Parasitology Research (2019) 118:347–352
https://doi.org/10.1007/s00436-018-6129-7

ARTHROPODS AND MEDICAL ENTOMOLOGY - SHORT COMMUNICATION

Seroprevalence of vector-borne pathogens in dogs from Croatia

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Received: 29 June 2018 / Accepted: 23 October 2018 / Published online: 31 October 2018

Abstract

Canine vector-borne pathogens (VBPs) are a group of globally distributed and rapidly spreading microorganisms transmitted by arthropods. In the present survey, we investigated *Anaplasma* spp., *Ehrlichia canis*, *Dirofilaria immitis* and *Borrelia burgdorferi* sensu lato seroprevalence between three groups of dogs (asymptomatic, suspected and deceased) from continental and coastal regions of Croatia with the commercial point-of-care SNAP®4Dx®Plus for VBPs. The overall prevalence for tested pathogens in dogs was 6.1% with the highest prevalence detected for *Anaplasma* spp. (4.5%), while the remaining pathogens were found at a prevalence of less than 1% (*E. canis* 0.6%, *B. burgdorferi* s.l. 0.4%, *D. immitis* 0.6%). No statistically significant differences in VBP detection between dog cohorts could be found with the exception of *D. immitis* in the deceased group. Interestingly, no evidence of *D. immitis* could be found in the hearts of dogs in this group at necropsy, however. This study provides the first data on the seroprevalence of selected VBPs between dogs of different health statuses in Croatia. The results demonstrate that serological evidence of VBPs alone or in combination with co-infections were found just as frequently in asymptomatic dogs as those with suspected or confirmed evidence of VBP disease, raising questions about the pathogenic potential of these organisms in domesticated dogs.

Keywords Dog · Vector-borne pathogens · Serology · Croatia

Section Editor: Charlotte Oskam

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Introduction

Vector-borne pathogens (VBPs) are a group of globally distributed and rapidly spreading microorganisms that are transmitted by arthropods, including ticks, fleas, mosquitoes and phlebotominae sand flies (Otranto et al. 2009). In dogs, bacteria such as *Anaplasma platys*, *Anaplasma phagocytophilum*, *Ehrlichia canis* and *Borrelia burgdorferi* (sensu lato) complex, as well as protozoans *Babesia canis*, *Hepatozoon canis* and *Leishmania infantum* and the nematode *Dirofilaria immitis*, are among the major VBPs (Day 2011). The prevalence of these VBPs in dogs appears to vary across different European regions. Unfortunately, a confounder to comparing these studies is the choice of detection methods. Different serological methods (immunofluorescent antibody test (IFAT), enzyme-linked immunoadsorbent assay (ELISA), SNAP®4Dx®) have been used to assess the distribution of VBPs among European countries showing that the prevalence can vary depending on the method used (Sainz et al. 2015; Maggi et al. 2014).

Using the SNAP®4Dx® commercial point-of-care test, the most frequently detected VBPs in dogs throughout Europe belong to the bacterial genus *Anaplasma*, including the species *Anaplasma phagocytophilum*, the causative agent of granulocytic anaplasmosis and *Anaplasma platys*, a potential agent of canine infectious cyclic thrombocytopenia (Sainz et al. 2015). *Anaplasma* spp. have been reported in dogs from northern (Finland; Pérez Vera et al. 2014), central (Hungary; Farkas et al. 2014), western (France; Pantchev et al. 2009) and south-eastern (Romania; Mircean et al. 2012) Europe, with prevalences ranging from 2.7% (France; Pantchev et al. 2009) to 7.9%, (Hungary; Farkas et al. 2014). In southern Europe, several studies (Italy, Portugal, Spain) have revealed that the prevalence in asymptomatic dogs was lower than in dogs clinically suspected to have canine vector-borne diseases (VBDs) (Cardoso et al. 2012; Miró et al. 2013; Piantedosi et al. 2017).

Ehrlichia canis is the bacterial pathogen responsible for acute and chronic canine monocytic ehrlichiosis (Sainz et al. 2015). The reported seroprevalence of *E. canis* in dogs varies throughout Europe. Lower prevalences, of up to 0.3%, were reported in Hungary (Farkas et al. 2014), Finland (Pérez Vera et al. 2014) and France (Pantchev et al. 2009), while higher in Romania (2.1%, Mircean et al. 2012). Higher prevalences are largely limited to Southern Europe and, in particular, the Mediterranean basin, which is considered to be endemic for *E. canis* (Sainz et al. 2015). In asymptomatic dogs, the *E. canis* seroprevalence ranges from 4.1% (Portugal, Cardoso et al. 2012) to 7.6% (Italy, Piantedosi et al. 2017), while in dogs with suspected VBD, seroprevalences from 7.5% (Spain, Miró et al. 2013) to 16.4% (Portugal, Cardoso et al. 2012) have been reported.

Helminthic *D. immitis* antigen is typically detected in very low levels in European countries with reported prevalences of < 1% in France (Pantchev et al. 2009) and Italy (Piantedosi et al. 2017), while higher in Hungary (Farkas et al. 2014) and Romania (Mircean et al. 2012). Exceptions to this include studies from Spain (Miró et al. 2013) and Portugal (Cardoso et al. 2012), where higher prevalences (e.g. > 8%) were reported in clinically suspected dogs over asymptomatic (~ 4.0%) dogs.

B. burgdorferi s.l. is a spirochaete and potential cause of Lyme disease in mammals (Chomel 2015). This VBP has the lowest prevalence rates reported from all European regions with decreasing prevalence from North to South (Finland, Pérez Vera et al. 2014; Italy, Piantedosi et al. 2017; France, Pantchev et al. 2009). In Portugal (Cardoso et al. 2012), the seroprevalence of *B. burgdorferi* s.l. was slightly higher in dogs with clinically suspected VBDs (0.5%) than in asymptomatic dogs (0.2%).

Co-infections of one or more VBPs in European countries are frequent in dogs in areas where canine VBDs are endemic (Otranto et al. 2009). While co-infection prevalence rates of < 1.0% have been described in Romania (Mircean et al. 2012) and Hungary (Farkas et al. 2014), higher prevalences have been reported in some countries in Southern Europe including Italy (2.3%; Piantedosi et al. 2017) and Portugal, the latter including detection of 2% in asymptomatic dogs while clinically suspected dogs had co-infection rates of 11.6% (Cardoso et al. 2012).

In Croatia, molecular methods have confirmed the presence of a range of VBPs in dogs including several *Babesia* species (Beck et al. 2009), *Hepatozoon canis* and *Hepatozoon* sp. (Vojta et al. 2009), and *A. platys* and *A. phagocytophilum* (Huber et al. 2017). Further, antibodies to *L. infantum* (Živičnjak et al. 2005; Mrljak et al. 2017), *E. canis*, *Anaplasma* spp., *B. burgdorferi* s.l. and *D. immitis* antigen (Mrljak et al. 2017) have been confirmed in asymptomatic dogs. To expand our knowledge and potential health impact of VBPs in Croatia, in the current study, we investigated the seroprevalence of VBPs between three groups of dogs (asymptomatic, suspected and deceased dogs) from different Croatian regions.

Materials and methods

Serological investigation for canine VBPs was conducted on blood samples from 1433 dogs collected in the period between 2012 and 2016. Samples originated from three groups of dogs (Table 2). The first group (asymptomatic) included 753 asymptomatic dogs (52.6%, 753/1433). The second group (suspected) was comprised of 617 dogs (43.1%, 617/1433) presented to private veterinary clinics due to clinical signs and/or haematological abnormalities (anaemia, thrombocytopenia, vomiting, anorexia, pale mucous membranes). The third group (deceased) consisted of 63 dogs (4.4%, 63/1433) with suspected canine VBD. The necropsy investigations and sampling of these animals is further described in Huber et al. (2017). Animals in each group could be further subdivided based on their geographic location of origin into continental (783/1433, 54.6%) or coastal regions (650/1433, 45.4%) of Croatia (Table 1). Since the groups of dogs were heterogeneous in their geographic location and/or health status, comparisons were limited to the following: (i) overall prevalence among groups, (ii) prevalence among groups from the continental region of Croatia and (iii) prevalence among asymptomatic groups from both regions.

Blood samples for animals in the asymptomatic and suspected groups were collected by veterinarians during clinical examination by cephalic venipuncture using 3 mL EDTA-anticoagulated vacuum tubes. Blood samples from deceased group dogs were collected from the right ventricle of the heart into an EDTA-containing tube as previously described (Huber et al. 2017).

The presence of *D. immitis* antigen and antibodies against *A. phagocytophilum*/*A. platys*, *B. burgdoferi* s.l. and *E. canis* were determined by use of the commercial point-of-care SNAP®4Dx®Plus (IDEXX Laboratories Inc., Westbrook, ME, USA) as per the manufacturer's instructions.

One-way ANOVA was performed to analyse differences in seroprevalence among detected VBPs and groups of dogs. When ANOVA assumptions were not met, after testing for data normality and homoscedasticity by Shapiro-Wilk W test and Levane's test, the non-parametric Kruskal-Wallis test was used. Statistical analyses were performed using Stata 13.1 (StataCopr. 2016. Stata Statistical Software: Release 13.1, College Station, TX). A level of $p < 0.05$ was considered significant.

Results and discussion

In order to investigate the prevalence and impact of VBPs on the health of dogs in Croatia, we conducted large-scale screening of 1433 dogs divided by health status. This screening revealed an overall prevalence of 6.1% to the range of pathogens tested (*Anaplasma* spp., *E. canis*, *D. immitis*, *B. burgdorferi* s.l.) (88/1433; Table 2). The observed seroprevalence was similar across all three groups of animals with 6.8% of asymptomatic dogs, 5.2% of suspected dogs and 7.9% of deceased dogs revealing positivity to one or more of the pathogens tested (Table 2). The overall seroprevalence is notably lower than the previous investigations of VBPs in asymptomatic dogs in Croatia (Mrljak et al. 2017) where the recorded prevalence was four times higher (25.7%). This may be explained in the previous study where the seroprevalence of the piroplasm *B. canis* (> 20% reported) was also assessed. With the removal of the *B. canis* seroprevalence, Mrljak et al. (2017) study reports an overall prevalence of VBPs in Croatian dogs of ~ 5–8%, which is consistent with the present study.

The canine VBPs most frequently detected in this study were *Anaplasma* spp. (4.5%; Table 2) and at seroprevalence rates similar to that reported in studies of dogs in other European countries (Mircean et al. 2012; Pantchev et al. 2009; Pérez Vera et al. 2014; Piantedosi et al. 2017). In these investigations, the prevalence is usually attributed to *A. phagocytophilum* despite the fact that the SNAP®4Dx test or SNAP®4Dx®Plus cannot distinguish between *A. phagocytophilum* and *A. platys* antibodies (Chandrashekar et al. 2010). Indeed, molecular detection of both pathogens in Croatian dogs suggested a higher prevalence of *A. platys* in different regions of the country (Huber et al. 2017). Surprisingly, the overall prevalence was the highest in the group of asymptomatic dogs (5.4%) than in suspected (3.4%) or in deceased dogs (3.2%) (Table 2), although these differences were not statistically significant. In contrast to results from our study, the prevalence in Portugal (Cardoso et al. 2012) and Spain (Miró et al. 2013) was higher in suspected (9.2%, Portugal; 3.8% Spain) than in asymptomatic dogs (4.5%, Portugal; 2.1% Spain).

The seroprevalence for the remaining VBPs detected in dogs in this study was typically < 1% (Table 2). Antibodies to *E. canis* were present in 0.6% of dogs (asymptomatic and suspected); however, deceased dogs were not seropositive (Table 2). No statistically significant differences could be observed in dogs from different regions of Croatia (Table 3). These results raise further questions over the significance of *E. canis* in Croatia since an even lower seroprevalence (0.5%) was detected in asymptomatic Croatian dogs (Mrljak et al. 2017) and PCRs failed to detect pathogen from 1080 dogs (Huber et al. 2017). Similar low prevalences were reported in asymptomatic dogs from countries where autochthonous cases are still lacking such as Hungary (0.2%, Farkas et al. 2014) and Finland (0.3%, Pérez Vera et al. 2014). Given that differences in the seroprevalence of *E. canis* have been reported between dogs with suspected monocytic ehrlichiosis and asymptomatic dogs in Portugal (Cardoso et al. 2012) and

Table 1 Geographical distribution of dogs in each cohort from the different regions of Croatia

Group of dogs	Continental region	Coastal region
Asymptomatic	146/1433; **10.2%** (95% CI 8.67–11.87)	607/1433; **42.4%** (95% CI 39.78–44.96)
Suspected	576/1433; **40.2%** (95% CI 37.64–42.79)	41/1433; **2.9%** (95% CI 2.06–3.86)
Deceased	61/1433; **4.3%** (95% CI 3.27–5.43)	2/1433; **0.1%** (95% CI 0.01–0.54)
Overall	783/1433; **54.6%** (95% CI 52.02–57.24)	650/1433; **45.4%** (95% CI 42.76–47.98)

Table 2 Summary of the screening results for VBPs detected in this study in Croatian dogs from each of the three cohorts (asymptomatic, suspected and deceased)

Pathogen	Asymptomatic dogs	Suspected dogs	Deceased dogs	Overall
Anaplasma	41/753; **5.4%** (95% CI 3.82–7.06)	21/617; **3.4%** (95% CI 1.97–4.83)	2/63; **3.2%** (95% CI 0–7.63)	64/1433; **4.5%** (95% CI 3.40–5.54)
E. canis	5/753; **0.7%** (95% CI 0.08–1.25)	4/617; **0.6%** (95% CI 0.01–1.28)	0/63; **0.0%**	9/1433; **0.6%** (95% CI 0.22–1.0)
B. burgdorferi	2/753; **0.3%** (95% CI 0.10–0.63)	3/617; **0.5%** (95% CI 0–1.04)	1/63; **1.6%** (95% CI 0–4.76)	6/1433; **0.4%** (95% CI 0.08–0.75)
D. immitis	3/753; **0.4%** (95% CI 0.05–0.85)	4/617; **0.7%** (95% CI 0.01–1.28)	2/63; **3.2%** (95% CI 0–7.63)	9/1433; **0.6%** (95% CI 0.03–1.04)
Overall	51/753; **6.8%** (95% CI 5.00–8.60)	32/617; **5.2%** (95% CI 3.57–7.24)	5/63; **7.9%** (95% CI 2.63–17.56)	88/1433; **6.1%** (95% CI 4.95–7.51)

Spain (Miró et al. 2013), further studies of dogs with suspected monocytic ehrlichiosis in Croatia are needed to resolve this issue.

The seroprevalence of *B. burgdorferi* s.l. in our study was similarly low (0.4%) with no statistically significant differences observed between dogs from either cohort (Table 2) or between geographic region of Croatia (Table 3). The prevalence in asymptomatic dogs was lower than that reported by Mrljak et al. (2017; 0.7%) but similar to that reported in other European countries such as Italy (0.3%, Piantedosi et al. 2017), Hungary (0.4%, Farkas et al. 2014) and Romania (0.5%, Mircean et al. 2012). Previous studies have shown that the dominant genospecies of *B. burgdorferi* s.l. in *Ixodes ricinus* ticks are *B. afzelii*, *B. garinii* and *B. valasiana* (Rijpkema et al. 1996; Tijsse-Klasen et al. 2013). *B. burgdorferi* s.s. is much rarer, however, but is so far the only genospecies found to be pathogenic in dogs after experimental infections (Krupka and Straubinger 2010). While studies have not been performed on the diversity of *B. burgdorferi* s.l. in ticks in Croatia, it is likely that pathogenic species such as *B. burgdorferi* s.s. are likely only found in extremely low levels.

The detection of *D. immitis* nematode prevalence in the deceased dog cohort was the only example where we observed statistically significant differences between dogs in this cohort versus those in the asymptomatic and suspected cohorts ($p = 0.0275$) (Table 2). The overall prevalence for *D. immitis* antigen was 0.6%, with a 0.6% (4/617) and 0.4% (3/753) prevalence found in suspected and asymptomatic dog cohorts, respectively, and in contrast to the prevalence of 3.2% (2/63) in the deceased dog cohort. Interestingly, no *D. immitis* parasites were found in the hearts of dogs in the deceased cohort during necropsy (data not shown). One potential explanation for this disparity may be the reported cross-reactivity of this *D. immitis* antigen assay with other helminths reported in Croatia (Venco et al. 2017). The seroprevalence rate in asymptomatic and deceased dogs is otherwise similar to that reported in other European countries (Pantchev et al. 2009; Mircean et al. 2012; Farkas et al. 2014; Piantedosi et al. 2017). Compared to other Croatian studies, the prevalence for *D. immitis* antigen in asymptomatic dogs (0.4%, Table 2) in this study was similar to the 0.5% previously reported by Mrljak et al. (2017). Higher rates of ~ 5–6% were previously reported in Croatian studies using modified Knott's test and SNAP 4Dx

Table 3 Geographical distribution of vector-borne pathogens in dogs in Croatia

Pathogen	Continental region			Coastal region		
	Asymptomatic	Suspected	Deceased	Asymptomatic	Suspected	Deceased
Anaplasma	9/146; **6.2%** (95% CI 2.86–11.37)	21/576; **3.7%** (95% CI 2.27–5.52)	2/61; **3.3%** (95% CI 0.40–11.34)	32/607; **5.3%** (95% CI 3.63–7.36)	0/41; **0.0%**	0/2; **0.0%**
E. canis	2/14; **1.4%** (95% CI 0.16–4.86)	4/576; **0.7%** (95% CI 0.18–1.77)	0/61; **0.0%**	3/607; **0.5%** (95% CI 0.10–1.44)	0/41; **0.0%**	0/2; **0.0%**
B. burgdorferi	1/146; **0.7%** (95% CI 0.02–3.76)	3/576; **0.5%** (95% CI 0.10–1.51)	1/61; **1.6%** (95% CI 0.04–8.79)	1/607; **0.2%** (95% CI 0.04–0.91)	0/41; **0.0%**	0/2; **0.0%**
D. immitis	2/146; **1.4%** (95% CI 0.16–4.86)	4/576; **0.7%** (95% CI 0.18–1.77)	2/61; **3.3%** (95% CI 0.40–11.34)	1/607; **0.2%** (95% CI 0.04–0.91)	0/41; **0.0%**	0/2; **0.0%**
Overall	14/146; **9.6%** (95% CI 5.80–15.45)	32/576; **5.6%** (95% CI 3.96–7.74)	5/61; **8.2%** (95% CI 2.72–18.10)	37/607; **6.1%** (95% CI 4.33–8.30)	0/41; **0.0%**	0/2; **0.0%**
	51/783; **6.5%** (95% CI 4.89–8.47)			37/650; **5.7%** (95% CI 4.04–7.76)		

test (Jurić et al. 2007; Holler et al. 2010) however. On this basis, the overall prevalence of *D. immitis* detected would appear to be lower than expected.

Serological or antigenic detection of at least two VBPs were found in 0.5% of dogs screened (0.5%, 7/1433, 95% CI 0.24–1.00). In the asymptomatic dog cohort, 4/753 (0.5%; 95% CI 0.21–1.36) were found to be positive for both *Anaplasma* spp. and *E. canis* (continental and coastal region). Two animals (2/617, 0.32%, 95% CI 0.09–1.17) in the suspected cohort from the continental region were found to be positive for *Anaplasma* spp. and either *B. burgdorferi* s.l. or *E. canis*. In the deceased cohort, one animal (1/63, 1.59%, 95% CI 0.28–8.46) was positive for *Anaplasma* spp. and *D. immitis* (continental region). Based on geographical distribution, co-infections were more prevalent in dogs from the continental region (0.6%, 5/783, 95% CI 0.27–1.49) than the coastal region (0.31%, 2/650, 95% CI 0.08–1.11). No differences were statistically significant, however. Co-infections are known to cause more severe clinical signs, resulting in complicating disease outcomes than infections with either agent alone (De Tommasi et al. 2013). In a Portuguese study (Cardoso et al. 2012), a higher prevalence of co-infections was found in animals with clinical signs and/or that are suspected to have VBDs (11.6%) than in asymptomatic dogs (2%). Interestingly, in our survey, co-infections were more frequent in asymptomatic than in suspected and deceased dogs, which further raises a question over the seriousness of co-infections in disease pathogenesis.

To our knowledge, this is the first survey that compares the seroprevalence of VBPs between three groups (asymptomatic, suspected and deceased) of dogs from different regions in Croatia. The results of this study demonstrate that the investigated canine VBPs were more frequent in apparently healthy dogs raising questions about the pathogenic potential of many of these organisms in canine hosts.

Acknowledgements We are grateful for the excellent technical support of Marija Stublić and Kristina Skrbin from the Croatian Veterinary Institute. We also wish to acknowledge company VOXA for their kind provision of assays to support the completion of this project.

Funding The research was financed by the Croatian Scientific Foundation Grant number 1957, acronym GENOTICKTRECK.

Compliance with ethical standards

The authors declare that the research complies with current ethical standards and laws of the country where the study was conducted. This study was approved by the institutional ethics committee of the Faculty of Veterinary Medicine, University of Zagreb (permit numbers 251–61-01/139–15-2) and the ethics committee of the Croatian Veterinary Institute (permit 2544 from 26-05-2014).

Conflict of interest The authors declare that they have no conflict of interest.

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