



Leptospira seroprevalence in owned dogs from Spain

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ABSTRACT

Leptospira is a widespread zoonosis that has been linked to transmission between dogs and humans. The main purposes were to determine the seroprevalence of anti-*Leptospira* serum antibody and to identify the most common serovars in dogs in Spain. This is a cross-sectional study with 1,310 records of canine *Leptospira* testing data from Spain since 2015 to 2017. Inclusion criteria were individual cases with MAT test results for 8 serovars (Bratislava, Icterohaemorrhagiae, Australis, Pomona, Grippityphosa, Autumnalis, Canicola and Saxkoebing) and to have the zip code data. Three hundred and thirty-eight samples (25.8%; 95%CI 23.6–28.4) were seropositive ($\geq 1:100$). According to geographic areas, North had the highest seroprevalence (38.0%; 95%CI 28.9–47.1) followed by South (29.4%; 95%CI 20.1–38.8), Center (28.6%; 95%CI 24.3–33.0), Mediterranean (22.3%; 95%CI 19.1–25.6) and Northwest (22.2%; 95%CI 7.9–36.4). Seropositivity (MAT $\geq 1:100$) was most common to serovars Icterohaemorrhagiae (19.4%; 95%CI 17.2–21.5) and Bratislava (8.5%; 95%CI 7.0–10.0), followed by Grippityphosa (7.2%; 95%CI 5.8–8.6), Australis (6.4%; 95%CI 5.0–7.7), Autumnalis (5.0%; 95%CI 3.8–6.2), Pomona (4.5%; 95%CI 3.3–5.6), Canicola (3.4%; 95%CI 2.4–4.4) and Saxkoebing (0.8%; 95%CI 0.3–1.3). An association was found between positivity (MAT $\geq 1:100$) and males ($P = 0.003$) and dogs that were 6 years old or older were at higher risk of exposure ($P = 0.001$; OR 4.61; 95%CI 1.86–11.43). This study has shown that dogs in Spain are commonly exposed to *Leptospira* infection and points out the necessity to control the prevalence of this severe widespread zoonosis in dogs and humans.

1. Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes with worldwide distribution affecting most mammalian species (Bharti et al., 2018).

Several studies have reported variable prevalence in dogs ranging from 1.8% in Australia to 71.1% in India (Davis et al., 2008; Zwijsenberg et al., 2008; Shi et al., 2012; Ambily et al., 2013; Bier et al., 2013; Ojha et al., 2018). In Europe, the prevalence ranges from 6% in Ireland to 49% in Italy (Scanziani et al., 2002; Burriel et al., 2003; Schuller et al., 2015b; Llewellyn et al., 2016; Habus et al., 2017).

Leptospirosis in dogs has been associated with serovars Canicola and Icterohaemorrhagiae but it is now clear that dogs are susceptible to infection with multiple serovars (Schuller et al., 2015a). In Europe, Grippityphosa and Bratislava have emerged as major causes of canine leptospirosis (Ellis, 2010; Mayer-Scholl et al., 2013; Renaud et al., 2013). Based on the available antibody prevalence data in Europe, most prevalent serovars are Icterohaemorrhagiae, Grippityphosa, Australis, Hardjo, and Canicola (Ellis, 2010). Seropositivity to the serogroup

Grippityphosa is common in mainland Europe but appears to be rare in the UK and Ireland, perhaps associated with the distribution of relevant reservoir hosts (Ellis, 2010). In Germany, the most common serovars in dogs are Grippityphosa and Sejroe (Geisen et al., 2007), while the serovar Australis appears to be common in Italy (Mastrolilli et al., 2007; Tagliabue et al., 2016). Infection with serovar Javanica has been described in dogs and humans in Europe, however, no reports have been found correlating infection with this serovar and clinical signs in dogs (VandenBroek et al., 1991; Cacciapuoti et al., 1994). As currently available anti-leptospiral vaccines are primarily serovar specific, the recognition of leptospires commonly involved in canine exposure in specific areas is crucial in order to keep vaccination strategies up to date. Canine vaccines used in Spain contain *L. interrogans* serovars Canicola and Icterohaemorrhagiae, although since 2012 vaccines also include *L. interrogans* serovar Bratislava and *L. kirschneri* serovar Grippityphosa (Klaasen et al., 2013). The rate of use of these vaccines has not been described in Spain, however multiple positive titers in MAT to vaccine serovars could be an indication of recent vaccination.

In contrast to other European countries, there have been limited

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canine seroprevalence studies in Spain. In dogs, prevalence data is available for two regions of Spain; Andalusia with 35.7% (Millán et al., 2009) and Comunidad Valenciana with 19.8% (Benito et al., 2005). Furthermore, serovars were only determined in Andalusia by indirect microscopic agglutination test (MAT), considered 1:100 the cut-point to positive sera, detecting Canicola, Icterohaemorrhagiae, and Australis (Millán et al., 2009).

A recent meta-analysis study identified being male sex and an urban dogs as major risks factors for leptospirosis (Azócar-Aedo and Monti, 2016). Dog ownership has also been identified as a risk factor for human leptospirosis in Nicaragua (Trevejo et al., 1998), Barbados (Douglin et al., 1997) and Germany (Jansen et al., 2005) suggesting transmission of *Leptospira* spp. from dogs to humans. Dogs could serve as an important sentinel specie for human infection, as well as indicator of the presence of leptospires in specific environments (Ghneim et al., 2007; Major et al., 2014; Schuller et al., 2015a).

The aims of this study were: 1) to determine the seroprevalence of anti-*Leptospira* serum antibody in owned dogs from Spain; 2) to know the most common canine serovars and if there is any relation between the region and serovars; and 3) to determine if geographic area, season, age and sex could be risk factors for exposure to pathogenic leptospires in dogs.

2. Material and methods

A cross-sectional study design was used to determine the prevalence of antibodies against various *Leptospira* serovars in owned dogs from Spain. Sampling was done by convenience with serological canine leptospirosis MAT results in Spain obtained through an agreement with IDEXX Laboratories (Ludwigsburg). The results of 1,310 individual tests were obtained from the proprietary database. The clinical history, vaccination status and whether samples were submitted for paired MATs were unknown. All MAT results for tests conducted from January 2015 to July 2017 were included. The laboratory is accredited according to ISO 17025 and regularly participates in the International Leptospirosis Society MAT Proficiency Testing Scheme. Source of cultures was the national reference laboratory (NRL) for leptospirosis (Bundesinstitut für Risikobewertung, BfR). MAT was performed according to OIE standards (Office International des Epizooties OIE, 2008). Samples were tested for the presence of antibodies to a panel of 8 serovars (Table 1). Dogs tested against the eight serovars with zip code of origin and dates of the test done, were eligible for inclusion. MAT positivity was defined as positive reaction to at least one serovar included in the 8 serovars panel at reciprocal titers of $\geq 1:100$ (ALL 100) or $\geq 1:400$ (ALL 400). Two instead of one cut-off titers were chosen in the absence of a consensus to what represents an ideal cut-off titer to document exposure in a population of dogs. A dog could be classified as having more than one positive serovar titer.

Samples analyzed were grouped by month of diagnosis (spring - March 1st, summer - June 1st, autumn - September 1st, and winter - December 1st), by sex (female, male), and by age (categorized as 0 to <2 years, 2 to <6 years, 6 to <10 years, and 10–15 years). Cut-offs for age categories were chosen to create 4 age groups approximately corresponding to those in previous studies (Grayzel and DeBess, 2016)

Table 1
Panel of 8 *Leptospira* spp. used as live antigens for microscopic agglutination testing.

Genomospecies	Serogroup	Serovar
<i>L. interrogans</i>	Australis	Bratislava
	Australis	Australis
	Autumnalis	Autumnalis
	Icterohaemorrhagiae	Icterohaemorrhagiae
	Pomona	Pomona
<i>L. borgpetersenii</i>	Sejroe	Saxkoebing
<i>L. kirschneri</i>	Grippothyphosa	Grippothyphosa

although no consistent agreement on age groupings was found among studies. For association and risk factor analysis, age groups were regrouped in <6 years and ≥ 6 years.

Geographic mapping was used to characterize the distribution of the disease in dogs throughout Spain by zip code. Spain is geographically divided into five domains (Estrada-Peña et al. 2017). These are: 1–2) North and Northwest, which are humid, with mild winters and summers, and the rough features of natural vegetation are similar in both North and Northwest, but weather is warmer and humid in the latter; 3) Center, which is a large area at high altitude (average above 800 m above the sea level and therefore has a continental type climate); 4) Mediterranean, which is also wet because of the influence of the Mediterranean Sea, with mild winters and hot summers; and 5) South, which is the warmest and driest region.

Descriptive statistics, relative prevalence calculations, univariate and multivariate analyses were performed using IBM SPSS Statistic version 25 for Mac. All statistical analyses were performed on data from 1,310 dogs and to the serovars they were exposed without exclusion of vaccinal serogroups (Icterohaemorrhagiae, Canicola, Grippothyphosa and Australis). Prevalence of different serovars in seropositive dogs was computed by geographic region. Season prevalence was determined for complete year data samples. In order to identify risk factors, the independent variables as sex (male, female), season (winter, spring, summer and autumn), age (<6 years and ≥ 6 years) and region (Mediterranean, Northwest, North, Center and South) with MAT seropositivity at reciprocal titers of $\geq 1:100$ (ALL 100) or $\geq 1:400$ (ALL 400) were assessed. Moreover, independent variables were also assessed with positive titers (≥ 100) to each serovar tested in MAT.

Univariate analysis was performed using χ^2 or Fisher's exact test for categorical variables. A value of $P < 0.05$ was considered as the critical level of significance. For multivariate analysis a logistical regression was performed. Risk factor was statistically significant at a p-value <0.05.

Geographic mapping was used to characterize the distribution of the disease in dogs throughout Spain by zip code using QGIS 3.4 (QGIS Development Team, 2018. QGIS Geographic Information System. Open Source Geospatial Foundation Project).

3. Results

A total of 1,310 samples met the inclusion criteria. All dogs included in this study were privately owned pet dogs. Three hundred thirty-eight out of 1,310 dogs (25.8%; 95%CI 23.6–28.4) were positive titer (≥ 100) to at least one serovar tested. One hundred and twenty-two (9.3%; 95%CI 7.8–10.9) were positive for ≥ 400 . One hundred and fifty-one dogs out of 338 (44.6%; 95%CI 39.3–50.0) had more than one positive titer (≥ 100) with a total of 723 different positive titers. The geographical distribution of positive sera across Spain is shown in Fig. 1.

As described in Table 2, seropositivity for MAT titer ≥ 100 ($n = 338$) was most common to serovar Icterohaemorrhagiae followed by Bratislava, Grippothyphosa, Australis, Autumnalis, Pomona and Canicola with similar prevalence, and Saxkoebing with the lowest percentage. A MAT titer ≥ 400 ($n = 122$) was quite similar among Icterohaemorrhagiae, Bratislava and Grippothyphosa, and between Pomona, Australis, Autumnalis and Canicola, with lowest prevalence of Saxkoebing. For a higher MAT titer ≥ 1600 ($n = 44$) distribution kept same patten where Icterohaemorrhagiae was the most common serovar, followed by Grippothyphosa and Bratislava (Table 2).

Serogroup analyses was also performed for all samples, at lower titers Icterohaemorrhagiae was the most common serogroup found followed by Australis and Grippothyphosa but at higher titers, Grippothyphosa was more common than Australis (Table 3).

Total co-reactivity prevalence of positive dogs with ≥ 100 and ≥ 400 cut-off was analyzed (Table 4). The most common serovars with double exposure (≥ 400) were Bratislava and Icterohaemorrhagiae (7/25, 28.0%), Icterohaemorrhagiae and Canicola (4/25, 16.0%), and Bratislava and Australis (3/25, 12.0%). Co-reactivity for serogroups in multivalent

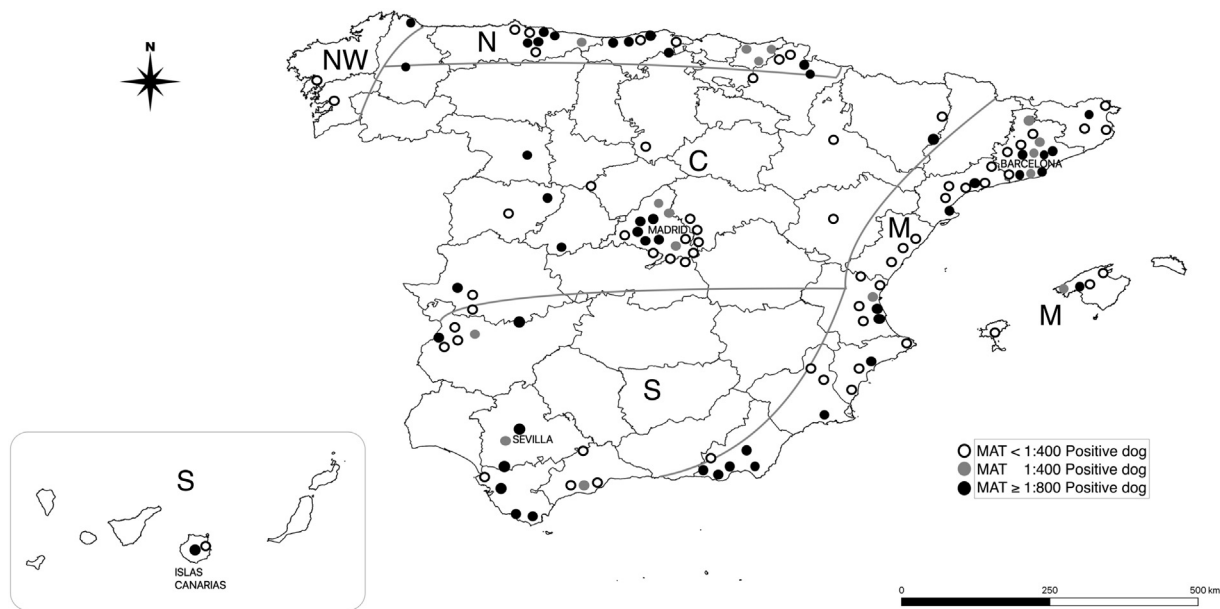


Fig. 1. The geographical distribution of positive dogs tested for serum antibodies *Leptospira* spp. with MAT (n = 338). Geocoding was performed based on the zip code. Geographic areas: Mediterranean (M), Northwest (NW), North (N), Center (C), and South (S).

Table 2
Number of individuals (n), relative prevalence and 95% confidence interval (CI) per *Leptospira* serovar for all dogs (n = 1310). Tested serovars: Bratislava (BRA), Icterohaemorrhagiae (ICT), Australis (AUS), Pomona (POM), Grippityphosa (GRI), Autumnalis (AUT), Canicola (CAN) and Saxkoebing (SAX).

MAT Titer		All Serovars	BRA	ICT	AUS	POM	GRI	AUT	CAN	SAX
≥1:100	n	338	111	254	84	59	94	66	44	11
	%	25.8	8.5	19.4	6.4	4.5	7.2	5.0	3.4	0.8
	CI	23.6–28.4	7.0–10.0	17.2–21.5	5.0–7.7	3.3–5.6	5.8–8.6	3.8–6.2	2.4–4.4	0.3–1.3
≥1:200	n	223	84	155	56	43	67	41	21	9
	%	17.0	6.4	11.8	4.3	3.3	5.1	3.1	1.6	0.7
	CI	15.0–19.1	5.1–7.7	10.1–13.6	3.2–5.4	2.3–4.2	3.9–6.3	2.2–4.1	0.9–2.3	0.2–1.1
≥1:400	n	122	54	72	29	30	35	25	13	8
	%	9.3	4.1	5.5	2.2	2.3	2.7	1.9	1.0	0.6
	CI	7.8–10.9	3.0–5.2	4.3–6.7	1.4–3.0	1.5–3.1	1.8–3.5	1.2–2.6	0.4–1.5	0.2–1.0
≥1:800	n	77	35	39	14	16	23	15	3	4
	%	5.9	2.7	3.0	1.1	1.2	1.8	1.1	0.2	0.3
	CI	4.6–7.2	1.8–3.5	2.1–3.9	0.5–1.6	0.6–1.8	1.0–2.5	0.5–1.7	0.0–0.5	0.0–0.6
≥1:1600	n	44	11	21	7	6	16	5	1	2
	%	3.4	0.8	1.6	0.5	0.5	1.2	0.4	0.1	0.2
	CI	2.4–4.4	0.3–1.3	0.9–2.3	0.1–0.9	0.1–0.8	1.6–1.8	0.1–0.7	0.0–0.2	0.0–0.4
≥1:3200	n	19	2	8	2	1	11	-	1	-
	%	1.5	0.2	0.6	0.2	0.1	0.8	-	0.1	-
	CI	0.8–2.1	0.0–0.4	0.2–1.0	0.0–0.4	0.0–0.2	0.3–1.3	-	0.0–0.2	-
≥1:6400	n	11	2	4	-	1	5	-	1	-
	%	0.8	0.2	0.3	-	0.1	0.4	-	0.1	-
	CI	0.3–1.3	0.0–0.4	0.0–0.6	-	0.0–0.2	0.1–0.7	-	0.0–0.2	-

vaccine (Australis, Icterohaemorrhagiae, Canicola, Grippityphosa) for titer ≥100 and ≥400 was 4/183 and 3/67, respectively.

Age was known in 209/1310 (15.9%) of dogs and ranged from 0 to 15 years old (median 5.1 IQR 2–8). Distribution of age groups was 49/209 (23.4%) from 0 to <2 years, 64/209 (30.6%) from 2 to <6, 71/209 (34.0%) from 6 to <10, and 25/209 (12%) for ≥10. Prevalence for titers ≥100 was 8.2% (4/49) in <2 years, 21.8% (14/64) from 2 to <6, 32.3% (23/71) from 6 to <10, and 32.0% (8/25) in ≥10. Dogs with ≥6 years in ≥100 cut-off were in major risk of being seropositive (OR 4.6; 95% CI 1.9–11.4, P = 0.001) as well as for Icterohaemorrhagiae (OR 3.7; 95% CI 1.4–9.7, P = 0.007) (Table 6). Sex was available for 309/1310 (23.6%) dogs and was equally distributed with 180/309 (58.3%) male and 129/309 (41.7%) female. A total of 51/180 (28.3%) males and 18/129 (13.9%) females were positive at least to one serovar. Evidence of positive serovars were more frequently found in males (87.5%) than females (12.5%, P = 0.015) for MAT titers ≥400. This association was also found

for Bratislava, Icterohaemorrhagiae and Autumnalis serovars (Table 5).

Seasonality was analyzed only for 2015 and 2016 samples. Autumn had the major prevalence with 82 positive dogs (37 and 45, respectively); followed by summer 60 (29 and 31), winter 57 (29 and 28) and spring 54 (30 and 24). Statistically significant relation was found between Grippityphosa positive titers and season (P = 0.039), higher prevalence was found in autumn and winter (9.0% and 9.6%, respectively) compared to spring (5.4%) and summer (4.0%).

According to geographic areas with a ≥100 cut-off, association is described in Table 5, statistically significant association was found with higher prevalence in North area (P = 0.001). For a ≥400 cut-off this relation was also found statistically significant (P = 0.042) with prevalence of 15.9% in North, 13.7% in South, 9.0% in Center, 8.3% in Northwest and 7.8% in Mediterranean region. In multivariate analyses no statistically significant risk factor was found between region and positivity (Table 6).

Table 3

Number of individuals (n), relative prevalence and 95% confidence interval (CI) per *Leptospira* serogroup for all dogs (n = 1310). Tested serogroups: Australis (AUS), Icterohaemorrhagiae (ICT), Pomona (POM), Grippotyphosa (GRI), Autumnalis (AUT), Canicola (CAN) and Sejroe (SAX).

MAT Titer		All Serogroups	AUS	ICT	POM	GRI	AUT	CAN	SEJ
≥1:100	n	338	117	254	59	94	66	44	11
	%	25.8	8.9	19.4	4.5	7.2	5.0	3.4	0.8
	CI	23.6–28.4	7.4–10.5	17.2–21.5	3.3–5.6	5.8–8.6	3.8–6.2	2.4–4.4	0.3–1.3
≥1:200	n	223	89	155	43	67	41	21	9
	%	17.0	6.8	11.8	3.3	5.1	3.1	1.6	0.7
	CI	15.0–19.1	5.4–8.2	10.1–13.6	2.3–4.2	3.9–6.3	2.2–4.1	0.9–2.3	0.2–1.1
≥1:400	n	122	57	72	30	35	25	13	8
	%	9.3	4.4	5.5	2.3	2.7	1.9	1.0	0.6
	CI	7.8–10.9	3.3–5.5	4.3–6.7	1.5–3.1	1.8–3.5	1.2–2.6	0.4–1.5	0.2–1.0
≥1:800	n	77	37	39	16	23	15	3	4
	%	5.9	2.8	3.0	1.2	1.8	1.1	0.2	0.3
	CI	4.6–7.2	1.9–3.7	2.1–3.9	0.6–1.8	1.0–2.5	0.5–1.7	0.0–0.5	0.0–0.6
≥1:1600	n	44	15	21	6	16	5	1	2
	%	3.4	1.1	1.6	0.5	1.2	0.4	0.1	0.2
	CI	2.4–4.4	0.6–1.7	0.9–2.3	0.1–0.8	1.6–1.8	0.1–0.7	0.0–0.2	0.0–0.4
≥1:3200	n	19	3	8	1	11	-	1	-
	%	1.5	0.2	0.6	0.1	0.8	-	0.1	-
	CI	0.8–2.1	0.0–0.5	0.2–1.0	0.0–0.2	0.3–1.3	-	0.0–0.2	-
≥1:6400	n	11	2	4	1	5	-	1	-
	%	0.8	0.2	0.3	0.1	0.4	-	0.1	-
	CI	0.3–1.3	0.0–0.4	0.0–0.6	0.0–0.2	0.1–0.7	-	0.0–0.2	-

Table 4

Number of individuals positive against one, two or more serovars at a MAT Titer of ≥100 and ≥400.

N° seropositive serovars	MAT ≥1:100 n (%)	MAT ≥1:400 n (%)
1	183 (54.2)	55 (45.0)
2	54 (16.0)	25 (20.5)
3	32 (9.5)	20 (16.4)
4	27 (8.0)	11 (9.0)
5	24 (7.1)	9 (7.4)
6	14 (4.1)	2 (1.6)
7	4 (1.2)	0 (0)
8	0 (0)	0 (0)

Dogs with MAT titer ≥100 were divided by serovar and region as described in Table 7, only one statistically significant association was found between Grippotyphosa serovar and region (P = 0.003), with higher prevalence in Center (47/422, 11.1%) followed by South (7/95, 7.4%), North (10/113, 8.8%), Northwest (2/36, 5.4%) and Mediterranean (28/644, 4.3%).

4. Discussion

The analyses conducted in this study evidenced that Spain's dogs are exposed to eight pathogenic *Leptospira* serovars. Our prevalence results (25.8% at ≥100 and 9.3% at ≥400 cut-off) were similar to previous data of seroprevalence of anti-*Leptospira* antibodies reported in Europe (Scanziani et al., 2002; Burriel et al., 2003; Krawczyk, 2005; Schuller et al., 2015b; Llewellyn et al., 2016; Delaude et al., 2017; Habus et al., 2017).

Leptospira prevalence in dogs from Spain has only been previously described in free-roaming dogs (10/28, 35.7%) in Andalusia (Millán

et al., 2009) and in a mix (owned and un-owned) population of dogs (167/864, 16.7%) in Comunidad Valenciana (Benito et al., 2005). The prevalence in Andalusia was higher than the mean of the present study, even if it is compared with our South region's prevalence (29.4%). A possible explanation for this difference is that Millán's study (2009) evaluated rural and un-owned dog's population. However, in Comunidad Valenciana the described prevalence was lower than in the present study. A plausible explanation to this would be that dogs were randomly taken for analyses in the previous study (Benito et al., 2005). It should be noted that in our study a possible bias could raise the prevalence because samples were collected from a laboratory and we hypothesized that probably received most of them from veterinary clinicians in order to confirm or reject leptospirosis as a potential diagnosis of sick dogs.

Although there was some variability between the different geographic areas, the most frequent serovars found in this study were Icterohaemorrhagiae, Bratislava, Grippotyphosa and Australis. Serovar Icterohaemorrhagiae was the most prevalent especially when ≥100 cut-off was used. This could be explained as a result of vaccination bias; however, it is also remarkable that this high prevalence was kept in higher cut-offs (≥400) when vaccination bias could be more unlikely. This serovar has been previously described as the most frequent in wild and domestic animals in southern Spain (Millán et al., 2009).

Conversely, our study showed a low rate of positivity for Canicola serovar consistent with the wide use of vaccination could be responsible of the decrease of positivity in Italy (Scanziani et al. 1994, 2002; Tagliabue et al., 2016) and Europe (André-Fontaine, 2006). Although both vaccine serogroups (Icterohaemorrhagiae and Canicola) are expected to induce agglutinating antibodies, in our study the seroprevalence of Icterohaemorrhagiae antibodies was higher than those of Canicola similar to a previous study with canine serum samples in France

Table 5

Analysis of association (χ^2) between season, sex, region or age with positive microscopic agglutination test against any of the tested serovars at MAT cut-offs ≥1:100 (ALL 100) and ≥1:400 (ALL 400); and against each serovar at MAT cut-off ≥1:100 for serovars Bratislava (BRA), Icterohaemorrhagiae (ICT), Australis (AUS), Grippotyphosa (GRI), Autumnalis (AUT), Canicola (CAN) and Saxkoebing (SAX).

Variable	ALL100	ALL400	BRA100	ICT100	AUS100	POM100	GRI100	AUT100	CAN100	SAX100
SEASON	0.187	0.060	0.232	0.254	0.512	0.149	0.039*	0.09	0.373	0.426
SEX	0.003*	0.015*	0.015*	0.023*	0.072	0.407#	1.000#	0.023# *	0.085#	1.000#
REGION	0.001*	0.042*	0.373	0.301	0.402	0.208	0.003*	0.257	0.241	-
AGE	0.001*	0.735#	0.729#	0.000*	1.000#	0.689#	0.456#	1.000#	0.663#	-

* Statistically significant (p < 0.05).

Fisher's test used.

Table 6

Multivariable analysis of the association between sex, age, season and region with positive against any serovar at MAT titers ≥ 100 , ≥ 400 and Icterohaemorrhagiae (ICT) serovar for titer ≥ 100 .

Covariate	Levels	Cut-Off	Odds ratio	95%CI	p-value
Sex	Female	Ref.			
	Male	$\geq 1:100$	2.16	0.90–5.19	0.086
		$\geq 1:400$	4.03	0.44–37.14	0.218
Age		ICT	1.53	0.60–3.87	0.367
	<6y	Ref.			
	$\geq 6y$	$\geq 1:100$	4.61	1.86–11.43	0.001*
		$\geq 1:400$	2.73	0.41–18.17	0.300
Season		ICT	3.73	1.42–9.77	0.007*
	W	Ref.			
	SP	$\geq 1:100$	0.72	0.21–2.44	0.602
		$\geq 1:400$	1.37	0.09–20.15	0.818
		ICT	0.62	0.18–2.22	0.467
	SU	$\geq 1:100$	0.50	0.14–1.83	0.295
		$\geq 1:400$	1.21	0.09–16.31	0.890
		ICT	0.73	0.20–2.67	0.634
	A	$\geq 1:100$	0.52	0.15–1.87	0.319
Region		$\geq 1:400$	0.87	0.04–37.14	0.926
		ICT	0.36	0.09–1.48	0.160
	S	Ref.			
	M	$\geq 1:100$	0.33	0.08–1.37	0.126
		$\geq 1:400$	0.15	0.01–2.70	0.195
		ICT	0.40	0.08–1.86	0.242
	NW	$\geq 1:100$	-	-	-
		$\geq 1:400$	-	-	-
		ICT	-	-	-
	N	$\geq 1:100$	1.27	0.18–8.93	0.813
		$\geq 1:400$	2.04	0.09–46.21	0.654
		ICT	0.90	0.10–7.83	0.242
	C	$\geq 1:100$	0.55	1.86–11.43	0.423
		$\geq 1:400$	0.68	0.06–8.11	0.760
		ICT	0.67	0.14–3.21	0.618

*Statistically significant ($p < 0.05$).

(André-Fontaine, 2006). Perhaps, this could be explained by the higher infection pressure in the environment of the former strain, compared to the latter.

The second most prevalent serovar reported in this study was Bratislava. This was consistent with previous studies in dogs in Italy (Scanziani et al., 2002) and Scotland (VandenBroek et al., 1991). In Spain, Bratislava was considered the most prevalent serovar in cattle (Alonso-Andicoberry et al., 2001; Guitián et al., 2001; Atxaerandio et al., 2005).

Our study showed that the third most frequent co-reactivity was to Australis and Bratislava serovars (12.0%), both belong to the serogroup Australis. That co-reactivity could be due to a cross-reactivity in MAT because this test is good detecting serogroups, being a bias in the analysis. Moreover, it was described that Bratislava antibody titers often

increase with titers to Grippotyphosa and Pomona in dogs (Brown et al., 1996; Greenlee et al., 2004) making the analysis of the infecting serovar even more challenging. Co-reactivity results for titers ≥ 400 also showed a high prevalence of Bratislava and Icterohaemorrhagiae (28.0%) as well as Icterohaemorrhagiae and Canicola (16.0%), all these serovars are found in multivalent vaccines and that could be an indicator of recent vaccination. Positive titers for only the four vaccinal serogroups was found in four dogs, while multiple co-reactivities with no vaccinal serogroups were found, this fact could be explained by cross-reaction in MAT test which is a challenge for MAT interpretation for vaccination without previous history of vaccination status.

In order to assess areas of increased risk, the prevalence of *Leptospira* serovars amongst 5 different geographic regions was compared. This geographic classification was based on weather variations found in different areas of Spain but does not consider the potentially significant regional variations as a flora and fauna or the presence of stagnant water and the wildlife populations. The prevalence was markedly higher in North region (38.9%) compared with quite similar prevalence found in the rest of areas of Spain. Furthermore, a significant relation between region and seropositive samples was found but no significant risk factor with any region was found. All this data could be explained due to the humid weather and more water sources available in North area. However, this high prevalence was not found in Northwest with similar climatological features, but the sample size from this region was too small to draw any conclusions and also the different sample size from different regions could be a bias for our study.

Higher prevalence could be biologically plausible in regions with lower altitudes due to less extreme climatic conditions throughout the year (Adler, 2015). Moreover, a recent study about *Leptospira* infection in Mediterranean periurban micro-mammals reported a prevalence of 12% that could be a high risk factor for infection in dogs and humans in this area (Millán et al., 2018). In our study, no risk association with Mediterranean area was found.

Leptospirosis in the United States has been characterized as having a seasonal distribution that favors late summer and early fall (Ward, 2002). A similar pattern can be found across Europe (Jansen et al., 2005; Barranton and Postic, 2006; Habus et al., 2017). In this study, only a significant association between Grippotyphosa and season was found (Table 5) with higher prevalence in autumn.

The high prevalence in male dogs observed in our study agreed with previous results described for canine leptospirosis (Azócar-Aedo and Monti, 2016). Similar to previous studies in dogs, our study also revealed correlation between age and MAT seropositivity, where being younger than 1-year-old was a protective factor and, in contrast, being 6 years old or older increased the risk of seropositivity (Harland et al., 2013; Grayzel and DeBess, 2016; Delaude et al., 2017). This relationship could be plausible as antibodies related to infection can persist for years and the

Table 7

Number of titers (n) and relative prevalence for MAT cut-off ≥ 100 divided by geographic areas and serovar. Serovars tested: Bratislava (BRA), Icterohaemorrhagiae (ICT), Australis (AUS), Pomona (POM), Grippotyphosa (GRI), Autumnalis (AUT), Canicola (CAN), and Saxkoebing (SAX).

Region		All Serovars	BRA	ICT	AUS	POM	GRI	AUT	CAN	SAX
Mediterranean	n	147	48	115	40	20	28	27	21	3
	%	22.3	7.4	17.9	6.2	3.1	4.3	4.2	3.2	0.5
	CI	19.1–25.6	5.4–9.5	14.9–20.8	4.3–8.1	1.7–4.4	2.7–5.9	2.6–5.7	1.9–4.6	0.0–1.0
Northwest	n	8	3	4	2	2	2	2	1	0
	%	22.2	8.3	11.1	5.6	5.6	5.6	5.6	2.8	-
	CI	7.9–36.4	0.0–17.8	0.3–21.9	0.0–13.4	0.0–13.4	0.0–13.4	0.0–13.4	0.0–8.4	-
North	n	44	13	25	12	7	10	8	1	6
	%	38.0	11.5	22.1	10.6	6.2	8.8	7.1	0.8	5.3
	CI	28.9–47.1	5.5–17.5	14.3–29.9	4.8–16.4	1.7–10.7	3.5–14.2	2.3–11.9	0.0–2.6	1.1–9.5
Center	n	125	36	88	23	25	47	23	18	1
	%	28.6	8.5	20.8	5.4	5.9	11.1	5.4	4.2	0.2
	CI	24.3–33.0	5.8–11.2	17.0–24.7	3.3–7.6	3.3–7.6	8.1–14.1	3.3–7.6	2.3–6.2	0.0–0.7
South	n	27	11	22	7	5	7	6	3	1
	%	29.4	11.5	23.2	7.4	7.4	7.4	6.3	3.2	1.0
	CI	20.1–38.8	5.0–18.1	14.5–31.8	2.0–12.7	2.0–12.7	2.0–12.7	1.3–11.3	0.0–6.7	0.0–3.1

probability of having contacted with *Leptospira* increases over time (Delaude et al., 2017).

The most important limitation of this study was the fact that vaccination status of the dogs was unknown, limiting the interpretation of the results of MAT. While the MAT is far from being a perfect method, it is currently the only tool available to document exposure in dogs (Adler, 2015). Non-infected dogs vaccinated with bivalent or quadrivalent whole cell anti leptospiral vaccines can have post-vaccinal titers of 1:6400 or higher to both vaccinal and non-vaccinal serovars (Barr et al., 2005; Midence et al., 2012; Martin et al., 2014). Although the majority of vaccinated dogs have been shown to become antibody negative by week 15 post-vaccination, vaccinal titers can persist for 12 months in a small percentage of dogs (Martin et al., 2014). Despite its limitations the MAT is well accepted and extensively used to derive information about the presence of exposition to *Leptospira* in sick (Boutillier et al., 2003; Ward et al., 2004; Ghneim et al., 2007; Hennebelle et al., 2013; Mayer-Scholl et al., 2013; Major et al., 2014) and healthy dogs (Davis et al., 2008; Arent et al., 2013; Schuller et al., 2015a; Llewellyn et al., 2016). In the absence of a consensus of what represents an ideal cut off titer for MAT positivity, our study used two different cut offs (≥ 100 and ≥ 400) to calculate seroprevalence in order to compare the results with previous publications worldwide. Furthermore, MAT does not discriminate between vaccination titers and titers due to exposure, thus adding further difficulty to the interpretation of canine tests, as a large proportion of dogs are vaccinated with either bivalent or multivalent *Leptospira* vaccines (Sykes et al., 2011). Moreover, multivalent vaccines were available in Spain since the study started which made the interpretation of the results even more challenging. In dogs with confirmed leptospirosis the sensitivity of MAT has been estimated to be 50–67% at 1:400 dilution and 22–67% at 1:800 dilution, while the specificity ranges from 69–93% at 1:400 dilution and 69–100% for 1:800 dilution (Miller et al., 2011). However, the sensitivity and specificity of the MAT in detecting chronic subclinical infection or post exposure titers has not been reported (Delaude et al., 2017). In the present study all statistical analyses were performed using both cut-off ≥ 100 and ≥ 400 in order to minimize the important limitation of vaccine interference. Finally, multiple seroconversions to two or more serovars contained in the current vaccines were detected in many dogs in this study, probably also related to vaccination. In this case the MAT was also not able to identify the real etiological agent as has been previously described (Cerri et al., 2003; Tagliabue et al., 2016).

5. Conclusions

This study shows that owned dogs in Spain are commonly exposed to *Leptospira* spp., with Icterohaemorrhagiae, Bratislava, Grippotyphosa and Australis being the most common serovars. Sex is associated with seropositivity and being older than 6 years old was a risk factor for seropositivity. Multiple positive serovars occurrence is very frequent and this produces difficulties to identify the real circulation of *Leptospira* in dogs. Since dogs are considered as sentinel of human zoonosis, it makes sense to consider a regular monitoring of them.

Declarations

Author contribution statement

M.C. López: Analyzed and interpreted the data; Wrote the paper.
A. Vila, X. Roura: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
J. Rodón: Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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