VECTOR-BORNE AND ZOONOTIC DISEASES
Volume XX, Number XX, 2017

DOI: 10.1089/vbz.2016.1990

ORIGINAL ARTICLE

Prevalence and Geographic Distribution of Vector-Borne Pathogens in Apparently Healthy Dogs in Croatia

Vladimir Mrljak,[1] Josipa Kuleš,[2] Željko Mihaljević,[3] Marin Torti,[1] Jelena Gotić,[1] Martina Crnogaj,[1] Tatjana Živičnjak,[4] Iva Mayer,[1] Iva Šmit,[1] Mangesh Bhide,[1,5] and Renata Barić Rafaj[2]

Abstract

Vector-borne pathogens (VBPs) are a group of globally extended and quickly spreading pathogens that are transmitted by various arthropod vectors. The aim of the present study was to investigate the seroprevalence against *Babesia canis*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi sensu lato*, *Leishmania infantum*, *Dirofilaria immitis*, and *Ehrlichia canis* in dogs in Croatia. We investigated 435 randomly selected apparently healthy dogs in 13 different locations of Croatia for antibodies to *B. canis* by indirect immunofluorescence using a commercial IFA IgG Antibody Kit. All samples were also tested for qualitative detection of *D. immitis* antigen and for antibodies to *A. phagocytophilum*, *B. burgdorferi sensu lato*, *L. infantum*, and *E. canis* with two point-of-care assays. Overall, 112 dogs (25.74%, 95% confidence interval [CI] 21.70–30.12) were serologically positive for one or more of the pathogens. *B. canis* was the most prevalent pathogen (20.00%, 95% CI 16.34–24.07), followed by *A. phagocytophilum* (6.21%, 95% CI 4.12–8.90), *L. infantum*, (1.38%, 95% CI 0.51–2.97), and *B. burgdorferi sensu lato* (0.69%, 95% CI 0.01–2.00). The lowest seroprevalence was for *D. immitis* and *E. canis* (0.46%, 95% CI 0.01–1.65). Coinfection was determined in 12 dogs (2.76%, 95% CI 1.43–4.77), of which 10 were positive to two pathogens (7 with *B. canis* and *A. phagocytophilum* and 1 *B. canis* with *B. burgdorferi sensu lato* or *L. infantum* or *E. canis*). One dog was positive to three pathogens and another dog to four pathogens. Seroprevalence for babesia was age, breed, and lifestyle/use dependent. Purebred dogs had almost half the chance of developing disease than crossbred (OR = 0.58, $p < 0.026$, 95% CI 0.37–0.94). Seropositivity to *B. canis* was 3.41 times higher for dogs that lived outdoors/shelter ($p < 0.006$) or 4.57 times higher in mixed/hunting ($p < 0.001$) compared to indoor/companion dogs. This is the first comprehensive survey of VBP seropositivity conducted in Croatia. Some of these VBPs are zoonotic and represent a potential risk to public health.

Keywords: *Anaplasma phagocytophilum*, *Babesia canis*, *Borrelia burgdorferi sensu lato*, *Dirofilaria immitis*, *Ehrlichia canis*, *Leishmania infantum*, vector-borne pathogens

Introduction

Vector-borne pathogens (VBPs) are a group of globally distributed and rapidly spreading pathogens that are transmitted by arthropods, including ticks, fleas, mosquitoes, and phlebotomine sand flies (Otranto et al. 2009).

Protozoans *Babesia canis*, *Leishmania infantum*, bacteria *Ehrlichia canis*, *Borrelia burgdorferi sensu lato*, *Anaplasma phagocytophilum*, and the nematode *Dirofilaria immitis* are among the major VBPs that can infect dogs (Day 2011). The vast majority of these pathogens, except *B. canis*, are also zoonotic, causing disease in humans, thus representing a great veterinary and public health threat (Alho et al. 2016). It appears that the frequency of some vector-borne diseases is increasing in Europe and that pathogens are circulating more easily (Beugnet and Marié 2009). Important drivers for the emergence and spread of vector-borne parasites include atmospheric and climate changes, habitat changes, deforestation

[1]Clinic for Internal Diseases and [2]Department of Chemistry and Biochemistry, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia.
[3]Croatian Veterinary Institute, Zagreb, Croatia.
[4]Department of Parasitology and Parasitic Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia.
[5]Department of Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Kosice, Slovakia.

and urbanization, globalization and increased trade, travel, aerial transport by migratory birds, and human movement (Zell 2004, Harrus and Baneth 2005).

As a result of global warming and the rapid spread overall of vector-borne diseases observed in the last decade, there is a need of a constant update of data on disease distribution and prevalence (Gray et al. 2009, Mills et al. 2010). Furthermore, the existence of public kennels for stray dogs, regardless of their health conditions, in areas where VBPs are endemic, contributes to the maintenance of the endemicity of many diseases, *e.g*., canine leishmaniasis (Otranto et al. 2007). In addition, some arthropod species, particularly ticks, act as vectors of more than one agent and coinfection of individual arthropods can occur (Shaw et al. 2001). The overall factors stated above underscore the importance of periodic surveillance of VBPs. There is little information about the prevalence of many VBPs or the comparative seroprevalence of different pathogens in specific geographical areas (Trotz-Williams and Trees 2003).

Among the abovementioned pathogens, canine babesiosis is the most commonly recognized tick-borne disease by clinicians in Croatia (Matijatko et al. 2009, Barić Rafaj et al. 2013) and at the same time is of worldwide importance. The main vectors for babesiosis, *Dermacentor* spp. and *Rhipicephalus* spp., ticks have been recorded in Croatia (see Integrated Consortium on Ticks and Tick-borne disease (ICTTD-3), www.icttd.nl). In another survey, the vectors *Ixodes ricinus* and *Dermacentor reticulatus* were identified in public gardens in Zagreb, Croatia (Beck et al. 2010). In the central and southern part of the coastal area (Dalmatia region), canine leishmaniasis (CanL) was recognized as a reemerging disease since 1997 (Živičnjak et al. 1998). Some studies indicate that *Phlebotomus neglectus*, *Phlebotomus perfiliewi*, and *Phlebotomus tobbi*, which are proven vectors of the protozoan agent *L. infantum*, are found as well in the coastal region of Dalmatia (Miščević et al. 1998, Killick-Kendrick 1999).

Surveys carried out in the Zagreb area have revealed the existence of antibodies to *B. burgdorferi sensu lato* in dogs (Turk et al. 2000). *B. burgdorferi sensu lato* DNA was detected in 45% of ticks collected in the Lyme borreliosis (LB) endemic region of the northwest part of continental Croatia (Golubić et al. 1998). In another research study, the tick *I. ricinus* was confirmed in the same area of Croatia (Rijpkema et al. 1996). In a seroepidemiological study conducted in Slovenia from 1999 to 2006, infection with *A. phagocytophilum* was confirmed by serology in 70.5% (481/682) of dogs considered to have an infection (Ravnik et al. 2009). The survey among 600 adult dogs detected *D. immitis* only in littoral Croatia, especially the Istrian peninsula and Dubrovnik-Neretva County (Živičnjak et al. 2007). There is no research on *E. canis* or canine monocytic ehrlichiosis in Croatia.

With this background, the objectives of the present study were to investigate the seroprevalence and geographic distribution of *B. canis*, *A. phagocytophilum*, *B. burgdorferi sensu lato*, *L. infantum*, *D. immitis*, *and E. canis* in canine populations in Croatia. The significance of several risk factors (age, sex, breed, use, lifestyle, and location) was also included in the investigation.

Materials and Methods

Study area

The Republic of Croatia is located at the crossroads of Central Europe, Southern Europe, and the Mediterranean. It lies mostly between latitudes 42° and 47° N and longitudes 13° and 20° E. Administrative Croatia is divided into 20 counties and the capital city is Zagreb. Considering the climate conditions, Croatia is divided into two predominant climate regions—continental and coastal. The survey was carried out in 13 different study areas of Croatia, 6 in the continental part (Varaždin, Zagreb, Karlovac, Slavonski Brod, Đakovo, and Osijek) and 7 in the coastal area (Poreč, Pula, Rijeka, Krk, Zadar, Split, and Dubrovnik) (Table 1).

Study population and blood samples

Over a period of 1 year, 435 randomly selected apparently healthy dogs living in different parts of Croatia were sampled with verbal consent from their owners or shelter managers.

Table 1. Seroprevalence Rates of Canine Vector-Borne Pathogens by Geographic Region

		Positive/total (%)					
Geographic region	n *tested*	Babesia canis	Anaplasma phagocytophilum	Borrelia burgdorferi sensu lato	Leishmania infantum	Dirofilaria immitis	Ehrlichia canis
Karlovac	41	0/41	0/41	0/41	0/41	0/41	0/41
Krk	69	14/69 (20.29)	6/69 (8.70)	0/69	0/69	0/69	0/69
Pula	15	4/15 (26.67)	1/15 (6.67)	0/15	0/15	0/15	1/15 (1.45)
Slavonski Brod	39	9/39 (23.08)	2/39 (5.13)	0/39	0/39	0/39	0/39
Zagreb	19	5/19 (26.32)	3/19 (15.79)	0/19	0/19	0/19	0/19
Dubrovnik	36	10/36 (27.78)	2/36 (5.56)	1/36 (2.78)	6/36 (16.67)	2/36 (5.56)	0/36
Osijek	18	6/18 (33.33)	3/18 (16.67)	0/18	0/18	0/18	0/18
Đakovo	22	9/22 (40.91)	1/22 (4.55)	0/22	0/22	0/22	0/22
Poreč	20	9/20 (45.00)	1/20 (5.00)	0/20	0/20	0/20	0/20
Rijeka	34	5/34 (14.71)	3/34 (8.82)	1/34 (2.94)	0/34	0/34	1/34 (2.94)
Split	29	2/29 (6.90)	0/29	0/29	0/29	0/29	0/29
Varaždin	41	7/41 (17.07)	3/41 (7.32)	1/41 (2.44)	0/41	0/41	0/41
Zadar	52	7/52 (13.46)	2/52 (3.85)	0/52	0/52	0/52	0/52
Total	435	87/435 (20.00)	27/435 (6.21)	3/435 (0.69)	6/435 (1.38)	2/435 (0.46)	2/435 (0.46)
χ^2; *p* value		40.047; 0.001	15.455;0.218	8.275; 0.763	30.879;0.002	10.071;0.610	6.043; 0.914

With previously unknown seroprevalence (set at 50%), an accepted precision of 5%, and population size of more than 100,000 dogs, the sample size necessary to estimate the prevalence was calculated at 385 dogs (Petrie and Watson 2013). It was estimated that some dog owners would be reluctant to allow testing; therefore the sample size was increased to 435 dogs. Data regarding age (*i.e.*, months 6–11, 12–35, 36–59, 60–83, and >84), gender, breed (crossbred or purebred), lifestyle (indoors versus outdoors, or mixed), use (companion, shelter, or hunting dogs), and location (the continent versus the coast) were recorded. The age of the dog was given by the owner or calculated by dental examination. Travel histories were not available for shelter dogs.

Blood samples were collected by cephalic venepuncture, using 3 mL vacuum tubes without additives. This study was approved by the Committee on Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit–Class: 322-01/07-22/5; Registration Number: 61-01/139-07-2).

Testing for serum antigen and antibodies

Serum samples from dogs were screened for simultaneous qualitative detection of circulating *D. immitis* antigen, and antibodies, both immunoglobulin G and M, to *E. canis*, *B. burgdorferi sensu lato*, and *A. phagocytophilum* with the SNAP® 4Dx® test (IDEXX Laboratories, Westbrook, ME). The same samples were further qualitatively tested for antibodies to *L. infantum* with SNAP *Leishmania* (IDEXX Laboratories). Both tests were performed according to the manufacturer's instructions. The SNAP 4Dx *D. immitis* analyte is derived from polyclonal antibodies specific to the heartworm antigen.

The commercially available point-of-care kit detects antibodies against peptides from p30 and p30-1 outer membrane proteins of *E. canis*. The specific peptide antigen of the *B. burgdorferi sensu lato* analyte detects antibodies specific to the C_6 synthetic peptide. The *A. phagocytophilum* analyte detects antibodies generated against a synthetic peptide from the major outer surface protein (p44/MSP2). Reported sensitivities/specificities of the SNAP 4Dx test are 99.2/100% for *D. immitis*, 96.2/100% for *E. canis*, 98.8/100% for *B. burgdorferi sensu lato*, and 99.1/100% for *A. phagocytophilum* (Chandrashekar et al. 2010). The sensitivity and specificity of SNAP *Leishmania* were 91.1/93.4% and 98.3/99.2%, respectively, compared with an immunofluorescence antibody (IFAT) or Western blot test (Ferroglio et al. 2007).

Each sample was also screened for the presence of specific IgG class antibodies against *B. canis* by indirect immunofluorescence, using the commercial *B. canis* IFA IgG Antibody Kit (Fuller Laboratories, Fullerton, CA) according to the manufacturer's instructions. The positive control included one dilution above the stated end point and one dilution below the stated end point (1:200–1:800). The end point titer of the positive control had to be from 1:100 to 1:800. The fluorescence intensity at 1:400 was used as the cutoff level required for a test reaction to be called positive. The kit manufacturer states that titers of 50 or more suggest recent or current infection. Slides were examined by the same reader using a fluorescence microscope. Samples with clearly fluorescent *B. canis* in the viewing field were considered to be positive following comparison with positive and negative controls. Patterns of reactivity different from those seen in the positive control were considered nonspecific.

Spatial distribution of VBPs

To understand the spatial distribution of VBPs among dogs in Croatia, we used Kernel Density estimation. The Kernel Density calculates the density of positive cases in a neighborhood around those cases. Conceptually, a smoothly curved surface is fitted over each point. The surface value is highest at the location of the point and diminishes with increasing distance from the point. This function is based on the quadratic kernel function described by Silverman (1998). Point GPS data of each seropositive dog were plotted on a map, and the analysis was performed in the ArcGIS 9.3. software (ESRI 2014) environment, using *Arc Tool Box>Spatial Analyst Tools>Kernel Density.* The default search radius (bandwidth) was used applying the following formula:

$$SearchRadius = 0.9 * \min\left(SD, \sqrt{\frac{1}{\ln(2)}} * D_m\right) * n^{-0.2}$$

(SD is the standard distance, D_m is the median distance, and n is the number of points.)

Statistical analyses

Univariable and multivariable logistic regression models were used to identify risk factors for VBPs in dogs. Initially, a univariable analysis was performed using a logistic regression model using epidemiological data (age, sex, breed, use, lifestyle, and location) as independent variables and serological status as dependent (outcome) variable. The outcome variable for our model was a positive/negative animal according to serological test cutoff values preset by the producer. Univariable analysis identified potential covariates for the multivariable model at the 0.25 alpha level based on the Wald chi-square statistic. A stepwise selection approach was applied to choose the final model. During the iterative multivariable fitting, covariates were eliminated one at a time because they were not significant in the multivariable model at the alpha level of 0.05, and when taken out, did not change any remaining parameter estimates by more than 20%. Statistical analyses were performed using Stata 13.0 (Stata Corp. 2013; Stata Statistical Software: Release 13.0, College Station, TX). A level of $p < 0.05$ was considered significant.

Results

Seroprevalence of investigated VBPs in dogs from Croatia

Overall, 112 dogs (25.74%, 95% confidence interval [CI] 21.70–30.12) were serologically positive for any of the six tested pathogens. In the study there were 235 (54.02%) female and 200 (45.98%) male dogs aged between 6 months and 16 years. Nearly half of the dogs were crossbred (246, 56.55%). In the study, based on their lifestyle/use, 98 (22.53%) dogs were categorized as indoor/companion, 159 (36.59%) dogs were categorized as mixed/hunting dogs, and 178 (40.92%) dogs were kept outdoors/shelters. Dogs included in the study originated from 13 different locations (180 dogs from continental parts of Croatia and 255 from the coastal areas). Seroprevalence rates obtained by geographic region are shown in Table 1.

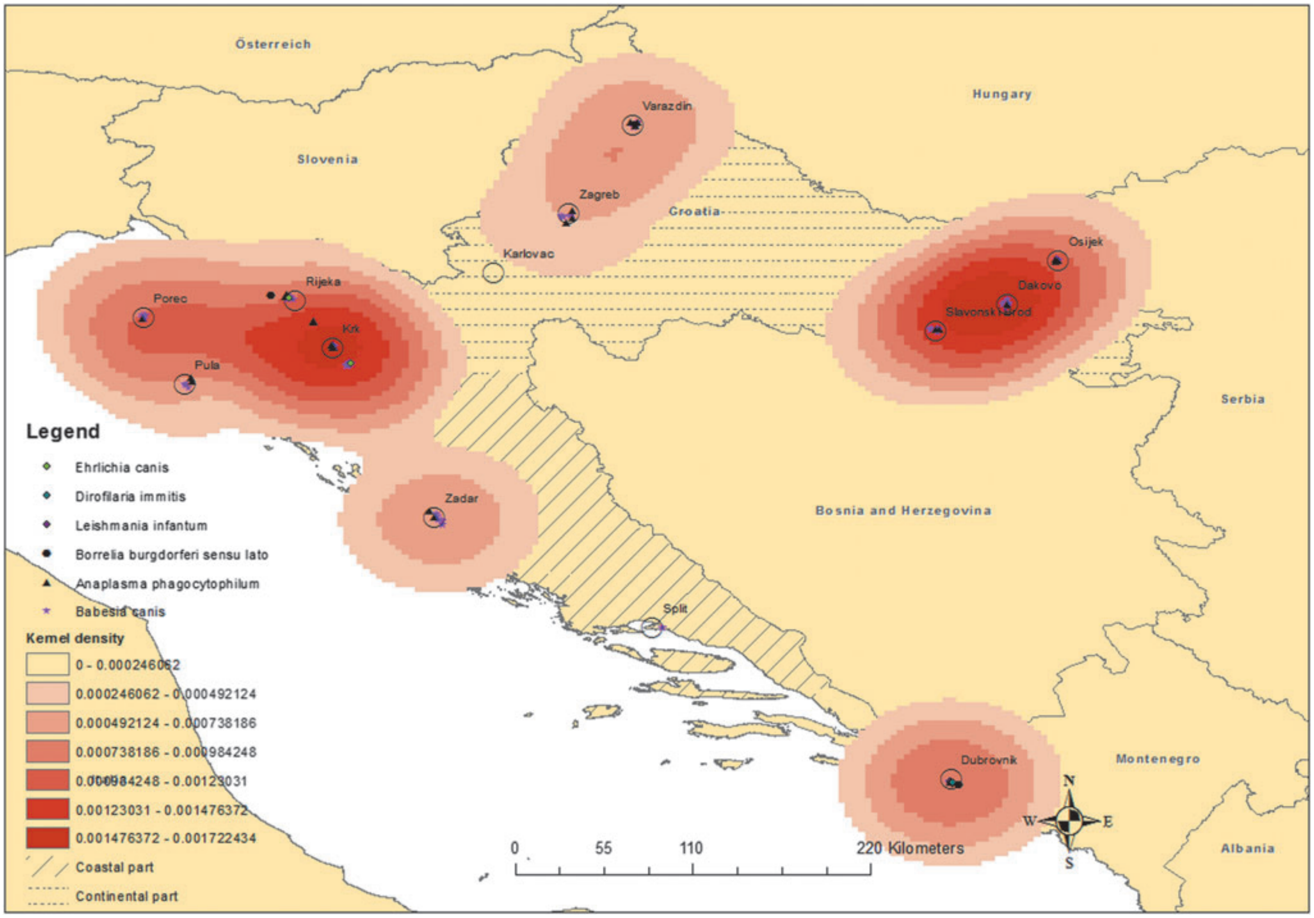

FIG. 1. Spatial distribution of vector-borne pathogens in dogs in Croatia.

Geographical analysis

Statistical analysis of infection with *B. canis* showed a significant difference with respect to the sampling area ($p < 0.001$). Seropositivity to *B. canis* and *A. phagocytophilum* was higher in the continental part; with foci of *B. canis* in the coastal part (Poreč, Dubrovnik), the rate for *L. infantum* antibodies was significantly higher in the Dubrovnik area compared to other cities ($r = 0.166$, $p = 0.001$).

D. immitis antigen was present only in the Dubrovnik region, while *B. burgdorferi sensu lato* was in the continental part (Varaždin region), as well as the coastal part (Rijeka and Dubrovnik) and *E. canis* was only in Rijeka and the island of Krk. Kernel density analysis indicated the highest density of dogs positive for *B. canis* to be in the eastern and western part of Croatia (Fig. 1).

Seroprevalence of B. canis

B. canis was the most prevalent VBP in Croatia, with 87 (20.00%, 95% CI 16.34–24.07) positive dogs throughout the country (Table 2). Out of 44 purebred dogs positive for *B. canis*, Posavac hound (11/24) and Alpine Dachsbracke (10/34) were the most frequently infected breeds. Seroprevalence for *B. canis* was age, breed, and use/lifestyle dependent (Tables 3 and 4). Different age factors tested have a significant impact on babesiosis ($p < 0.05$) except for 12–35 months which show only a tendency ($p = 0.055$). Dogs older than 36 months have 10–20 times more chance to develop disease than 6–11 months dogs, being at greatest risk between 60 and 83 months. Seroprevalence was similar in both sexes ($p = 0.16$). Additional analysis showed that if we take into account age, gender, and use/keeping of dogs, purebred dogs had 1.78 times less chance of developing disease than crossbred. We also found that the seroprevalence for *B. canis* was dependent on the lifestyle/use of dogs. Namely, seropositivity to *B. canis* was 2.56 times higher ($p < 0.024$, 95% CI 1.13–5.78) for dogs that lived outdoors/shelter, and when we controlled for age and breed, 3.41 times higher

Table 2. Seroprevalence for the Canine Vector-Borne Pathogens in Croatia ($N = 435$)

Pathogen	*No. of positive dogs*	*Prevalence (%)*	*95% CI*
B. canis	87	20.00	16.34–24.07
A. phagocytophilum	27	6.21	4.13–8.90
B. burgdorferi sensu lato	3	0.69	0.01–2.00
L. infantum	6	1.38	0.51–2.97
D. immitis	2	0.46	0.05–1.65
E. canis	2	0.46	0.05–1.65

CI, confidence interval.

Table 3. Results of Univariate Logistic Regression Analysis of Risk Factors for Seropositivity of *Babesia canis* in Croatia (n=435)

Epidemiological variable	*Factors*	*OR*	p *value*	*95% CI*
Age	6–11 months	Reference variable		
	12–35 months	7.28	0.055	0.96–55.18
	36–59 months	10.51	0.023	1.37–80.51
	60–83 months	20.03	0.004	2.55–157.22
	>84 months	17.73	0.007	2.22–141.42
Breed	Purebred	Reference variable		
	Crossbreed	0.59	0.027	0.37–0.94
Sex	Female	Reference variable		
	Male	1.44	0.13	0.9–2.30
Use/lifestyle	Companion/indoor	Reference variable		
	Hunting/mix	4.57	0.001	2.06–10.19
	Shelter/outdoor	2.56	0.024	1.13–5.78
Location	Continent (inland)	Reference variable		
	Coast	0.95	0.843	0.59–1.53

($p<0.006$, 95% CI 1.41–8.24), or 4.57 times higher in mixed/hunting ($p<0.001$, 95% CI 2.06–10.19) compared to indoor/companion dogs (Tables 3 and 4).

Seroprevalence of A. phagocytophilum

Specific antibodies against *A. phagocytophilum* were detected in 27 dogs (6.21%, 95% CI 4.13–8.90) (Table 2). Seroprevalence of *A. phagocytophilum* was not dependent on age, breed, sex, use/lifestyle, or location ($p>0.05$) (Table 5).

Seroprevalence of E. canis, B. burgdorferi sensu lato, D. immitis, *and* L. infantum

Only two dogs (0.46%, 95% CI 0.05–1.65), both males, one crossbred and one purebred, one younger than 1 year and the other 4 years old, and both from the coastal part of Croatia, were found to be positive for *E. canis* (Table 2). All three seropositive animals for *B. burgdorferi sensu lato* were female, crossbred dogs, originating from shelters. Two female dogs were 3 years old, and one dog was 18 months old. One was from inland and two from the coastal part of Croatia. Two seropositive dogs (0.46%, 95% CI 0.05–1.65) for *D. immitis* were crossbred male and female, age 30 and 60 months, originating from shelters in the coastal part of Croatia. Six dogs seropositive (1.38%, 95% CI 0.51–2.97) for *L. infantum* were detected during the survey. Three dogs were aged 12–35 months, two 36–59 months, and one dog was older than 84 months. All of them were crossbred from shelters and from the coastal region of Croatia (Dubrovnik). Fifty percent was male.

Seroprevalence of coinfection

Coinfection with two or more pathogens was detected in 12 dogs (seroprevalence 2.76%, 95% CI 1.43–4.77) in all the studied areas. The coinfection was mostly with *B. canis* and *A. phagocytophilum* (1.61%, 95% CI 0.65–2.793.28; $n=7$ dogs). Furthermore, concurrent infections of *B. canis* with *B. burgdorferi sensu lato* ($n=1$ dog) or *L. infantum* ($n=1$ dog) or *E. canis* ($n=1$ dog) were determined. In the coastal region of Croatia (Dubrovnik), two dogs had coinfections with multiple pathogens. In one dog, coinfection was (0.23%, 95% CI 0.05–1.27) with *B. canis*, *A. phagocytophilum*, and *L. infantum* and in the other (0.23%, 95% CI 0.05–1.27) with *B. canis*, *A. phagocytophilum*, *B. burgdorferi sensu lato*, and *L. infantum.*

Discussion

This is the most complete and comprehensive survey of VBP seropositivity that has been conducted in Croatia. Our results confirmed that 25.74% (95% CI 21.70–30.12) of dogs were seropositive for one of six VBPs.

The highest seroprevalence against *B. canis* in our study is in agreement with seroprevalences reported for dogs from other European countries. Thus, the prevalence of *B. canis* in France was from 14.1% to 20.0%, while in Western Romania it was 19.8% (Mas 1990, Cabannes et al. 2002, Imre et al. 2013). In other European countries the prevalence was 7.3% and 13% in Albania, 5.7% in Hungary, and from 0.8% to 17% in Italy (Traldi et al. 1988, Trotz-Williams and Trees 2003, Hornok et al. 2006, Lazri et al. 2008, Hamel et al. 2009). The highest seroprevalence (average 34%) was obtained by

Table 4. Results of Multivariate Logistic Regression Analysis of Risk Factors for Seropositivity of *Babesia canis* in Croatia (Adjusted for Confounding Variables Age and Breed) (n=435)

Epidemiological variable	*Factors*	*OR*	p *value*	*95% CI*
Use/lifestyle	Companion/indoor	Reference variable		
	Hunting/mix	4.57	0.001	2.06–10.19
	Shelter/outdoor	3.41	0.006	1.41–8.24
Confounding variable	Breed	0.80	0.538	0.40–1.61
Confounding variable	Age	1.14	0.001	1.05–1.24

Table 5. Results of Univariable Logistic Regression Analysis of Risk Factors for Seropositivity of *Anaplasma phagocytophilum* in Croatia ($N=435$)

Epidemiological variable	*Factors*	*OR*	p *value*	*95% CI*
Age	0–11 months	Reference variable		
	12–35 months	1.92	0.408	0.41–8.83
	36–59 months	2.29	0.299	0.48–10.99
	60–83 months	1.5	0.664	0.24–10.34
	>84 months	1.15	0.89	0.16–8.51
Breed	Purebred	Reference variable		
	Crossbreed	0.55	0.132	0.25–1.20
Sex	Female	Reference variable		
	Male	0.546	0.15	0.24–1.23
Use/lifestyle	Companion/indoor	Reference variable		
	Hunting/mix	1.10	0.86	0.39–3.11
	Shelter/outdoor	1.18	0.753	0.43–3.23
Location	Continent (inland)	Reference variable		
	Coast	0.90	0.788	0.41–1.95

Cassini et al. (2009), with a reduction in the prevalence in central to northern Italy. The authors found in the central regions of Italy the seroprevalence to be 52–57%. The highest recorded seroprevalence is probably overestimated, considering that titers were generally low and cross-reaction is commonly reported for IFAT (Taylor et al. 2007).

Related to our research, it is reasonable to assume that the sensitivity and specificity of serological tests is not 100%, therefore further validation of serological tests used in our study in field conditions is needed to obtain a true prevalence of the investigated diseases in Croatia. In addition, evaluation of population dynamics of ticks responsible for the dispersal of pathogens should be performed in the future. The seroprevalence obtained in the present study indicates the majority of Croatia to be an endemic region. A total of 21 species of hard tick were registered to occur in Croatia (Krčmar 2012). The continental Croatian area is populated mainly by *Dermacentor reticulatus* and *I. ricinus* (Dobec et al. 2009, Beck et al. 2010, Krčmar 2012).

Antibodies to *B. canis* were confirmed in the majority of Croatia to be related to the tick *D. reticulatus.* An expanding area of *D. reticulatus* distribution has been observed in central Europe up to the Baltic countries and in the Netherlands, Belgium, Germany, Hungary, and the southwest of England (Matjila et al. 2005, Sreter et al. 2005, Dautel et al. 2006). Probably the region of Croatia is also included due to the expanding area of *D. reticulatus* distribution. Generally, for tick-borne diseases, the emergence of clinical cases and detection of seropositivity correspond to the distribution and activity of arthropod vectors. Empirically, clinical cases of tick-borne diseases in Croatia occur at the end of winter until the end of spring, and also in autumn due to favorable conditions for Ixodid tick activity, although during mild winters and rainy summers tick-borne diseases are common as well.

The dynamics of spreading of canine babesiosis in Europe has markedly changed in the last decade. However, the prevalence rates may vary considerably depending on the presence and density of potential arthropod vectors and hosts (Hamel et al. 2011). The cause of these changes is probably due to the effect of global warming, shifting use of the landscape, the increase of wild animal populations, spreading of vectors by wild birds and animals, and the change of habitat structure of wildlife (Leschnik et al. 2008). At the same time, the occurrence of *B. canis* in asymptomatic dogs is very important, because these animals may serve as reservoirs if moved to nonendemic regions (Beck et al. 2009).

Our results confirm that seroprevalence for *B. canis* is dependent on use/lifestyle, and it was, respectively, significantly higher in hunting/mixed dogs ($p<0.001$) and shelter/outdoor dogs ($p<0.006$), compared to companion/indoor animals. When it comes to dogs living in shelters, they are often outdoors and, thus, have a higher risk of exposure to ticks. They are also provided with limited healthcare management and are consequently more exposed to the vectors and the agents these vectors transmit (Cassini et al. 2009). At the same time, shelter dogs, as dogs of promiscuous origin, kennelled in an environment with a high animal and a high tick density, are significantly more at risk to become infected with tick pathogens (Pennisi et al. 2012).

The results of other researchers confirm our results on hunting and kennel dogs compared to companion animals (Solano-Gallego et al. 2008, Imre et al. 2013). The survey conducted in Western Romania confirmed that hunting lifestyle is a major risk for acquiring *B. canis* infection (Imre et al. 2013). Our results from the Mediterranean part, particularly Poreč and the island of Krk, also confirmed this hypothesis. The reason could be that in the survey a large proportion of hunting dogs were included. These dogs are often involved in hunting in the continental part of Croatia and, thus, have greater contact with ticks. The available evidence shows that the *Rhipicephalus sanguineus* tick is abundant in coastal and insular Croatia (Mikačić 1965, Tovornik and Vesenjak-Hirjan 1988). It is important to note that in the Croatian coastal area the presence of pathogen *Hepatozoon canis* (Vojta et al. 2009) and its vector *R. sanguineus* was identified. Therefore, we may assume that the protozoan parasite *B. vogeli* is also present in this area, since both *B. vogeli* and *H. canis* are transmitted through the same vector *R. sanguineus.*

Our data also showed that significant differences ($p<0.05$) were present in the seroprevalence of infected animals with regard to age. Seropositivity was significantly higher in dogs older than 1 year. Similar results were obtained by some investigations (Yamane et al. 1994), while another survey indicated that different age groups did not differ significantly from each other (Imre et al. 2013). The increase in the prevalence of seropositive dogs with age could be related to the cumulative increase of the exposure period to arthropod vectors over the years. Our results also confirmed that the seroprevalence for *B. canis* was breed dependent (OR = 0.59; 95% CI 0.37–0.94; $p < 0.026$). Additional analyses have shown that purebred dogs have a 1.78 times greater chance of developing disease than crossbred dogs. The higher prevalence of antibodies to *B. canis* was present in Posavac hound (11/24) and Alpine Dachsbracke (10/34) purebreds.

Similar results were obtained in other studies as well (Bizzeti et al. 1997, Adaszek et al. 2011). Authors have found that breed was associated with the seropositive reaction to *B. canis*, but more often in purebred dogs than in crossbred dogs. Some authors have observed that the prevalence of antibodies to *B.canis* was significantly higher among German Shepherds and Komondors, while in another survey *B. gibsoni* was typically associated with American Pit Bull Terriers. At the same time, there are surveys that show breed to not be associated with seropositive reactions to *B. canis* in the dog populations tested (Yamane et al. 1994, Costa-Júnior et al. 2009, Imre et al. 2013).

It is difficult to biologically interpret purebred dogs as risk factors for babesiosis. However, in our research, hunting dogs were mainly purebred dogs and probably more likely to be infected with VBPs. In addition, in our survey dogs from shelters were living mostly outdoors, unlike companion dogs that are living mainly indoors. Adaszek et al. (2011) have considered that despite the statistical significance, babesiosis is not connected with predispositions of particular breeds, but with the dogs' living conditions and the nature of their work (Bourdoiseau 2006).

In our survey, no significant gender predisposition to disease was found, in contrast to another investigation that observed babesiosis more frequently in male animals (Adaszek et al. 2011). There is clear evidence that *B. canis* prevalence is different among areas ($p < 0.001$) and Kernel density analysis indicated two areas with the highest density of dogs positive for *B. canis* in the western part of Croatia around Rijeka and in the eastern part around Đakovo (Fig. 1). Observed differences could be due to vector density and duration of the hunting season, which should be further investigated.

This study provides the first serological evidence for canine exposure to *A. phagocytophilum.* The seroprevalence of antibodies was 6.21% with a geographical distribution of seropositive cases homogeneous throughout the country. This could be explained by the ubiquitous character of *I. ricinus*, the main tick vector of *A. phagocytophilum* in Europe (Strle 2004). In addition, research studies indicate that migrating birds may be important in the dispersal of *A. phagocytophilum* infected *I. ricinus* in Europe (de la Fuente et al. 2005, Skoracki et al. 2006). In the investigation conducted in Hungary, circulating antibodies to *A. phagocytophilum* showed it to be the most prevalent pathogen, after babesiosis, with a positive antibody titer frequency of 7.9% (Farkas et al. 2014). Our study confirmed that antibodies against *A. phagocytophilum* were not age, breed, sex, use/lifestyle, or location dependent ($p > 0.05$). A similar result has been reported in Hungary (Farkas et al. 2014).

In Europe, *A. phagocytophilum* is transmitted by the tick *I. ricinus*, whereas *A. platys* could be transmitted by *R. sanguineus*, the brown dog tick (Dantas-Tores 2008, Chomel 2011). In the continental part of Croatia Varaždin region has confirmed *A. phagocytophilum* and *B. burgdorferi sensu lato.* Both are transmitted by ticks of the genus *Ixodes* (Parola and Raoult 2001).

The available evidence shows that the *R. sanguineus* tick is abundant in the coastal Croatia. We had identified *E. canis* and *Anaplasma* spp. infection along the Croatian coast. Recent research confirmed that the *A. phagocytophilum* analyte could cross-react with the *Anaplasma platys*-infected dog sera samples by the SNAP 4Dx ELISA (Bowman et al. 2009, Chandrashekar et al. 2010, Pantchev 2010). We assume that the parasite *A. platys* could also be present in the region, since both *E. canis* and *A. platys* are transmitted by the same tick vector species *R. sanguineus.* Therefore, more specific diagnostic methods are required to distinguish *A. phagocytophilum* from *A. platys* because of serological cross-reactivity. It is important to keep in mind that positive serological results presented in this study might be due to either evidence of prior exposure to the corresponding pathogen at some point and some location in the dog's history or on-going infection. Serological testing detects basically chronic or inconspicuous infections and is limited by a reduced ability to identify acute infections (Menn et al. 2010).

Antibodies against *B. burgdorferi sensu lato* were detected in three dogs (0.67%), one dog in the continental part (Varaždin region) and two dogs in the coastal part in coinfection with other vector borne diseases (Rijeka and Dubrovnik regions). In Europe, at least six different genospecies that belong to the *B. burgdorferi sensu lato* complex have been found: *Borrelia afzelii*, *Borrelia garinii*, *B. burgdorferi sensu stricto*, *Borrelia valaisiana*, *Borrelia lusitaniae*, *and Borrelia spielmanii* (Derdakova and Lenčakova 2005, Richter et al. 2006). In another study from Croatia, Rijpkema et al. (1996) identified four genomic groups of *B. burgdorferi sensu lato* (*Borrelia afzelii*, *Borrelia garinii*, group VS116, and *B. burgdorferi sensu stricto*) in *I. ricinus* ticks collected in a LB endemic region.

In Croatia, antibodies to *B. burgdorferi sensu lato* were detected in six (5%) dogs from the Zagreb area (Turk et al. 2000). Our findings suggest that exposure to *B. burgdorferi sensu lato* exists in dogs in Croatia not only in an endemic region (Varaždin region) but also in Rijeka and Dubrovnik region. Our results may also indicate that LB foci are restricted to small areas, but due to the small number of dogs further studies on *Borrelia* prevalence are necessary to confirm this hypothesis. Very low seroprevalence, similar to our result, was found in some countries such as Spain (Miró et al. 2013), France (Pantchev et al. 2009), Romania (Mircean et al. 2012), and Hungary (Farkas et al. 2014). The differences in seroprevalence rates could arise from variability in tick densities or the proportion of infected ticks (Kybicova et al. 2009, Farkas et al. 2014). Some authors reported a higher prevalence of LB in older dogs, while others claim the same for younger dogs (<1 year) (Amusategui et al. 2008, Miró et al. 2013, Farkas et al. 2014).

CanL caused by *L. infantum* is a widespread endemic disease in the Mediterranean basin, and its seroprevalence ranges from 10% up to 53.1% in some foci in Italy (Brandonisio et al. 1992, Fisa et al. 1999, Miró et al. 2012). In the southern parts of Croatia, CanL was recognized as a problem for the first time in the first part of the 20th century (Tartaglia 1937). In our research, seropositivity toward *L. infantum* was detected in Dubrovnik regions in dogs from shelters (1.38%), while antibodies were not detected in dogs from Split and Zadar regions. For the coastal city of Split and in the hinterland of Split regions, another research has confirmed seroprevalence ranging from 0% to 42.85%, depending on the location (Živičnjak et al. 2005).

In central and southern parts of Dalmatia, pathologic and parasitologic analysis confirmed a case of visceral leishmaniasis (*L. infantum*) in a gray wolf (Canis lupus) (Beck et al. 2008). Such differences between studies could be attributable to the different population analyzed, the sampling season, arthropod vector distribution and density, the increased exposure to phlebotomines due to spending more time outdoors, as well as the diagnostic technique used (Miró et al. 2013). Generally, leishmaniasis is endemic/enzootic only in the middle and south Dalmatia area, and phlebotomine sandy flies are active during the warmest period of the year. Because of the chronic course of the disease, according to our clinical experience with dogs living in a nonenzootic area, exposure to sand flies happened typically 1, 2, or 3 years before clinical signs appeared.

Entomological surveys carried out in Dalmatia revealed sand fly species of the *Phlebotomus* genus, among which three belonged to the *Larroussius* subgenus, being proven to be *L. infantum* vectors (*Phlebotomus tobbi*, *Phlebotomus Neglectus*, and *Phlebotomus perfi liewi*) (Bosnić et al. 2006). Recent entomological studies evidenced two competent *Leishmania* vectors, *P. neglectus* and *P. tobbi*; the first was prevalent (75.9%), being also the species much more associated with habitats where dogs are present. Moreover, the prevalence of *P. neglectus* females feeding on human blood reached 30% (Živičnjak et al. 2011). Possibly due to global warming, cases of canine leishmaniasis have been reported in foci outside the Mediterranean countries, for example, autochthonous cases in Hungary and Germany (Mencke 2011, Tanczos et al. 2012).

The seroprevalence of *D. immitis* was 0.46% with a focal region in the southern part of the coast (Dubrovnik). The results from the coastal part of Croatia, particularly the Istrian Peninsula, may be somewhat unexpected. Namely, we did not find circulating *D. immitis* antigen in two locations (Poreč, Pula) in the Istrian Peninsula. A possible explanation for this is the small number of dogs in our survey, but also the possibility that the ELISA test can give rise to false negatives in dogs with low heartworm burdens or in blood samples from dogs infected only by male worms (Atkins 2003).

Other studies confirmed the presence of *D. immitis* in the Istrian Peninsula (Jurić et al. 2007, Holler et al. 2010). Even in the former Yugoslavia, in Croatia between 1987 and 1989 several cases of canine dirofilariasis were reported, but were not considered autochthonous (Brglez and Senk 1987, Genchi at al. 2001). Živičnjak et al. (2007) confirmed the existence and spreading of *D. immitis* in dogs in the area of Dubrovnik and the Istrian Peninsula. One of the reasons for spreading of dirofilariasis in dogs in Europe is the fact that the mosquito of the *Aedes albopictus* genus has been spreading aggressively along the coasts of the Istrian peninsula and toward its hinterland (Klobučar et al. 2006, Živičnjak et al. 2006, Holler et al. 2010). Another reason may be the occurrence and spreading of *A. albopictus* in Italy and implications for its introduction into other parts of Europe (Knudsen et al. 1996).

Canine monocytic ehrlichiosis is a widespread tick-borne infection in the world and is the only *Ehrlichia* species that has been isolated in dogs from Europe (Keysary et al. 1996, Aguirre et al. 2004). In the present study we found an overall seroprevalence of 0.46%. The low prevalence may be explained by several factors, such as the absence of *E. canis*, rare exposure of pet dogs to *R. sanguineus*, or inefficient transmission of *E. canis* by *R. sanguineus* in colder parts of Croatia (Gary et al. 2006).

In the areas where VBPs are endemic, coinfection is a frequent event in dogs, especially in the environment in which the vector population density is high. At the same time, Solano-Gallego et al. (2016) suggest that coinfection with *Babesia* spp. is not well documented and rarely reported in dogs. In our survey, coinfection was found in 12 animals (2.76%), of which 10 were seropositive for two pathogens. The coinfection was mostly with *B. canis* and *A. phagocytophilum* (1.61%). In general, coinfections are present in continental and coastal parts of Croatia, particularly in the region of Dubrovnik. Namely, dog populations from coastal areas of the Mediterranean show high infection rates with various VBPs. In addition, our results show that coinfections were more frequently found in shelter dogs, especially in the Dubrovnik region. This is consistent with other studies (Pennisi et al. 2012).

In Hungary, coinfection was found in eight dogs (0.61%), of which seven were seropositive for two pathogens, and one dog was serologically positive for three pathogens (Farkas et al. 2014). In the Mediterranean region, there have been many reports of coinfections with *L. infantum* and *E. canis* (Trotz-Williams and Trees 2003), while in our study we had only two coinfections with *L. infantum* and *E. canis*.

Conclusions

This is the first comprehensive survey of VBP seropositivity conducted in Croatia. The incidence and geographic distribution indicate that dogs in Croatia are at risk of developing infections with VBPs. Humans are also susceptible to many of these VBPs. The results of this study demonstrate that *B. canis* is the most prevalent among the studied VBPs. Coinfections are present in the continental and coastal parts of Croatia, which is very important for the clinical management of canine patients. This study will be useful for veterinary and public health authorities with the increased importance of VBPs in Croatia.

Acknowledgments

The study was supported, in part, by VOXA IDEXX Laboratories, Croatia, and European Union grant FP7-ERA Chair project ''VetMedZg'' (Grant Agreement 621394).

Author Disclosure Statement

No competing financial interests exist.

References

Adaszek L, Carbonero Martinez A, Winiarczyk S. The factors affecting the distribution of babesiosis in dogs in Poland. Vet Parasitol 2011; 81:160–165.

Aguirre E, Sainz A, Dunner S, Amusategui I, et al. First isolation and molecular characterization of *Ehrlichia canis* in Spain. Vet Parasitol 2004; 125:365–372.

Alho AM, Pita J, Amaro A, Amaro F, et al. Seroprevalence of vector-borne pathogens and molecular detection of *Borrelia afzelii* in military dogs from Portugal. Parasites Vectors 2016; 9:225.

Amusategui I, Tesouro MA, Kakoma I, Sainz A. Serological reactivity to *Ehrlichia canis, Anaplasma phagocytophilum, Neorickettsia risticii, Borrelia burgorferi and Rickettsia conorii* in dogs from Northwestern Spain. Vector Borne Zoonotic Dis 2008; 8:797–803.

Atkins CE. Comparison of results of three commercial heartworm antigen test kits in dogs with low heartworm burdens. J Am Vet Med Assoc 2003; 222:1221–1223.

Barić Rafaj R, Kuleš J, Selanec J, Vrkić N, et al. Markers of coagulation activation, endothelial stimulation, and inflammation in dogs with babesiosis. J Vet Intern Med 2013; 27: 1172–1178.

Beck A, Beck R, Kusak J, Gudan A, et al. A case of visceral leishmaniosis in a gray wolf (*Canis lupus*) from Croatia. J Wildl Dis 2008; 44:451–456.

Beck R, Habrun B, Bosnić S, Benić M, et al. Identification of pathogens in *Ixodes ricinus* and *Dermacentor reticulates* from public gardens in Zagreb, Croatia. *12th International Conference on Lyme Borreliosis and Other Tick-Borne Diseases*, September 26–29, 2010, Ljubljana Slovenia. Book of Abstracts 2010:95.

Beck R, Vojta L, Mrljak V, Marinculić A, et al. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. Int J Parasitol 2009; 39:843–848.

Beugnet F, Marié J-L. Emerging arthropod-borne diseases of companion animals in Europe. Vet Parasitol 2009; 163:298–305.

Bizzeti M, Corazza M, Mancianti F, Minori D, et al. Indirect fluorescent antibody test in the diagnosis of babesiosis in dogs. Ann Fac Med Vet 1997; 50:235–239.

Bosnić S, Gradoni L, Khouy C, Maroli M. A review of leishmaniasis in Dalmatia (Croatia) and results from recent surveys on phlebotomine sandfl ies in three southern counties. Acta Trop 2006; 99:42–49.

Bourdoiseau G. Canine babesiosis in France. Vet Parasitol 2006; 138:118–125.

Bowman D, Little SE, Lorentzen L, Shields J, et al. Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: Results of a national clinic-based serologic survey. Vet Parasitol 2009; 160:138–148.

Brandonisio O, Carelli G, Ceci L, Consenti B, et al. Canine leishmaniasis in the Gargano promontory (Apulia, South Italy). Eur J Epidemiol 1992; 8:273–276.

Brglez J, Senk L. *Dirofilaria immitis* (Leidy, 1856) Railliet et Henry, 1911, in a dog *Dirofilaria immitis* (Leidy, 1856) Railliet et Henry, 1911, pri psu. Zbornik Biotehniske Fakultete Univerze Edvardo Kardelja v Ljubljani. Veterinarstvo 1987; 24:69–72.

Cabannes A, Pelse H, Lucchese F, Appriou M. Séroprévalence de la babésiose canine dans le Sud-Ouest de la France. Rev Med Vet 2002; 153:27–28.

Cassini R, Zanutto S, Frangipane di Regalbono A, Gabrielli S, et al. Canine piroplasmosis in Italy: Epidemiological aspects in vertebrate and invertebrate hosts. Vet Parasitol 2009; 165: 30–35.

Chandrashekar R, Mainville CA, Beall MJ, O'Connor T, et al. Performance of a commercially available in-clinic ELISA for the detection of antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Borrelia burgdorferi* and *Dirofilaria immitis* antigen in dogs. Am J Vet Res 2010; 71: 1443–1450.

Chomel B. Tick-borne infections in dogs—An emerging infectious threat. Vet Parasitol 2011; 179:294–301.

Costa-Júnior LM, Ribeiro MFB, Rembeck K, Rabelo EMI, et al. Canine babesiosis caused by *Babesia canis vogeli* in rural areas of the State of Minas Gerais, Brazil and factors associated with its seroprevalence. Res Vet Sci 2009; 86:257–260.

Dantas-Tores F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): From taxonomy to control. Vet Parasitol 2008; 152:173–185.

Dautel H, Dippel C, Oehme R, Hartelt K, et al. Evidence for an increased geographical distribution of *Dermacentro reticulatus* in Germany and detection of *Rickettsia* sp. Int J Med Microbiol 2006; 296:149–156.

Day MJ. The immunopathology of canine vector-borne diseases. Parasites Vectors 2011; 4:48.

de la Fuente J, Naranjo V, Ruiz-Fons F, Hőfle U, et al. Potential vertebrate reservoir hosts and invertebrate vectors of *Anaplasma marginale* and *A. phagocytophilum* in central Spain. Vector Borne Zoonotic Dis 2005; 5:390–401.

Derdakova M, Lenčakova D. Association of genetic variability within the *Borrelia burgdorferi* sensu lato with the ecology, epidemiology of Lyme borreliosis in Europe. Ann Agric Environ Med 2005; 12:165–172.

Dobec M, Golubić D, Punda-Polić V, Kaeppeli F, et al. *Rickettsia helvetica* in *Dermacentor reticulatus* ticks. Emerg Infect Dis 2009; 15:98–100.

ESRI. *ArcGIS Desktop: Release 10.2.2*. Redlands, CA: ESRI, 2014.

Farkas R, Gyurkovszky M, Lukacs Z, Aladics B, et al. Seroprevalence of some vector-borne infections of dogs in Hungary. Vector Borne Zoonotic Dis 2014; 14:256–260.

Ferroglio E, Centaro E, Mignone W, Trisciuoglio A. Evaluation of an ELISA rapid device for the serological diagnosis of *Leishmania infantum* infection in dog as compared with immunofluorescence assay and Western blot. Vet Parasitol 2007; 144:162–166.

Fisa R, Gállego M, Castillejo S, Aisa MJ, et al. Epidemiology of canine leishmaniosis in Catalonia (Spain): The example of the Priorat focus. Vet Parasitol 1999; 83:87–97.

Gary AT, Webb JA, Hegarty CH, Breitschwerdt EB. The low seroprevalence of tick-transmitted agents of disease in dogs from southern Ontario and Quebec. Can Vet J 2006; 47:1194–1200.

Genchi C, Kramer LH, Prieto G. Epidemiology of canine and feline dirofilariasis, a global view. In: Simón F, Genchi C, eds. *Heartworm Infection in Humans and Animals.* Salamanca: Ediciones Universidad de Salamanca, 2001:121–134.

Golubić D, Rijpkema S, Tkalec-Makovec N, Ruzić E. Epidemiologic, ecologic and clinical characteristics of Lyme borrelliosis in northwest Croatia. Acta Med Croatica 1998; 52:7–13.

Gray JS, Dautel, H, Estrada-Pena A, Kahl O, et al. Effects of climate change on ticks and tick-borne diseases in Europe. Interdiscip Perspect Infect Dis 2009; Article ID 593232. F.

Hamel D, Röhrig E, Pfister K. Canine vector-borne disease in travelled dogs in Germany-a retrospective evaluation of laboratory data from the years 2004–2008. Vet Parasitol 2011; 181:31–36.

Hamel D, Silaghi C, Knaus M, Visser M, et al. Detection of *Babesia canis* subspecies and other arthropod-borne diseases in dogs from Tirana, Albania. Wien Klin Wochenschr 2009; 121:42–45.

Harrus S, Baneth G. Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. Int J Parasitol 2005; 35:1309–1318.

Holler D, Racz A, Bošnir J, Petrak O. The prevalence of dirofilariasis in the hinterland of the Istrian peninsula. Med Jad 2010; 40:67–74.

Hornok S, Edelhofer R, Farkas R. Seroprevalence of canine babesiosis in Hungary suggesting breed predisposition. Parasitol Res 2006; 99:638–642.

Imre M, Farkas R, Ilie M, Imre K, et al. Seroprevalence of *Babesia canis* infection in clinically healthy dogs from Western Romania. J Parasitol 2013; 99:161–163.

Jurić B, Beck R, Martinković F, Milosavljević A, et al. The prevalence of dirofilaria infections among truffle dogs in Istria, Croatia. *Proceedings of the First European Dirofilaria Days*, FEDD 2007 Zagreb, Croatia, February 22–25, 2007:33–34.

Keysary A, Waner T, Rosner M, Warner CK, et al. The first isolation, in vitro propagation, and genetic characterization of *Ehrlichia canis* in Israel. Vet Parasitol 1996; 62:331–340.

Killick-Kendrick R. Phlebotomine vectors of the leishmaniases: A review. Med Vet Entomol 1990; 4:1–24.

Klobučar A, Merdić E, Benić N, Blaklaić Z, et al. First record of *Aedes albopictus* in Croatia. J Am Mosq Control Assoc 2006; 22:147–148.

Knudsen AB, Romi R, Majori G. Occurrence and spread in Italy of *Aedes albopictus*, with implications for its introduction into other parts of Europe. J Am Mosq Control Assoc 1996; 12(2 Pt 1):177–183.

Krčmar S. Hard ticks (Acari, Ixodidae) of Croatia. ZooKeys 2012; 234:19–57.

Kybicova K, Schanilec P, Hulinska D, Uherkova L, et al. Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi sensu lato* in dogs in the Czech Republic. Vector Borne Zoonotic Dis 2009; 9:655–661.

Lazri T, Duscher G, Edelhofer R, Bytyci B, et al. 2008. Infektionen mit arthropodenübertragenen Parasiten bei Hunden im Kosovo und in Albanien unter besonderer Berücksichtigung der Leishmanieninfektionen. Wien Klin Wochenschr 2008; 120(S4):54–58.

Leschnik M, Kirtz G, Tichy A, Leidinger E. Seasonal occurrence of canine babesiosis is influenced by local climate conditions. Int J Med Microbiol 2008; 298:243–248.

Mas JP. Séroépidémiologie de la babésiose canine en région d'endémie. Thčse Doct Vét Lyon, 1990:n849.

Matijatko V, Kiš I, Torti M, Brkljačić M, et al. Septic shock in canine babesiosis. Vet Parasitol 2009; 162:263–270.

Matjila TP, Nijhof AM, Taoufik A, Houwers D, et al. Autochthonous canine babesiosis in The Netherlands. Vet Parasitol 2005; 131:23–29.

Mencke N. The importanceof canine leishmaniosis in non-endemic areas, with special emphasis on the situation in Germany. Bern Munch Tierarztl Wochenschr 2011; 124:434–442.

Menn B, Lorentz S, Naucke TJ. Imported and travelling dogs as carriers of canine vector-borne pathogens in Germany. Parasites Vectors 2010; 3:34.

Mikačić D. Ticks of the coastal area of Yugoslavia. III: the distribution and dynamics of certain species during the year. Vet Archiv 1965; 35:23–27.

Mills JN, Gage KL, Khan AS. Potential influence of climate change on vector-borne and zoonotic diseases: A review and proposed research plan. Environ Health Perspect 2010; 118: 1507–1514.

Mircean V, Dumitrache MO, Györke A, Pantchev N, et al. Seroprevalence and geographic distribution of *Dirofilaria immitis* and tick-borne infections (*Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, and *Ehrlichia canis*) in dogs from Romania. Vector Borne Zoonotic Dis 2012; 12:595–604.

Miró G, Checa R, Montoya A, Hernandez L, et al. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. Parasites Vectors 2012; 5:60.

Miró G, Montoya A, Roura X, Galvez R, et al. Seropositivity rates for agents of canine vector-borne diseases in Spain: A multicentre study. Parasites Vectors 2013; 6:117.

Miščević Z, Milutinović M, Ivović V. Fauna and distribution of sandflies (Diptera, Phlebotomidae) in Yugoslavia, Croatia Macedonia and their role in the transmission of parasitic and viral diseases. Acta Vet 1998; 48:163–172.

Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: Part one. Trends Parasitol 2009; 25:157–163.

Otranto D, Paradies P, Lia RP, Latrofa MS, et al. Efficacy of a combination of 10% imidacloprid/50% permethrin for the prevention of leishmaniasis in kennelled dogs in an endemic area. Vet Parasitol 2007; 144:270–278.

Pantchev N. C-reactive protein as a marker in canine granulocytic anaplasmosis. Vet Rec 2010; 166:632.

Pantchev N, Schaper R, Limousin S, Norden N, et al. Occurrence of *Dirofilaria immitis* and tick-borne infections caused by *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato and *Ehrlichia canis* in domestic dogs in France: Results of a countrywide serologic survey. Parasitol Res 2009; 105: S101–S113.

Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: Emerging infections threat. Clin Infect Dis 2001; 32: 897–928.

Pennisi M-G, Capri A, Solano-Gallego L, Lombardo G, et al. Prevalence of antibodies against *Rickettsia conorii*, *Babesia canis*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* antigens in dogs from the Stretto di Messina area (Italy). Ticks Tick Borne Dis 2012; 3:315–318.

Petrie A, Watson P. *Statistics for Veterinary and Animal Science*, 3rd edition. Oxford: Wiley-Blackwell, 2013.

Ravnik U, Tozon N, Strasek K, Zupanc TA. Clinical and haematological features in *Anaplasma phagocytophilum* seropositive dogs. Clin Microbiol Infect 2009; 15:39–40.

Richter D, Postic D, Sertour N, Livey I, et al. Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol 2006; 56: 873–881.

Rijpkema S, Golubić D, Molkenboer M, Verbeek-De Kruif N, et al. Identification of four genomic groups of *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks collected in a Lyme borreliosis endemic region of northern Croatia. Exp Appl Acarol 1996; 20:23–30.

Shaw SE, Day MJ, Birtles RJ, Breitschwerdt EB. Tick-borne infectious diseases of dogs. Trends Parasitol 2001; 17:74–80.

Silverman BW. *Density Estimation for Statistics and Data Analysis*. London: Chapman and Hall/CRC, 1998.

Skoracki M, Michalik J, Skotarczak B, Rymaszewska A, et al. First detection of *Anaplasma phagocytophilum* in quill mites (Acari: Syringophilidae) parasitizing passerine birds. Microbes Infect 2006; 8:303–307.

Solano-Gallego L, Sainz A, Roura X, Estrada-Pena A, et al. A review of canine babesiosis: The European perspective. Parasites Vectors 2016; 69:336.

Solano-Gallego L, Trotta M, Carli E, Carcy B, et al. *Babesia canis canis* and *Babesia canis vogeli* clinicopathological findings and DNA detection by means of PCR-RFLP in blood from Italian dogs suspected of tick-borne disease. Vet Parasitol 2008; 157:211–221.

Sreter T, Szell Z, Varga I. Spatial distribution of *Dermacentor reticulatus* and *Ixodes ricinus* in Hungary: Evidence for change? Vet Parasitol 2005; 128:347–351.

Strle F. Human granulocytic ehrlichiosis in Europe. Int J Med Microbiol 2004; 293 Suppl 37:27–35.

Tanczos B, Balogh N, Kiraly L, Biksi I, et al. First record of autochtonous canine leishmaniasis in Hungary. Vector Borne Zoonotic Dis 2012; 12:588–594.

Tartaglia P. La leishmaniose canine a Split. Bull.de l'Office Intern d'Hyg. Publ. XXIV:1937; 9.1927.

Taylor MA, Coop RL, Wall RL (eds.). *Veterinary Parasitology*, 3rd ed. Oxford: Blackwell Publishing, 2007:874.

Tovornik D, Vesenjak-Hirjan J. A revision of ticks belonging to the *Rhipicephalus sanguineus* complex (Latreille), collected in the Yugoslav coastal region. Biol Vestn 1988; 36:77–84.

Traldi G, Ahmed M H, Mazzucchelli M. Diffusione di *Babesia canis* in 2 province del nord Italia. Parassitologia 1988; 30(Suppl 1):209–210.

Trotz-Williams LA, Trees AJ. Systemic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Vet Rec 2003; 152:97–105.

Turk N, Marinculić A, Modrić Z. Serologic studies of canine Lyme borreliosis in the Zagreb area (Croatia). Vet Arhiv 2000; 70:39–45.

Vojta L, Mrljak V, Čurković S, Živičnjak T, et al. Molecular epizootiology of canine hepatozoonosis in Croatia. Int J Parasitol 2009; 39:1129–1136.

Yamane I, Gardner IA, Ryan CP, Levy M, et al. Serosurvey of *Babesia canis, Babesia gibsoni*, and *Ehrlichia canis* in pound dogs in California, USA. Prev Vet Med 1994; 18:293–304.

Zell R. Global climate change and the emergence/re-emergence of infectious diseases. Int J Med Microbiol 2004; 293 Suppl 37:16–26.

Živičnjak T, Martinković F, Beck R. Canine dirofilariosis in Croatia: Let's face it. Proceedings of the First European Dirofilaria Days, FEDD 2007 Zagreb, Croatia, February 22–25, 2007:35.

Živičnjak T, Martinković F, Beck R. Dirofilariosis in Croatia, spread and public health impact. In 5th Croatian Congress on Infective Diseases, Zadar, 2006.

Živičnjak T, Martinković F, Khoury C, Bongiorno G, et al. Serological and entomological studies of canine leishmaniosis in Croatia. Vet Arhiv 2011; 81:99–110.

Živičnjak T, Martinković F, Marinculić A, Mrljak V, et al. A seroepidemiologic survey of canine visceral leishmaniosis among apparently healthy dogs in Croatia. Vet Parasitol 2005; 131:35–43.

Živičnjak T, Stojčević D, Marinculić A, Ramadan P, et al. Epizootiological survey for canine visceral leishmaniasis and the isolation of the protozoa leishmania infantum. In: Abstract Book from 1st Croatian Congress on Infectious Diseases. October 1–3, Dubrovnik, Croatia, 1998:106.

Address correspondence to:
Vladimir Mrljak
Clinic for Internal Diseases
Faculty of Veterinary Medicine
University of Zagreb
Heinzelova 55
Zagreb 10000
Croatia

E-mail: vmrljak@vef.hr