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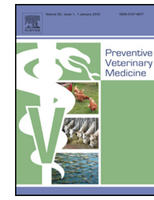

A cross-sectional study of the impact of regular use of insecticides in dogs on Canine Leishmaniosis seroprevalence in southeast Spain

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ABSTRACT

The relationship between Canine Leishmaniosis (CanL) seroprevalence and regular use of topical insecticides was investigated in 800 pet dogs with no visible signs of CanL in Murcia, southeast Spain in 2011. Dogs were clients to 17 veterinary practices and were analyzed for *Leishmania infantum* antibodies in blood plasma using two commercial ELISAs (Ingezim, Ingenasa®, Spain; Leishcan, Hipra®, Spain). Owners were interviewed to gather data on dog related variables. They included date of birth, home address and frequency, duration and timing of insecticide treatments used to prevent ectoparasite infestations. The dog's residence was georeferenced and environmental data potentially associated with the dog's risk of *L. infantum* infection was obtained. A mixed logistic regression model was then developed to analyze the relationship between the dog's serological status and insecticidal treatment adjusted for demographic and environmental variables. Overall, CanL seroprevalence (95% confidence limits) was 18% (16–21%) including 11% in dogs not using insecticide treatments ($n = 60$) and 19% in those using them ($n = 740$) ($p > 0.05$). At least 16 different insecticide products were used and 73%, 26% and 1% of dogs received 1, 2 and 3 products a year. The most frequent commercial brands used and the only ones in the market claiming anti-sandfly activity, were Scalibor collars (deltametrin 40 mg/g; MSD®), Advantix pipettes (permethrin 500 mg/ml and imidacloprid 100 mg/ml; Bayer®) and Exspot spot-on pipettes (permethrin 715 mg/ml; MSD®). Seroprevalence was 9%, 16%, 20%, 22% and 25% for dogs with Scalibor collars plus Advantix pipettes, Scalibor collars plus ExSpot pipettes, Advantix pipettes alone, Scalibor collars alone and Exspot pipettes alone, respectively. The multivariable model confirmed a significant reduction in the risk of *Leishmania* spp. seropositivity in dogs using the Scalibor and Advantix combination compared to those using either product alone and provided evidence of greatly increased risk of CanL in rural areas situated at 300–500 m altitude and average March–July temperatures of 18.6–19 °C. The study highlights the difficulty in controlling CanL infection by means of insecticide use alone and that it could be improved by using the Scalibor and Advantix combination and identifying and targeting specific geographical areas.

1. Introduction

Canine Leishmaniosis (CanL) caused by *Leishmania infantum* is a major vector borne zoonotic infection in Mediterranean Spain causing mortality and a spectrum of severe clinical outcomes (Baneth et al., 2008). Its impact in endemic areas is further exacerbated by the difficulty in preventing infection (Otranto and Dantas-Torres, 2013). The efficacy of available vaccines is still under assessment and treatment of infected animals doesn't achieve parasitological cure. Moreover, it's not implemented on asymptomatically infected animals, which represent 50–80% of dogs living in some endemic areas in Spain (Solano-Gallego et al., 2001; Chitimia et al., 2011). In European countries CanL control efforts primarily focusses on preventing phlebotomine sandfly vector biting by using insecticides with residual activity in the dog (Otranto and Dantas-Torres, 2013).

When this study was carried out there were no leishmania vaccines in Spain and three commercial insecticide products specifically marketed to prevent phlebotomine sandfly vector

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infection were available: Scalibor insecticide-impregnated collars (deltametrin 40 mg/g; MSD®), Advantix topical (spot-on) pipettes (permethrin 500 mg/ml and imidacloprid 100 mg/ml; Bayer®) and Exspot spot-on pipettes (permethrin 715 mg/ml; MSD®). In laboratory conditions all three products have a sandfly repellent (blood feeding avoidance on a treated dog) efficacy greater than 90% and an insecticidal efficacy (additional mortality of fed and unfed sandflies exposed to a treated dog) of 30–99%, during the first three weeks after application (Killick-Kendrick et al., 1997; Mencke et al., 2003; Miró et al., 2007; Molina et al., 2012). Controlled field trials in pets and kenneled dogs in places with a high CanL prevalence corroborate that both Scalibor collars and Advantix and Exspot pipettes can be efficacious at preventing infection in dogs. However, the reduction in annual CanL seroconversión ranged from 50 to 100% depending on the year and the product used (Maroli et al., 2001; Foglia Manzillo et al., 2006; Otranto et al., 2007, 2010; Ferroglio et al., 2008). The residual activity of these products is time dependent, lasting 3 to 4 weeks for pipettes and around six months for the collar however, collar losses can be a major problem (Foglia Manzillo et al., 2006). Other factors conditioning the practicality of collars as a tool for controlling CanL is the percentage of the dog population collared and the infection incidence (the greater, the more useful collars are) (Maroli et al., 2001; Reithinger et al., 2004).

There is however a lack of information on the performance of dog insecticides to prevent CanL infection in the wide dog population in endemic areas in uncontrolled field conditions. Such studies maybe hampered by the difficulty in comparing the risk of infection in heterogeneous dog populations with widely variable exposure to infected sandflies. In the present study we employed multivariable regression modeling to investigate the relationship between regular use of anti-sandfly insecticide products and dogs CanL serological antibody status in endemic Murcia Region, to control for critical dogís demographic and residential environmental variables. CanL seroprevalence in Murcia was recently estimated at 7% for dogs from periurban residential areas (Chitimia et al., 2011).

Table 1
Canine Leishmaniosis seroprevalence (95% confidence intervals) according to dogís signalment and body weight. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011.

Variable	Level	*N*	ELISA			*P* value
			% Positive	95−	95+	
Gender	Female	426	15	11	18	0.0041
	Male	375	23	18	27	
Age (years)	0.5–1	172	9	5	14	0.0484
	2	112	16	9	23	
	3	103	16	9	23	
	4	72	21	11	30	
	5	57	19	9	30	
	6	69	22	12	31	
	7	44	25	12	38	
	8	25	24	7	41	
	9	33	27	12	42	
	10	27	30	12	47	
	>10	78	23	14	32	
Breed	Mixed	306	18	14	22	0.3659
	Spanish	37	27	13	41	
	Other countries	457	18	14	21	
Body weight	1.5–7.0	210	9	5	13	<0.0001
	7.1–15.0	189	18	13	23	
	15.2–27.0	177	19	13	25	
	27.5–73.0	216	27	21	33	

2. Materials and methods

2.1. Study population and data collection

The study was carried out on up to 800 pet dogs aged 6 months-old or older, with no visible clinical signs of CanL, clients to 17 veterinary practices across Murcia Region, in November 2011. This is a traditionally endemic southern Mediterranean area (de Ybáñez et al., 2009) situated 38°N and 1°50′W, ranging 11300 km^2, with approximately 1470000 inhabitants. There is no recent information on sandfly abundance in the region. Studies in the 1980s showed that *Phlebotomus perniciosus* was the main *L. infantum* vector in Murcia with peaks of maximum abundance in June and September and no activity recorded from mid-November until April (Martínez Ortega and Conesa Gallego, 1987).

Dogs selected attended the veterinary practice to participate in the study as they were offered a CanL serological analysis free of charge, or for a consultation unrelated to CanL. Upon selection, dogs were clinically examined to discard animals with visible CanL signs (Baneth et al., 2008) and were then blood sampled and weighed. Owners were asked to answer a brief questionnaire about the dogís date of birth, gender, breed, residential home address and type, presence of people and animals in their home, sleeping place and insecticide usage in the animal (Tables 1–3). The specific questions concerning insecticide use were: (i) do you use insecticides in the dog on a regular basis to protect it against insects, mites and ticks? if you do, (ii) which insectide brand or brands do you use on your dog on a regular basis? and (iii) how many months and when in the year is your dog under insecticide treatment?

2.2. Blood sampling and L. infantum ELISA antibody analysis

Blood samples were taken from the cephalic vein into EDTA vacuum tubes, plasma recovered by centrifugation and used to detect *L. infantum* IgG antibodies with two commercial antibody tests: Ingezim Leishmania (Ingensa®, Spain) and Civtest Canis Leishmania (Hipra®, Spain, later commercialized by Esteve®, Spain as Leiscan). Both tests use crude immunodominant *L. infantum* antigens and conjugates include a specific canine IgG monoclonal in the Ingezim test and a generic protein A/HRPO in the Civtest. The validity of these tests has been assessed by manufacturers using the indirect immunoflorescence test (IFI) as the reference test. The Ingezim showed a 95% and 80% results agreement for 1/100 and 1/160 IFI cut-offs, respectively, and the estimated Civtestís sensitivity (Se) and specificity (Sp) was 98% and 96%, respectively. Moreover, the performance of both tests was recently assessed in experimentally infected dogs by Rodríguez-Cortés et al. (2013). Sp was 100% for both tests whilst Se were 98% for Leiscan and 78% for Ingezim.

Samples were done in duplicate and antibody optical densities (OD) were read in a spectrophotometer. The mean of both readings were used to classify samples as positive,-negative or- inconclusive, following kit instructions.

2.3. Spatial characterization of the dogís residential area

The dogís residential environment was characterized using a geographical information system (ArcGIS v.10, ESRI, Redlands, USA), and used with other dog variables, as covariates in the analysis of the relationship between the dogs CanL serological status and insecticide use. The approach taken was described in detail in a recent human Leishmaniosis study in Murcia (Pérez-Cutillas et al., 2015). Briefly, the dogís home address was used to obtain the geographical coordinates (http://iderm.imida.es/) and define a 500 m radius circular area around the point location from which environmental data was extracted (Tables 4 and 5). Ecological variables considered were the average monthly mean temperature, rainfall and relative humidity, obtained from an interpolation using point data from 45 regional weather stations (http://siam.

Table 2
Canine Leishmaniosis seroprevalence (95% confidence intervals) according to dogís insecticide usage. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011.

Variable	Level	*N*	ELISA			*P* value
			% positive	95−	95+	
Regular use of insecticides	No	60	12	4	20	0.2218
	Yes	740	19	16	22	
No. of insecticides used in any one year	1 Product	522	20	17	24	0.6715
	2 Products	191	17	11	22	
	3 Products	10	10	0	29	
Insecticide application mode	Spot-on	334	19	14	23	0.1312
	Collar	192	20	14	25	
	Spot-on and collar	142	14	8	20	
	Spray	36	22	9	36	
	Lotion/shampoo	18	39	16	61	
Insecticidal product regularly used	Advantix spot-on[1] only	159	20	14	26	0.3721
	Exspot spot-on[2] only	40	25	12	38	
	Scalibor collar[3] only	127	22	15	9	
	Advantix spot-on and Scalibor collar	69	9	2	15	
	Advantix spot-on and other products	21	19	2	36	
	Exspot spot-on and Scalibor collar	25	16	2	30	
	Exspot spot-on and other products	16	31	9	54	
	Scalibor collar and other products	45	18	7	29	
	Other products[4]	161	17	12	23	
Insecticidal product; annual treatment period (months)	Advantix spot-on only; 1–7	49	16	6	27	0.2002
	Advantix spot-on only; 8–12	106	21	13	28	
	Exspot spot-on only; 8–12	29	24	9	40	
	Advantix spot-on; 8–12 and Scalibor collar; 12	53	6	0	12	
	Exspot spot-on; 8–12 and Scalibor collar; 12	12	17	0	38	
	Scalibor collar only; 3–9	32	22	8	36	
	Scalibor collar only; 12	88	22	13	30	

Advantix pipettes[1]: 50% permethrin and 10% imidacloprid, Exspot pipettes[2]: 65% deltamethrin,Scalibor collar[3]: 4% deltamethrin,other products[4]: included pipettes and collars other than Scalibor collars and Advantix and Exspot pipettes as well as insecticide body shampoos and lotions.

Table 3
Canine Leishmaniosis seroprevalence (95% confidence intervals) according to dogís home characteristics and sleeping place. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011.

Variable	Level	*N*	ELISA			*P* value
			% positive	95−	95+	
Home type	Modern terraced	70	19	9	28	<0.0001
	Traditional terraced	141	16	10	22	
	Farmhouse	313	26	21	30	
	Detached house	143	16	10	22	
	Apartment	128	5	2	9	
Sleeping place	Indoors	433	12	9	15	<0.0001
	Outdoors	362	26	21	30	
Other dogs at home	No	340	16	12	19	0.1004
	Yes	461	20	17	24	
Cats at home	No	648	18	15	21	0.4269
	Yes	153	21	14	27	
Other animals at home	No	665	18	15	21	0.6741
	Yes	135	20	13	27	
No. people at home	0	60	30	18	42	0.1325
	1	67	16	8	25	
	2	239	15	10	19	
	3	163	18	12	24	
	4	179	21	15	27	
	≥5	63	19	9	29	

imida.es/), the mean ground altitude (Digital Elevation Model, NASA TERRA mission, http://gdem.ersdac.jspacesystems.or.jp) and the CORINE Land Cover use (1:100000) (http://terrestrial.eionet.europa.eu/CLC2006). In addition, individual dogís residential environment were visually examined using orthoimages from WMS of IDERM and classified into one of eight habitat classes. They included the six previously defined human habitats (Pérez-Cutillas et al., 2015), i.e.: urban areas with a high or medium-low population density, periurban residential areas, and rural villages, hamlets and isolated farms, and two additional ones: industrial states and periurban orchard holdings (Table 4).

2.4. Statistical analysis

Seroprevalence was defined as the proportion of samples positive or inconclusive (ODs between testís negative and positive thresholds) to one or both ELISA tests. It was estimated globally and for specific explanatory variables (Tables 1–5). Seroprevalence and median log-transformed ODs across variable levels were compared using Yates-corrected Chi-squared test and the Kruskal–Wallis test, respectively (Kirwood and Sterne, 2003).

The relationship between the dogs serological status (seropositive or seronegative) and the insecticide product regularly used

Table 4
Canine Leishmaniosis seroprevalence (95% confidence intervals) according to the dogís residential setting and environment. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011.

Variable	Level	*N*	ELISA			*P* value
			% positive	95−	95+	
Residential setting	Urban high density	144	9	4	14	0.0054
	Urban low density	206	16	11	20	
	Periurban residential states	37	16	4	28	
	Periurban orchard holdings	186	23	17	29	
	Rural villages	38	21	8	34	
	Rural hamlets	41	29	15	43	
	Isolated ranches	47	28	15	40	
	Industrial states	8	0	0	0	
Predominant land coverα	Artificial surfaces[1]	232	12	8	16	0.0045
	Natural areas[2]	343	23	19	27	
	Mix artificial and natural	89	15	7	22	
	Green urbanized areas[3]	75	20	11	29	
Altitude	04–98	228	14	10	19	0.0007
	103–299	73	25	15	35	
	301–500	107	30	21	39	
	503–878	292	15	11	19	

Predominant land cover from Corine Land Codes (CLC): Artificial surfaces[1]: 111 (Continuous urban fabric), 1.2 (Industrial, commercial and transport units), 1.3 (Mine, dumps and construction sites). Natural[2]: 2 (Agricultural areas), 3 (Forest and seminatural areas), 4 (Wetlands), 5 (Water bodies), Green urbanized areas[3]: 112 discontinuous urban fabric: residential states with gardens, 1.4 (artificial non-agricultured vegetated areas).

was further explored using random effects logistic regression, to adjust for confounding by explanatory variables and consider the potential correlation between results of dogs from the same veterinary practice (Kleinbaum et al., 1998). The later was modelled as a random effect and other explanatory variables as fixed categorical variables. Categories were those in Tables 1–5 except age that was grouped according to seroprevalence, and altitude and average month temperature between March and July that was included as a single variable combining both (Table 6). The later variable was shown to be the main environmental predictor of human *L. infantum* infection in Murcia (Pérez-Cutillas et al., 2015).

A step-wise model building strategy was used using Maximum Likelihood estimation method. Variables remaining in the final model were those associated with the outcome or known to be important risk factors of *L. infantum* infection. Adjusted odds ratios were used to quantify the relationship between explanatory variables and the dogs CanL serological status. Significance was taken for $p < 0.05$ for a two-tailed test. All analysis were carried out in the R program (http://cran.r-project.org/).

3. Results

3.1. CanL seroprevalence and univariate relationship with sex, age, body weight and insecticide usage

The percentage of Civtest and Ingezim positive dogs were 9% (75/800) and 16% (125/800), respectively, and 147/800 dogs were positive to one or both tests. Accordingly, the estimated CanL seroprevalence (95% confidence interval) was 18% (16–21%). Seroprevalence varied with gender, age and body weight. It was 15% in females compared to 23% in males and increased from 9% among 0.5–1 year old dogs to 30% among 10 year olds and from 9% in 1–7 kg dogs compared to 27% in 27.5–73.0 kg dogs ($p < 0.05$) (Table 1). In contrast, seroprevalence was 12% in insecticide-free dogs ($n = 60$) compared to 19% in those regularly having insecticides ($n = 740$) ($p > 0.05$) (Table 2). The age and sex distribution and the proportion of dogs living in an urban or rural environment was similar for dogs having and not having insecticides ($p > 0.05$). In contrast, the mean weight of the latter was significantly lower ($p < 0.05$). Among dogs having insecticides, seroprevalence was 9% ($n = 69$) in dogs with Scalibor collars plus Advantix pipettes, 16% ($n = 25$) in those with

Table 5
Canine Leishmaniosis seroprevalence (95% CI) according to climatic variables in the area around the dogís residence. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011.

Period	Temperature (°C)				Rainfall (mm)				Relative humidity (%)			
	Range	*N*	% ELISA positive	*P*	Range	*N*	% ELISA positive	*P*	Range	*N*	% ELISA positive	*P* value
Annual	13.6–15.0	222	16	0.0622	288–329	150	24	0.0546	61–65	463	19	0.0910
	15.1–16.5	88	16		342–370	367	17		66–70	122	21	
	16.6–17.5	146	25		371–413	185	14		71–76	117	11	
	17.6–18.3	246	15									
January–February	05.8–8.0	267	16	0.2270	37–51	197	15	0.1435	66–69	36	28	0.0774
	08.1–10.0	76	24		52–67	157	23		70–73	557	18	
	10.1–10.5	98	22		68–86	348	17		74–79	109	12	
	10.6–11.3	261	16									
March–July	16.4–17.5	155	17	0.1244	079–104	287	18	0.4619	56–61	461	18	0.1014
	17.6–18.5	142	15		105–130	120	21		62–67	126	21	
	18.6–19.0	120	23		131–154	120	21		68–75	115	11	
	19.1–19.5	185	21									
	19.6–20.5	100	11									
September–October	16.1–18.5	279	16	0.0294	053–81	166	24	0.0145	66–69	482	20	0.1497
	18.6–20.0	165	25		082–109	453	17		70–73	110	16	
	20.1–21.0	53	9		110–137	83	10		74–77	110	12	
	21.1–21.9	205	17									

Table 6
Estimates of the multivariable logistic regression model of the relationship between the dogís Canine Leishmaniosis serological status and dog demographic and residence variables. A cross-sectional study in dogs from Murcia, southeast Spain in autumn 2011 ($n = 588$ dogs).

Variable	Level	ELISA Odd ratio	95−	95+	P value
Age (years)	1	1			
	2–3	1.21	0.55	2.69	0.6343
	4–6	1.99	0.93	4.26	0.0763
	7–15	2.78	1.32	5.85	0.0073
Bodyweight (kg)	1.5–7.0	1			
	7.1–15.0	2.91	1.36	6.25	0.0061
	15.2–27.0	2.00	0.85	4.70	0.1136
	27.5–73.0	3.82	1.73	8.40	0.0009
Sleeping place	Indoors	1			
	Outdoors	1.72	0.94	3.16	0.0784
Residential setting	Urban high density and industrial states	1			
	Urban low density	2.56	1.08	6.11	0.0337
	Periurban residential states	2.23	0.62	7.94	0.2172
	Periurban orchard holdings	1.92	0.81	4.56	0.1403
	Rural villages	2.50	0.71	8.80	0.1538
	Rural hamlets	3.56	1.02	12.48	0.0473
	Isolated ranches	3.58	1.09	11.82	0.0360
Altitude (m); Average March–July Ambient temperature (°C)	04–98; 19.1–19.5	1			
	04–98; 19.6–20.5	0.80	0.32	2.01	0.6362
	103-–299; 18.6–19.0	0.32	0.06	1.77	0.1918
	103–299; 19.1–19.5	2.80	0.79	9.97	0.1116
	301–500; 18.6–19.0	4.48	1.87	10.75	0.0008
	503–878; 16.4–17.5	1.56	0.67	3.59	0.2998
	503–878; 17.6–18.5	1.15	0.45	2.95	0.7668
Regular insecticide preventive Treatment	No treatment	1			
	Advantix pipettes	2.14	0.73	6.27	0.1661
	Advantix pipettes[1] and Scalibor collar[2]	0.65	0.17	2.59	0.5458
	Advantix pipettes and other products[3]	3.06	0.66	14.23	0.1542
	Exspot pipettes[4]	3.62	1.02	12.84	0.0464
	Exspot pipettes and other products	14.45	2.15	97.26	0.0060
	Scalibor collar	1.83	0.62	5.36	0.2712
	Scalibor collar and Exspot pipettes	4.39	0.96	20.10	0.0568
	Scalibor collar and other products	1.94	0.47	8.05	0.3605
	Other products	2.32	0.76	7.07	0.1370

Insecticides: Advantix pipettes[1]: 50% permethrin and 10% imidacloprid, Scalibor collar[2]: 4% deltamethrin, other products[3]: included pipettes and collars other than Scalibor collars and Advantix and Exspot pipettes as well as insecticide body shampoos and lotions, Exspot pipettes[4]: 65% deltamethrin.

Scalibor collars plus ExSpot pipettes, 20% ($n = 159$) in those with Advantix pipettes alone, 22% ($n = 127$) in dogs with Scalibor collars alone and 25% ($n = 40$) in those with Exspot pipettes alone ($p > 0.05$) (Table 2). Differences in seroprevalence between dogs using Scalibor collars plus Advantix pipettes and those using Scalibor collars or Exspot pipettes alone were significant ($p < 0.05$).

Treatment duration ranged from 1 to 12 months a year and was always administered from spring onwards when used for less than 12 months.The group of dogs with the lowest seroprevalence was 6% corresponding to those using Advantix pipettes and Scalibor collars for 8–12 months and 12 months a year, respectively (Table 2). Median ODs among seropositive dogs was similar for dogs using or not insecticides and for different insecticide brands and combinations ($p > 0.05$).

3.2. Univariate relationship between CanL seroprevalence and dogís residential environment

There were marked differences in CanL seroprevalence according to the dog's place of residence and it was 5% in dogs living in apartments compared to 26% among dogs living in a farm. Also, seroprevalence was 26% in dogs sleeping outside compared to 12% in those sleeping indoors ($p < 0.05$) (Table 3). Sharing a home with other dogs was also associated to higher prevalence although differences were not significant (Table 3).

The strong association between seroprevalence and the dog's residence is further exemplified when considering environmental variables in the dog's wider home surroundings. Seroprevalence was 9% in dogs in highly densely populated urban areas, 16% in urban areas with a lower population density and in periurban residential states, 21% in rural villages, 23% in periurban orchard holdings, 28% in isolated ranches and 29% in rural hamlets ($p < 0.05$) (Table 4). Similarly, seroprevalence was 12% in areas dominated by artificial surfaces, 20% in green urbanized areas, 23% in predominantly natural areas and 15% in areas with a mixture of artificial and natural areas ($p < 0.05$) (Table 4).

CanL seroprevalence also varied depending on climatic variables. Areas with the highest prevalence had moderate or moderately high annual and seasonal temperatures, lower annual and autumn rainfall and lower relative humidity compared to areas with less seroprevalence, although differences in some cases where not significant (Table 5).

3.3. Relationship between CanL seroprevalence and insecticide use adjusted for dog demographic and environmental variables in the place of residence

The most parsimonious logistic regression model investigating the relationship between CanL seroprevalence and insecticides in dogs included age, body weight, sleeping place (outdoors or indoors), residential setting and a variable representing the combination between altitude and the average March–July temperature in the dog's residence (Table 6). There was no significant residual variation associated to the veterinary practice.

The model confirmed the lack of an association between dogs having insecticide treatment on a regular basis and reduced seroprevalence (Table 6). Among treated dogs, the combination of Advantix pipettes and Scalibor collars was associated with a significantly ($p < 0.05$) or marginally significantly ($p < 0.10$) lower risk of being seropositive compared to dogs having these products alone or in combination with others (OR not shown). The model also indicated that seropositivity was greatest for dogs living in the 300–500 m altitude range with average March–July temperatures of 18.6–19.0 °C than dogs living below 100 m and with average March–July temperatures of 19.1–19.5 °C (Table 6).

4. Discussion

The percentage of dog owners using insecticides on a regular basis was over 90%, probably higher than that in the overall population in Murcia. Owners taking their dogs to the veterinarian and willing to have a CanL investigation would be more concerned about preventing infection and using insecticides. They were also likely to provide accurate information. However, it was considered unrealistic to ask them about the dog's life-long insecticide use and obtain accurate data. It's probable that owners of most young and middle age dogs used the same ectoparasite control program as recommended by their veterinarian, throughout their dog's life. This may have not been the case for older animals particularly those outliving the products life in the Spanish market (Advantix, Scalibor and Exspot were available since 2004, 2000 and 1997, respectively). Insecticidal products efficacy cannot be accurately estimated unless dogs are treated from the youngest age, before being exposed to sandflies and manufacturer's indications are strictly followed. Some dogs in the present study may have been infected before using the products claimed. It's also likely that products were not always used as recommended since owners may not be fully aware or find difficult to strictly adhere to manufacturer's indications. Study results however, provide a moment in time picture of CanL seroprevalence in dogs in relation to regular insectide usage. They indicate that the availability of these products in an endemic area for a long time has not resulted in a significant reduction in CanL seroprevalence, although it was lowest in dogs using the combination of Scalibor collar and Advantix pipettes than either product alone or Exspot. This is an important finding with practical implications for the control of CanL. Experimental trials should be carried out to corroborate the efficacy of the double treatment with Advantix and Scalibor and to examine related dog health and safety issues. The latter is important because using the two antiparasitic formulations concomitantly may increase the risk of pyrethroid toxicity.

The intrinsical limitations associated to insecticidal products to prevent *Leishmania* spp. infection have been demonstrated experimentally. Advantix and Exspot's repellent efficacy against *P. perniciosus* is 87–99% during the first three weeks after application and is considerably reduced in the fourth and subsequent weeks (Miró et al., 2007; Molina et al., 2012). As mentioned previously, in controlled field trials Scalibor collars may only reduce the annual risk of seroconversion by 50% (Maroli et al., 2001; Foglia-Manzillo et al., 2006) hence, the risk of dogs becoming infected gradually increases year after year, possibly to the levels observed in this study. The dog's chance of becoming infected may be further increased if insecticidals are not used throughout the entire sandfly season. Some owners in the present study used them for less than 8 months a year and this would not always cover the adult sandfly season in Murcia. Spot-on formulations may be washed off if dogs are soaked in water before the product is absorbed. Incorrect mechanical application of the product can also reduce its ability to prevent infection. Study findings highlight the need for veterinary practices to keep owners informed of the risks of vector exposure, and on the importance of proper use and limitations associated with insecticidal products to prevent CanL.

The reasons for the low seroprevalence among insecticide-free dogs are unclear. It is possibly a chance finding given the relatively small number of dogs in this group ($n = 60$). *L. infantum* seroprevalence is an indirect measure of the extent of CanL susceptibility (Baneth et al., 2008). Two serological tests were used here to increase diagnostic sensitivity. In contrast to the study by Rodríguez-Cortés et al. (2013), Ingezim detected a greater proportion of seropositives compared to Civtest. Variability in diagnostic tests performance could be related to differences in the number and origin of the dogs investigated. Our seroprevalence estimation when considering only Civtest positives as seropositive is similar to that in a previous study in dogs from periurban areas in Murcia (Chitimia et al., 2011). Given that both tests are highly specific (Rodríguez-Cortés et al., 2013), it should be advisable to employ both tests to detect a greater number of seropositives and investigate associated risk factors.

The study also provided useful baseline CanL epidemiologic data for Murcia, particularly for dogs visiting veterinary practices and using insecticidal treatments. Murcia is a typical southern Mediterranean *L. infantum* endemic area. Like in other studies in this area, seroprevalence increased with age except in oldest dogs and with bodyweight. The former could be associated to a greater death rate among seropositive dogs due to CanL and other concomitant infections, and the latter to bigger dogs being used as guard dogs and living permanently outside (Alvar et al., 2004). The dogís residential setting, altitude and temperature also had a major influence in seroprevalence, presumably by affecting sandfly abundance and exposure to infection. Studies in Murcia and nearby areas showed a similar relationship between leishmaniosis and altitude and temperature (Alonso et al., 2010; Pérez-Cutillas et al., 2015).

As expected, taking into account environmental and other dog-related variables is an essential part of population-based studies investigating the role of insecticides against vector-borne infections. They can partly overcome lack of accurate information on vector abundance, which is costly and time demanding to obtain. Moreover, the epidemiological information in this article can help design rational, cost-effective entomological studies. Future cross-sectional studies in Murcia would be required for a more accurate indication of the role played by insecticides in the dog on CanL seroprevalence in the region.

5. Conclusion

Using preventive anti-sandfly insecticide products on a regular basis appears to have no effect on CanL seroprevalence except possibly for the combination of Scalibor collars (deltametrin 40 mg/g; MSD®) and Advantix pipettes (permethrin 500 mg/ml and imidacloprid 100 mg/ml; Bayer®). Stronger emphasis needs to be placed on informing dog owners of the best protocols to minimize sandfly infection and on identifying and targeting high prevalence areas to improve CanL control.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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