4)			
Spray					
No	28	(87.5)			
Yes	4	(12.5)			
Shampoo					
No	32	(100)			
Yes	0	(0)			
Preventive products specifically against sand flies					
No	5	(15.6)			
Yes	27	(84.4)			

Abbreviations: N, number of answers; (%), percentage of answers.

We received at least one questionnaire from each county, the numbers varying from 1 to 13, with the exception of La Cerdanya, where no veterinary clinic existed at the time of study (Fig. 1).

71.9% of veterinarians had clients from mixed environments (urban and rural) and over half the clinics received more than 20 dogs per week (56.3%) (Table 1). Approximately 40% had received more than 20 suspected cases of leishmaniasis in the last 12 months (40.6%). The range of confirmed and new cases is shown in Table 1. A large percentage of the veterinarians (93.8%) thought that dogs had been infected in the study area and 78.1% reported an increase in the disease in the last 10 years. All the veterinarians recommended preventive measures, specifically impregnated collars (100%). Most of the practitioners also recommended the use of preventive agents that specifically indicate repellent effects against phlebotomine sand flies (84.4%).

All the veterinarians used different methods to confirm clinical suspicion. Serology was applied in all cases, using any of three techniques (ICF, IFAT and ELISA). Other methods used were microscopic analysis (18.8%) or PCR (9.4%). Diagnosis was practiced in external private laboratories (78.1%) as well as in the veterinarians' own laboratories (65.6%).

The frequency and diagnostic value of the clinical signs described by the veterinarians are included in Table 2. Other signs, such as chronic diarrhoea (1 case) and arthritis (1 case), were considered.

3.2. Descriptive analysis of cross-sectional canine leishmaniasis survey

3.2.1. Defining the dog population and owner awareness of leishmaniasis

A total of 145 dogs were examined from 16 villages in the county of Pallars Sobirà in 2009 (Fig. 1). All dogs were autochthonous except 2 from France, 1 from central Spain and 3 from Andorra. The characteristics of the dog population are recorded in Table 4. Dogs were distributed at different altitudes, ranging from 601 to 1300 m a.s.l. The majority of the dogs came from rural areas (57.2%), lived in kennels (80.7%), slept outdoors (91.7%) and were hunting dogs (84.1%). The number of females (45.5%) and males (54.5%) was similar, as was the number of pure breed (57.9%) or crossbreed dogs (42.1%). Most of the dogs were ≤25 kg (71%). The majority lived with other dogs (95.2%) and only some of them lived with other animals (20%).

The answers of the owners to questions on leishmaniasis awareness revealed that most of them had heard of visceral leishmaniasis (85.5%) and more than half (59.3%) considered their dogs were not at risk of contracting leishmaniasis during their lifetime. Approximately half of them had knowledge of preventive measures against sand fly bites (50.3%). All the owners with awareness of preventive methods against CanL used collars on their dogs (42.1% of the totality).

Table 3
Serological results (% of positives) on CanL obtained from 145 dogs from Pallars Sobirà County (Lleida, NE Spain).

IFAT (%)	ELISA (%)	WB (%)	ICF (%)	Seropositive dogs (%)[a]
20	29.7	37.9	22.1	33.1

[a] Dogs were considered seropositive when they tested positive in at least two immunological methods.

3.2.2. Serological results

Based on the results obtained with the different serological techniques (Table 3), 48 (33.1%) dogs were considered probably infected and positive for the statistical analysis. Positive dogs were found at all altitudinal ranges (from 600 to 1300 m a.s.l.) in 12 of the 16 localities studied (75%) and all of them had been born in the area (Table 4). The prevalence ranged from 0% to 66% but the infection was not concentrated in any particular village.

Clinical examination of dogs revealed that 31.2% of seropositive animals had signs referable to CanL (Fig. 2). One or more of those clinical signs were observed in symptomatic dogs, which included weight loss, alopecia, dermatitis furfuracea, ocular lesions, onychogryphosys and lymph node swelling.

3.3. Bivariate analysis

The relationship between dog seropositivity and a series of individual and location variables are presented in Table 4. Significant differences in CanL risk ($p < 0.05$) were found

Table 2
Clinical signs reported in the veterinary questionnaire on CanL.

Clinical sign	Frequency (%)				Diagnostic value (%)			
	Very frequent	Frequent	Rare	No answer	High	Medium	Low	No answer
Loss of weight	46.9	53.1	0	0	56.3	21.9	12.5	9.4
Tiredness	9.4	68.8	21.9	0	15.6	21.9	50	12.5
Anaemia	21.9	43.8	34.4	0	12.5	31.3	46.9	9.4
Hyperthermia	0	25	71.9	3.1	9.4	15.6	65.6	9.4
Epistaxis	9.4	40.6	50	0	43.8	25	18.8	12.5
Alopecia	37.5	46.9	15.6	0	21.9	37.5	31.3	9.4
Squamosis	53.1	43.8	3.1	0	31.3	34.4	25	9.4
Onychogryphosis	28.1	40.6	28.1	3.1	34.4	34.4	18.8	12.5
Ulcers	21.9	53.1	25	0	18.8	43.8	28.1	9.4
Adenopathy	59.4	34.4	6.3	0	50	21.9	18.8	9.4
Ocular lesions	6.3	40.6	53.1	0	12.5	31.3	46.9	9.4
Renal failure	9.4	56.3	31.3	3.1	9.4	53.1	25	12.5
Splenomegaly	3.1	18.8	71.9	6.3	6.3	21.9	56.3	15.6

Table 4
Main characteristics of the Pallars Sobirà dog population and owner awareness of leishmaniasis. Serological results obtained and bivariate factors associated with canine leishmaniasis (two positive serological tests).

Pallars Sobirà county (145 dogs)	N. dogs analyzed	% of seropositive dogs	Bivariate	
			OR (95% CI)	*p*-Value
Village location[*]				
North Pallars Sobirà (817–1219 m a.s.l., mean: 1004 m a.s.l.)	63	22	Ref	
South Pallars Sobirà (619–1169 m a.s.l, mean: 792 m a.s.l.)	82	40	2.15(1.04–4.47)	0.039
Altitude (m)				
600–800	49	41	Ref	
801–1000	38	29	0.59(0.24–1.46)	0.253
>1000	58	29	0.60(0.27–1.34)	0.214
Habitat[*]				
Rural	83	41	Ref	
Urban-Periurban	62	22.6	0.42(0.20–0.88)	0.021
Abode				
Outdoors	16	25	Ref	
In the home	12	8.3	0.27(0.03–2.83)	0.276
In kennel	117	36.8	1.74(0.53–5.74)	0.361
Sleeping habits[*]				
Outdoors	133	35.3	Ref	
Indoors	12	8.3	0.17(0.02–1.33)	0.091
Use given				
Pet dog	11	27.3	Ref	
Hunting dog	122	36.1	1.50(0.38–5.96)	0.561
Others	12	12.5	0.24(0.02–2.78)	0.255
Age (in years)[a,*]			1.34(1.11–1.61)	0.002
<1	7	0		
1	23	8.7		
2	19	21.1		
3	24	45.8		
4	14	35.7		
5	18	38.9		
6	9	44.4		
>6	31	48.4		
Hair length				
Short	83	34.9	Ref	
Medium-long	62	75	0.82(0.41–1.66)	0.587
Sex[*]				
Male	79	38	Ref	
Female	66	27.3	0.61(0.30–1.24)	0.174
Breed size (kg)				
≤25	103	67	Ref	
>25	42	67	1.01(0.47–2.17)	0.970
Breed				
Pedigree dog	84	31	Ref	
Crossed dog	61	36.1	1.26(0.63–2.53)	0.519
Lives with other dogs				
Yes	138	34.1	Ref	
No	7	14.3	0.32(0.04–2.76)	0.302
Lives with other animals				
Yes	29	27.6	Ref	
No	116	34.5	1.38(0.56–3.40)	0.481
Symptomatology[*]				
Asymptomatic	113	29.2	Ref	
Symptomatic	32	46.9	2.14(0.96–4.78)	0.064
The owner has heard of VL[*]				
Yes	124	35.5	Ref	
No	21	19	0.43(0.13–1.35)	0.148
Risk of dogs having leishmaniasis according to the owner[a,*]			1.24(1.05–1.47)	0.013
0%	86	27.9		
5%	14	28.6		
10%	4	50		
20%	20	30		
50%	1	0		
50–90%	9	44.4		
90–100%	11	72.7		
The owner has knowledge of preventive measures against sand flies bites[*]				
Yes	73	38.4	Ref	
No	72	27.8	0.62(0.31–1.24)	0.177

Table 4 (*Continued*)

The owner has used prevention methods against VL (If YES, collars)[*]				
Yes	61	39.3	Ref	
No	84	28.6	0.62(0.31–1.24)	0.175
If NO, why?				
It doesn't work	4	25	Ref	
VL is not important	29	37.9	1.83(0.17–19.89)	0.618
Other	51	23.5	0.92(0.09–9.71)	0.947
Not proceed	61	39.3	–	–
The owner has used topical insecticides against arthropod bites				
Yes	124	33.9	Ref	
No	21	28.6	0.78(0.28–2.16)	0.634

[a] Variable used as continuous in the model.
[*] Variable included in the multivariate model ($p < 0.2$).

Table 5
Estimates of the mixed logistic regression model determining the risk factors for dogs being seropositive. Risks expressed as Odds Ratios (OR) and their 95% Confidence Intervals (95% CI). Location of dogs was set up as a random effect in the mixed model.

Variable	OR	95% CI	*p*-Value
Age	1.35	1.12–1.64	0.002
Urban-Periurban	0.17	0.06–0.52	0.002
South Pallars Sobirà	6.20	1.25–30.69	0.025
Probability of CanL according to the owner	1.26	1.03–1.54	0.024
Owner is unaware of anti-sand fly methods	11.6	1.83–73.86	0.009

for the variables of village location ($p = 0.039$), habitat ($p = 0.021$), age ($p = 0.002$), and risk of dogs contracting leishmaniasis according to their owners ($p = 0.013$).

3.4. Multivariate analysis

The results of the final multivariate mixed model are summarised in Table 5. The risk of dogs being seropositive increased with age (OR = 1.35, *p*-value = 0.002) (Table 5), among dogs living in the southern part of Pallars Sobirà (OR = 6.20, *p*-value = 0.025), and among those whose owners considered their animals to be at risk of leishmaniasis infection (OR = 1.26, *p*-value = 0.024) and who were unaware of anti-sand fly preventive methods (OR = 11.6, *p*-value = 0.009). The risk decreased when dogs lived in an urban-periurban habitat compared to rural areas (OR = 0.17, *p*-value = 0.002).

4. Discussion

4.1. Veterinarian questionnaire on canine leishmaniasis

The number of questionnaires sent to the practitioners corresponded to the number of veterinary pet clinics in the province of Lleida in 2009. The clinics are distributed among all the counties, with the exception of La Cerdanya, where no veterinary clinic existed when the study was performed. Lleida is mainly an agricultural and farming region with a low human population density and where the veterinarians work principally with farm animals. Despite the small number of questionnaires sent out, as explained above (41), the response (78%) was considerably greater than in other European studies using this methodology (23%, 31% and 47%) (de Ybáñez et al., 2009; Farkas et al., 2011; Gálvez et al., 2011) and similar to a study carried out in Italy (83.3%) (Morosetti et al., 2009), the percentage of response being independent of veterinary opinion on CanL trends. The high percentage of returned questionnaires could be related with the extent of veterinarian awareness of the disease and the widespread opinion that CanL has increased among patients during the last ten years (78.1%) or, as in the Italian study, that cases would increase in the future.

In our study, the dogs attending the veterinarian clinics were mainly from mixed areas (71.9%), reflecting that Lleida is a region composed of towns and villages with agricultural and farming activities. A high percentage of the veterinarians thought that CanL cases were new and acquired locally, which plays in favour of an active focus in the region.

The clinical diagnosis of CanL is hampered by the wide spectrum of clinical signs, including cutaneous or systemic signs, and asymptomatic or atypical forms (Gradoni, 2002; Gállego, 2004; Alvar et al., 2004; Baneth et al., 2008; Solano-Gallego et al., 2009). In agreement with other authors (Gálvez et al., 2011), the most frequent clinical signs observed by the veterinarians in the present study were adenopathies, squamosis, loss of weight and alopecia, the highest diagnostic value being given to loss of weight and adenopathies (56.3% and 50%, respectively). Clinical signs described as uncommon were hyperthermia, splenomegaly and ocular lesions, and these were considered of low diagnostic value, along with renal failure.

Possibly due to the difficulty in clinical diagnosis, all veterinarians used analytical methods to confirm CanL cases. Serology was the diagnostic method of choice to confirm a suspicious case (100%) and results were corroborated in the veterinarian's own laboratory (65.6%) as well as in external private laboratories (78.1%).

All the practitioners recommended actively preventive measures, particularly collars and spot-on products, as in other European regions (de Ybáñez et al., 2009; Morosetti et al., 2009; Gálvez et al., 2011). This could be related with the general veterinary opinion that local CanL cases had increased (78.1%) and that the infections were acquired in the area (93.8%), leading them to consider the disease as a real epidemiological problem.

The information gathered in the veterinary questionnaires, which proved to be a simple, cheap and feasible method for collecting data, helped us to rapidly define the knowledge, perception and awareness of the disease in the naïve region, as has previously been done in other parts of Europe. The questionnaire results support the hypothesis of an existing CanL focus in Pallars Sobirà put forward by Ballart et al. (2012a).

4.2. Cross sectional study of canine leishmaniasis

For a long time, seroepidemiological studies on CanL in Spain were able to take advantage of rabies vaccination campaigns to collect blood. Nowadays, the rabies vaccination is no longer mandatory in some regions, including our area of study, making it necessary to rely on the help of local veterinarians to search for dogs and encourage owner participation. The long tradition of hunting among the inhabitants of Pallars Sobirà explains why a high percentage of dogs in the study were hunting dogs living in kennels in a rural environment (54%). Dogs of this kind of population have been considered as sentinel indicators of diseases of veterinary and zoonotic interest (Cabezón et al., 2010).

The most useful diagnostic approaches to the study of infected dogs include serological and molecular techniques (Solano-Gallego et al., 2009). Many serological methods have been used to diagnose CanL, showing different degree of sensitivity and specificity that could influence in the seropositivity of CanL in an area as it shown in this study (Table 3) (Aisa et al., 1998; Iniesta et al., 2002; Riera et al., 1999). For this reason we decided to combine several techniques and considered as seropositive dogs that tested positive with at least two immunological methods according to Iniesta et al. (2002). Studies applying IFAT have used different cut-offs: 1:40 in areas considered non endemic (Morosetti et al., 2009; Baldelli et al., 2011), and 1:80 or 1:160 in endemic areas (Dereure et al., 2009; Martín-Sánchez et al., 2009; Gálvez et al., 2010; Morales-Yuste et al., 2012). We decided to use the intermediate cut-off (1:80) for the naïve area studied, which is also the most frequently used in Europe (Franco et al., 2011). All the dogs with IFAT values of 1:80 produced at least two other positive serological tests (data not shown), confirming the utility of the cut-off considered in the present study.

The seroprevalence reported in this study (33.1%), in an area where the presence of the disease was unknown, is high considering that the highest seroprevalence value found in a known focus in Spain is 34.6% (Morillas et al., 1996). However, our figure can be considered as an overestimation due to the biased nature of the sample. The analyzed dogs (57.2% from rural and 37.2% from periurban environments and 80.7% in kennels) lived outdoors in optimal conditions for disease transmission (Fisa et al., 1999; Amusategui et al., 2004; Martín-Sánchez et al., 2009; Gálvez et al., 2010; Morales-Yuste et al., 2011), due to the predominant exophilic and exophagic character of most sand fly species taking a blood meal at twilight or night (Killick-Kendrick, 1999).

The density of dogs living in kennels is another factor that facilitates parasite transmission both directly and by vector, as previously mentioned and reviewed (Riera and Valladares, 1996; Solano-Gallego et al., 2009).

The risk of seropositivity in dogs increased with age, contrary to the results reported by Morales-Yuste et al. (2011). Even if the seroprevalence may vary in different age groups and may decrease in older dogs (Fisa et al., 1999; Alonso et al., 2010) the risk of seropositivity tends to increase with age (Gálvez et al., 2010). All the analyzed dogs of <1 year included in the study had spent the summer period in the area and therefore could have been infected by a sand fly bite. With age, the risk of exposure to sand fly bites increases (Abranches et al., 1991) due to more transmission periods spent in endemic areas. The chronic evolution of CanL also favours the possibility of sand flies becoming infected in endemic areas.

Similarly to other studies (Martínez-Cruz et al., 1990; Acedo Sánchez et al., 1996; Alonso et al., 2010; Gálvez et al., 2010; Morales-Yuste et al., 2011), no significant differences were found regarding altitude (Table 4). Nevertheless, the multivariate analysis showed an increasing or decreasing risk for CanL depending on whether the dog location was southern or northern, respectively (Table 5). The odds of leishmaniasis infection increased 6.20 times in dogs living in southern Pallars Sobirà. Differences in prevalence could be related to the qualitative and quantitative composition of the vectors. The two vector species of *L. infantum* in Spain, *Phlebotomus ariasi* and *P. perniciosus*, are present in the area, but their relative abundance differs between the north and south (authors' unpublished data). *P. ariasi* prefers humid and sub-humid environments whilst *P. perniciosus* is favoured by periarid or subhumid conditions (Gállego et al., 1990). Also, differences in temperature between both areas could play a role in the activity period and phenological behaviour of the vectors, which may affect their vectorial ability. It has been observed that the risk period in a transmission season varies according to the age structure of the gonotrophically concordant sand fly population and is independent of the maximal population density. In addition, the impact of temperature on the life cycle of leishmania in the vector has been demonstrated experimentally (Rioux et al., 1985).

No differences were found related to the sleeping habits of the dogs (outdoors/indoors), in contrast with results reported by other authors (Zaffaroni et al., 1999; Gálvez et al., 2010; Morales-Yuste et al., 2011). Location of dogs in a rural environment increases CanL risk, as shown by the multivariate analysis. This factor has also been mentioned as influential by other authors (Acedo Sánchez et al., 1996; Biglino et al., 2010).

The proportion of asymptomatic dogs was statistically similar to that of symptomatic animals, as in numerous other studies (Acedo Sánchez et al., 1996; Fisa et al., 1999; Solano-Gallego et al., 2001; Gálvez et al., 2010; Cabezón et al., 2010). As mentioned earlier, one or more clinical signs compatible with CanL were observed in symptomatic dogs. Some of these signs (alopecia, adenopathies and squamosis) were also mentioned in the veterinarian questionnaire as those most frequently observed.

Two variables related with the owners were found to be risk factors for dog infection (Table 5): owner opinion about the risk of their dogs contracting CanL during their lifetime (OR: 1.26) and owner unawareness of prophylactic measures against CanL that protect dogs from sand fly bites (OR: 11.6) (Table 5). In the first variable, ownership of an ill dog could heighten the perceived risk of infection for the animals in the area. In the second variable, owner unawareness of preventive measures implies a high possibility of their dogs experiencing sand fly bites and consequently being infected. In rural areas most dog owners did not routinely visit a veterinarian, especially if their animals were not ill, and so were not exposed to recommendations for using the preventive measures mentioned by veterinarians in the questionnaire. Nevertheless, we found that 42.1% of the analyzed dogs used a preventive method, a collar, which is effective against sand fly bites (Molina et al., 2001).

The utility of sampling kennel dogs in studies on CanL has been discussed (Cabezón et al., 2010; Baldelli et al., 2011; Miró et al., 2012), referring mainly to kennels that collect free-roaming and abandoned dogs with no data on their origin. In our case, the kennels had private owners and the majority of the dogs were local and had identification cards. Thus, our study of kennel dogs of local origin proved useful for detecting an autochthonous focus of leishmaniasis through the analysis of a small number of animals (145 dogs). Nevertheless, it should be taken into account that this is a biased canine population and so the results obtained do not reflect the real prevalence of CanL in the area. Consequently, serological studies of a more representative population of dogs are necessary to obtain more accurate data.

4.3. Presence of a stable focus of canine leishmaniasis in Lleida province (Spain)

The idea that CanL has increased in Spain in the last decades is widespread among veterinarians as well as dog owners. Nevertheless, whether this perceived rise in CanL is real or is due to a greater awareness and improved diagnosis remains an open debate. The spread of CanL in some parts of Europe has been demonstrated in recent years (Maroli et al., 2008; Dereure et al., 2009; Miró et al., 2012). Data on CanL in Lleida province are very scarce and recent (Ballart et al., 2012a), and only 37 cases of HL were declared between 1986 and 2010 (updated from Ballart et al., 2012a), which could have been locally acquired or imported from other endemic areas. In Mediterranean *L. infantum* foci, dogs are considered the main reservoir for HL. An Iranian study showed that dog ownership is a major risk factor for *L. infantum* infection in humans (Gavgani et al., 2002).

Although no comparative data are available, environmental changes leading to an increase and new distribution of sand fly vectors have been proposed as responsible for the spread and/or increase of the disease (Desjeux, 2001; Ferroglio et al., 2005; Ready, 2008; Morosetti et al., 2009; Colwell et al., 2011; Ballart et al., 2012b). Furthermore, only one fragmentary study on sandflies in Lleida province has been reported (Gállego et al., 1990), so it is not possible to estimate if the distribution and density of the vector has changed in recent years.

The high percentage of seropositive asymptomatic dogs detected in the current study prompts us to hypothesize that the existence of CanL in Pallars Sobirà county is not very recent, which is supported by a seroprevalence threshold usually associated with steadily established CanL foci (2.5%) (Rioux et al., 1971). Also, a high percentage of asymptomatic cases among seropositive dogs is frequently found in endemic areas (Fisa et al., 1999; Gálvez et al., 2010), which would also support the idea that CanL is not new in the studied area. Thus, although there is no conclusive data about whether the disease is increasing or is now being diagnosed more efficiently, the present study shows the existence of a CanL focus in the Lleida province, particularly in the Pyrenean area. The observed seroprevalence of CanL complies with the criteria established by Maroli et al. (2008) to define an undisputable leishmaniasis focus: (i) the confirmation of one or more CanL cases, mostly by serological methods and in one case isolation of the parasite (Ballart et al., 2012a); (ii) seropositive dogs found amongst the asymptomatic residents, and (iii) two vector species present in the area of study (Gállego et al., 1990). In addition, *L. infantum* DNA has been found in sand flies of the region (Alcover et al., 2012). The partial data provided by our study using standardized methods (EDEN), could be the basis of future research to determine the extension and dynamics of the focus.

5. Conclusion

The response to the opinion poll carried out among veterinary practitioners in the province of Lleida (Spain) suggested the presence of an autochthonous focus of canine leishmaniasis in this region, which was confirmed by a cross-sectional serological study in Pallars Sobirà. Further serological studies on a more representative dog population are required to know the true extent and prevalence of the infection.

Conflicts of interest

The authors have no conflicts of interest concerning the work reported in this paper.

Ethics

Not required.

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