

Current Surveys of the Seroprevalence of *Borrelia burgdorferi*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Leishmania infantum*, *Babesia canis*, *Angiostrongylus vasorum* and *Dirofilaria immitis* in Dogs in Bulgaria

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Abstract

Canine vector-borne diseases (CVBDs) have increasingly become a focus of interest in recent years. Some of the CVBDs are zoonotic and may therefore also represent a risk for the human population. Different factors are in discussion to explain the expansion of vectors and pathogens into formerly unaffected areas. Knowledge of the prevalence and distribution of CVBDs in Bulgaria is scant overall and most data rely on single case descriptions. The aim of the present study was to determine the seroprevalence of important CVBDs in 167 dogs from central-southern Bulgaria (Stara Zagora), with special emphasis on hitherto

uninvestigated babesiosis and angiostrongylosis, on poorly investigated Lyme borreliosis and canine granulocytic anaplasmosis, and on the potentially zoonotic dirofilariosis and leishmaniosis. Relatively high prevalence rates were documented for anti-*Babesia canis* antibodies, *Dirofilaria immitis* antigen (16.2%; 27/167 each), anti-*Ehrlichia canis* (21%; 35/167) and anti-*Anaplasma phagocytophilum* antibodies (30.5–46.1%; 51–77/167), while *Borrelia burgdorferi* seroprevalence was low (2.4%; 4/167). All samples were negative for *Leishmania infantum* antibodies and *Angiostrongylus vasorum* antigen and antibodies. In total, 64.7% (108/167)

of the samples indicated infection or exposure to at least one agent and a high proportion of dual infections (39.8%; 43/108) was demonstrated. Multiple infections with up to four different organisms were also detected. Our data underline the importance of CVBDs and especially of co-infections which could influence the clinical outcome in dogs.

Introduction

Canine vector-borne diseases (CVBDs) have increasingly become a focus of interest in recent years. Climate change and biotic factors such as an increase in (wild) reservoir abundance, changing habitat structure, socio-political changes and, especially for dogs, increased travelling and dog imports for welfare reasons are discussed in this context as potential factors in the expansion of vectors and pathogens into formerly unaffected areas. Some of the CVBDs are zoonotic and therefore may also represent a serious risk for the human population. Knowledge of the prevalence and distribution of CVBDs in Bulgaria is scant overall and most data rely on case descriptions. Furthermore, data about some agents, such as the protozoan *Babesia* spp. or the metastrongyloid nematode *Angiostrongylus vasorum*, are completely lacking. The situation in this country is even more complicated as stray dogs are still common and are being imported for welfare reasons into other countries (e.g. Germany). Stray dogs, in particular, are not protected by preventive measures against infestation with ticks, mosquitoes, sand flies or against the development of the filarial heartworm *Dirofilaria immitis* and therefore pose a potentially considerable reservoir for vector-transmitted infections. In Bulgaria, canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis* was first reported in 2003 (Tsachev 2006). Subsequently, a seroprevalence of 37.5% in the northern part of the country and 30% in southern Bulgaria was determined (Tsachev et al. 2006a, b). Furthermore, a confirmed clinical case of a co-infection of *E. canis* with *Hepatozoon canis* in a

dog from Stara Zagora was documented (Tsachev et al. 2008a). Thereafter, several cases of CME were reported not only from Bulgaria but also from neighbouring Greece (Tsachev 2009; Tsachev and Petrov, 2009; Tsachev et al., 2013).

In contrast to *E. canis*, there are no published studies on the occurrence and prevalence of canine *Babesia* spp. from Bulgaria. This is surprising because babesiosis is well known in dogs in this country (Tsachev, personal communication). Recent investigation of 295 dogs for ticks revealed an infestations rate of 23.7% for ticks, with *Rhipicephalus sanguineus* and *Ixodes ricinus* found in 19.7% and 6.4% of the dogs respectively, while obviously no *Dermacentor reticulatus* ticks were identified (Kirkova et al. 2013). These data were in part confirmed by another study investigating ticks (n=472) collected from humans (n=32), animals (n=264) and environment (n=176) in nine major districts in Bulgaria (Ivanov et al. 2011). Latter study revealed *R. sanguineus* in 31.4% (148/472) and *I. ricinus* in 25.4% (120/472) of the cases, but also *D. reticulatus* was found, even though at a lower rate (3.6%; 17/472). Unfortunately, latter study provides no information about the exact source of *D. reticulatus* ticks identified. These data point to possible transmission of *Babesia vogeli* in dogs in this country through *R. sanguineus*, but the presence of *B. canis* with *D. reticulatus* as a vector cannot be excluded.

There are only restricted data on CVBDs transmitted by *I. ricinus* in Bulgaria. A single clinical case of canine Lyme borreliosis (LB) has been reported so far (Popova and Kamenov 1998), as has one case of a co-infection with *E. canis* and *Borrelia burgdorferi* (Tsachev et al. 2007a). Subsequent studies demonstrated a low incidence of dogs with compatible clinical signs of LB (0.8%; 4/490), mainly in the south (Tsachev and Dimov 2012). Canine granulocytic anaplasmosis (CAG) caused by *Anaplasma phagocytophilum* which is also transmitted by *Ixodes* spp., was demonstrated for the first time in Bulgaria in 2008 (Tsachev et al. 2008b). Subsequent studies showed its occurrence only

in southern regions (3.5% in Plovdiv and 17.4% in Stara Zagora) and not in northern Bulgaria (Tsachev 2009).

The first cases of dirofilariosis in dogs in Bulgaria were documented in 1997–1999. Microfilariae determined as larval stages of *Dirofilaria immitis* were found in both pet (1.4%) and stray dogs (12.5%; Georgieva et al. 2001). In studies conducted in southern Bulgaria from 2001 to 2007 (n=511 dogs) microfilariae of *D. immitis* were detected in 47 (9.2%) and of *Dirofilaria repens* in 7 (1.4%) of the dogs tested (Kirkova et al. 2008). Some potential mosquito vectors for filarial nematodes were shown to occur in this country (*Anopheles maculipennis*, *Anopheles cinereus*, *Culex pipiens*, *Culex perexiguus*, *Aedimorphus vexans* and *Coquillettidia richardii*; Mikov 2011).

The first confirmed clinical cases of canine visceral leishmaniosis in Bulgaria in 2006 were located on the border with Greece (Tsachev 2009; Tsachev et al. 2010). Sporadic cases were also reported from other southern parts of the country (Tsachev 2009). Potential vectors for *Leishmania* sp. present in Bulgaria are *Phlebotomus balcanicus*, *Ph. perniciosus*, *Ph. sergenti*, *Ph. papatasi* and *Ph. tobbi* (Mikov and Harizanov 2012).

The aim of the present study was to determine the seroprevalence of important CVBDs in dogs in Bulgaria with special emphasis on hitherto uninvestigated *Babesia* spp. and *Angiostrongylus vasorum*, poorly investigated *B. burgdorferi* and *A. phagocytophilum* and the potentially zoonotic *D. immitis* and *L. infantum*.

Material and methods

Samples

All samples (n=167) originated from dogs presented at the small animal clinic of the Faculty of Veterinary Medicine of Trakia University, Stara Zagora, for various clinical reasons only partly related to the infections investigated in the present study. It can be assumed that most dogs originated from

the region of Stara Zagora (central-southern Bulgaria). Information about gender was available for 166 dogs (n=98 female; n=68 male), and the age of all 167 dogs ranged from 1 to 16 years (median 5, mean 5.5 years). There was no information available about prophylactic measures against parasites. All dogs were pet animals (no stray dogs and no dogs with a specific type of use, e.g. hunting dogs). All samples were obtained and examined with the agreement of the owners.

Methods

All samples were examined by means of SNAP® 3Dx® enzyme immunoassay (EIA), *A. phagocytophilum* indirect immunofluorescence assay (IFA), *B. canis* microplate ELISA and *L. infantum* microplate ELISA. In total 150 of the samples (due to a shortage of material from the remaining 17 samples) were examined for the presence of circulating *A. vasorum* antigen and of specific antibodies against *A. vasorum* by validated ELISAs. Three samples (one with very low positive and two with borderline *L. infantum* ELISA test results and concurrent high *B. canis* ELISA results) were additionally investigated by means of *L. infantum* IFA to exclude potential cross-reactions with anti-*B. canis* antibodies, as reported for a similar *L. infantum* ELISA (according to Mettler et al. 2005; Wolf et al. 2014). Twenty-seven samples that reacted positively for anti-*Ehrlichia* antibodies within the SNAP® 3Dx® EIA were additionally examined by means of SNAP® 4Dx® Plus EIA in order to detect potential cross-reactions of anti-*E. canis* antibodies with *A. phagocytophilum* whole-cell antigen (as present on IFA slides), as described by Harrus and Waner (2011). All tests were performed in Ludwigsburg, Germany (IDEXX Laboratories), apart from *A. vasorum* antigen and antibody ELISAs that were performed in Zurich, Switzerland (Institute of Parasitology, University of Zurich). Differences between prevalence rates were analysed for significance using the Chi-square test (differences were regarded as significant at a level of $p < 0.05$; Bland 2000). Data was evaluated via Excel (MS).

***A. phagocytophilum* serology (IFA)**

Serological examinations for antibody detection were performed using *A. phagocytophilum* IFA (Mega-Screen FLUOANAPLASMA ph., cut-off = 1:50, MegaCor, Hoerbranz, Austria). The test uses slides coated with *A. phagocytophilum*-infected cells (see also Dyachenko et al. 2012). The instructions of the manufacturer were slightly modified. Thus, the positive cut-off was set at a clear green fluorescence of the cytoplasmic inclusion bodies (morulae) at dilutions of $\geq 1:200$. Titres of 1:50 and 1:100 were considered borderline. The kit contents were modified by using another FITC-conjugated secondary antibody at a dilution of 1:1600 (fluorescein-labelled affinity purified antibody to dog IgG (H+L) produced in goat, Kirkegaard and Perry Laboratories/KPL) with Evans Blue (2.5 ml, Bio-Rad) as the counter stain, and Biognost® mounting medium (Bios) to cover the wells prior to microscopy. Fluorescence was analysed using an Olympus BX41 microscope equipped with an Osram Mercury Short Arc HOB® lamp (103 W/2).

***Leishmania* serology (ELISA, IFA)**

Serological examinations for antibody detection were performed using a microplate *L. infantum* ELISA (*Leishmania*-ELISA Dog, Afosa GmbH, Dahlewitz, Germany). The reference range of the test score (TS; the test converts the colorimetric reaction through a formula into “test scores”) is negative below 7, borderline at 7–12 and positive above 12 according to Wolf et al. (2014). Test performance and evaluation in terms of test score, sensitivity, specificity as well as positive and negative predictive values as compared to IFA and another microplate ELISA are described by Wolf et al. (2014). *L. infantum* IFA was performed according to Wolf et al. (2014).

***Babesia* serology (ELISA)**

Serological examinations for antibody detection were performed using a microplate *B. canis* ELISA (*Babesia*-ELISA Dog, Afosa GmbH, Dahlewitz, Germany; the reference range of the test score (see

above) is negative < 14, borderline at 14–19 and positive > 19). According to the test manufacturer, the sensitivity and the specificity of the *B. canis* ELISA compared with the indirect immunofluorescence assay (IFA) (671 sera tested using four different IFAs) are 91.6% and 95.4%, respectively. In relation to a “gold standard” IFA validated at a university institute according to strict scientific criteria (287 sera), sensitivity and specificity are as high as 96.3% and 100%. This ELISA is currently the only test authorised by the German Friedrich Loeffler Institute for the detection of *B. canis*-specific antibodies in dogs. However, it shows cross-reactivity with other *Babesia* spp. (such as *B. vogeli* according to Dyachenko et al. 2012) and with *B. gibsoni* and *Rangelia vitalii* (Pantchev et al. 2015). This whole-cell based assay can be therefore rather regarded as piroplasma-specific.

***Angiostrongylus vasorum* serology (antigen and antibody detection; ELISAs)**

The ELISA for antigen detection with a sensitivity of 95.7% and a specificity of 94.4% was performed as previously described (Schnyder et al. 2011). For the detection of specific antibodies a sandwich ELISA using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) was used, with a sensitivity of 81% and a specificity of 98.8% (Schucan et al. 2012). Test cut-offs (optical density at 405 nm/OD₄₀₅: 0.208 for antibodies and 0.352 for antigen) were adjusted on the basis of canine study populations in Germany (Schnyder et al. 2013a) and Poland (Schnyder et al. 2013b).

***E. canis*, *B. burgdorferi*, *D. immitis* (antigen detection) serology (EIA)**

Serological examinations were performed using a rapid test system based on an EIA technique (SNAP® 3Dx®, IDEXX Laboratories, Inc., Westbrook, ME, USA) performed according to the manufacturer's directions. The in-clinic assay uses a proprietary device that provides reversible chromatographic flow of sample and automatic, sequential

flow of wash and enzyme substrate. Positive test results are detected visually as blue-coloured spots. Two spots are impregnated respectively with a specific peptide antigen of *B. burgdorferi* sensu lato/*Bbsl* (C6 peptide derived from the IR6 region within the *Borrelia* membrane protein VlsE; O'Connor et al. 2004) and *E. canis* (peptides from p30 and p30–1 outer membrane proteins; cross-reactive with *Ehrlichia chaffeensis*; Davoust et al. 2006; O'Connor et al. 2006). The *D. immitis* analyte is derived from two antibodies (one for capture and the other for detection) specific to heartworm antigens, which are primarily produced by adult females (Weil 1987; Bowman et al. 2009; Chandrashekar et al. 2010).

Anaplasma serology (EIA)

Serological examination was performed using a rapid test system based on an EIA technique (SNAP[®] 4Dx[®] Plus, IDEXX Laboratories, Inc., Westbrook, ME, USA). Similar to the earlier-generation EIA devices (SNAP[®] 3Dx[®]/4Dx[®]), this new in-clinic EIA detects antibodies against peptides derived from the major immunodominant p30 and p30–1 proteins of *E. canis* and from the VlsE protein-derived C6 peptide of *B. burgdorferi*, but it additionally detects antibodies to *Ehrlichia ewingii* (peptide derived from p28 outer surface protein family) and, compared to the SNAP[®] 3Dx[®]

used in the present study, specific antibodies to *A. phagocytophilum*/*Anaplasma platys* (peptide from the major surface protein p44/MSP2) are also detected. No cross-reaction between *Anaplasma* and *Ehrlichia* at genus level is observed within this EIA device based on utilisation of specific peptides in contrast to the whole-cell antigen present on *A. phagocytophilum* IFA slides (Diniz and Breitschwerdt 2012; Harrus et al. 2012). The SNAP[®] 4Dx[®] Plus test system has been validated for dogs. The sensitivity and specificity were 93.2% and 99.2% for *A. phagocytophilum*, 89.2% and 99.2% for *A. platys*, 96.7% and 98.8% for *Bbsl*, 97.8% and 92.3% for *E. canis*, and 98.9% and 99.3% for *D. immitis*, according to Stillman et al. (2014). This latter study furthermore showed a cross-reactivity of *E. canis* antigens with anti-*E. chaffeensis* antibodies as proposed for the earlier test generation (SNAP[®] 3Dx[®], see above).

Results

Overall positive and borderline results are shown in Table 1. One hundred and eight of 167 examined samples (64.7%; 95% CI: 56.9–71.9%) showed a positive result for at least one agent. The mean age for the positive tested dogs (n=108) was 5.3 years and for the dogs tested negative (n=59) 5.8 years.

Table 1 Occurrence of *Dirofilaria immitis* antigen and seroprevalence of tick-borne infections in dogs from Bulgaria (n=167)

Causative organism	Antigen (Di) or antibodies positive/all tested dogs	Antibodies borderline/all tested dogs	Percentage rate	95 % confidence interval
<i>Ehrlichia canis</i>	35/167	na	21 %	[15.1–27.9 %]
<i>Dirofilaria immitis</i>	27/167	na	16.2 %	[10.9–22.6 %]
<i>Babesia canis</i>	27/167		16.2 %	[10.9–22.6 %]
		7/167	4.2 %	[1.7–8.4 %]
<i>Borrelia burgdorferi</i>	4/167	na	2.4 %	[0.7–6 %]
<i>Anaplasma phagocytophilum</i>	77/167		46.1 %	[38.4–54 %]
		32/167	19.2 %	[13.5–26 %]

na: not applicable; Di: *Dirofilaria immitis*

Table 2 Proportion of single infections (n=56) considering all positive samples (n=108; 51.9%; 95% CI: 42–61.6%)

	Ap (alone)	Bc (alone)	Di (alone)	Ec (alone)
positive samples	31	14	10	1
positive tests (total)	108	108	108	108
percentage	28.7%	13.0%	9.3%	0.9%
95% CI	[20.4–38.2%]	[7.3–20.8%]	[4.5–16.4%]	[0.0–5.1%]

Ap: *Anaplasma phagocytophilum*, Bc: *Babesia canis*, Di: *Dirofilaria immitis*, Ec: *Ehrlichia canis*

Table 3 Proportion of dual infections (n=43) considering all positives samples (n=108; 39.8%; 95% CI: 30.5–49.7%)

	Ap + Ec	Ap + Di	Ap + Bc	Bc + Di	Bb + Ec	Ec + Di
positive samples	25	8	5	2	2	1
positive tests (total)	108	108	108	108	108	108
percentage	23.1%	7.4%	4.6%	1.9%	1.9%	0.9%
95% CI	[15.6–32.2%]	[3.3–14.1%]	[1.5–10.5%]	[0.2–6.5%]	[0.2–6.5%]	[0.0–5.1%]

Ap: *Anaplasma phagocytophilum*, Bc: *Babesia canis*, Bb: *Borrelia burgdorferi sensu lato*, Di: *Dirofilaria immitis*, Ec: *Ehrlichia canis*

Table 4 Proportion of triple and multiple infections (n=9) considering all positives samples (n=108; 8.3%; 95% CI: 3.9–15.2%)

	Ap + Ec + Di	Ap + Bc + Di	Ap + Bc + Ec	Bb + Ec + Bc	Bb + Ec + Ap + Bc
positive samples	3	3	1	1	1
positive tests (total)	108	108	108	108	108
percentage	2.8%	2.8%	0.9%	0.9%	0.9%
95% CI	[0.6–7.9%]	[0.6–7.9%]	0.0–5.1%	0.0–5.1%	0.0–5.1%

Ap: *Anaplasma phagocytophilum*, Bc: *Babesia canis*, Bb: *Borrelia burgdorferi sensu lato*, Di: *Dirofilaria immitis*, Ec: *Ehrlichia canis*

The female to male ratio in the whole population was 1.4 (98 females and 68 males), while the female to male ratio among positive and negative tested dogs was 1.8 (69 females and 39 males) and 1 (females and males each of 29), respectively ($p=0.083$).

The proportions of single, dual, triple and multiple infections are shown in Tables 2–4. Slightly more single than dual infections were encountered, but the difference was not statistically significant ($p=0.076$). However, significantly more single or dual infections than triple or multiple infections were found ($p<0.001$). Nine of 108 positive samples showed a multiple infection with three or four agents (Table 4).

Positive ($n=27$; 16.2%; 95% CI: 10.9–22.6%) and borderline ($n=7$; 4.2%; 95% CI: 1.7–8.4%) results for *B. canis* serology based on ELISA TS are shown in Table 5.

All samples tested by means of *L. infantum* ELISA were negative, apart from two borderline samples (TS of 9.1 and 11.2) and one positive (TS of 12.6) result. These three results were interpreted as cross-reactions based on high anti-*B. canis* antibody levels (samples 10, 11 and 114; Table 5), and on *L. infantum* IFA, since all three samples were negative in this alternative confirmatory test (anti-body titre < 1:50).

Table 5 *Babesia* spp. positive and borderline dogs based on test score results of the ELISA applied in the present study

sample number dog	TS* of positive samples	sample number dog	TS** borderline samples
3	42	6	15.3
8	26.2	12	17.5
10	60.9	43	17.5
11	54.9	52	15.9
27	23.8	78	14.3
31	62.8	145	18.4
37	23.3	147	17.9
40	24.2		
41	23.2		
45	27.4		
50	27.6		
57	26.4		
85	19.6		
92	19.6		
103	28.1		
108	19.4		
114	197.8		
115	56.9		
117	24.3		
133	23.3		
139	23.2		
141	38.3		
152	24.1		
155	19.7		
160	31.2		
161	21.8		
162	25.4		

* range of positive TS: 19.4–197.8; mean TS of positive samples 36.8; median TS of positive samples 25.4

** range of borderline TS: 14.3–18.4; mean TS of borderline samples 16.6; median TS of borderline samples 17

All 150 samples tested for the detection of *A. vasorum* circulating antigen by ELISA were negative (0%, 95% CI: 0.0–2.0%), while a single sample showed a reaction slightly above the cut-off (OD₄₀₅ of 0.211) in the ELISA for antibody detection (1/150; 0.7%; 95% CI: 0.0–3.7%).

The antibody titre of all *A. phagocytophilum*-positive samples (IFA, n=77) was compared to the antibody titre of *Anaplasma*-positive, *Ehrlichia*-negative samples (n=47; Table 6) in order to evaluate potential cross-reactions with anti-*E. canis*

antibodies. The *A. phagocytophilum* antibody titre of all *Ehrlichia* positive samples (n=35) was also analysed (Table 7). Thirty out of 35 *Ehrlichia* positive samples were indeed *A. phagocytophilum*-positive (IFA), in contrast to only five samples with a negative or borderline *A. phagocytophilum* antibody titre (highly significant difference; p < 0.001). Furthermore, the number of samples with an *A. phagocytophilum* antibody titre ≥ 1:3200 (22/35; Table 7) was significantly higher compared to all other titres (13/35; p < 0.031). Additionally, there

Table 6 Antibody titre (IFA) of all *Anaplasma phagocytophilum* positive samples in relation to *A. phagocytophilum* positive but *Ehrlichia canis* negative samples

<i>Anaplasma</i> positive (total n = 77; 46.1 %)		<i>Anaplasma</i> positive, <i>Ehrlichia</i> negative (n = 47*)	
antibody titre	number of samples	number of samples	significance (p < 0.05)
1:200	10	8	0.616
1:400	16	13	0.536
1:800	7	7	1
1:1600	12	9	0.481
1:3200	18	9	0.057
>1:3200	14	1	< 0.001

* 28.1 % (95 % CI: 21.5–35.6 %)

Table 7 *A. phagocytophilum* antibody titre (IFA) of all *E. canis* positive samples (n = 35)

<i>Anaplasma</i> titre	<i>E. canis</i> positive samples (n)
<1:50 (negative)	3
1:50 (borderline)	1
1:100 (borderline)	1
1:200	2
1:400	3
1:1600	3
1:3200	9
>1:3200	13

were 30 *A. phagocytophilum*-borderline samples (18 %; 95 % CI: 12.5–24.6 %; n = 17 at 1:50 and n = 13 at 1:100) with a concurrent *Ehrlichia*-negative result, in contrast to only two *A. phagocytophilum*-borderline samples with an *Ehrlichia*-positive result (1.2 %; 95 % CI: 0.1–4.3 %). This difference was statistically highly significant (p < 0.001).

Twenty-seven of 35 *Ehrlichia*-positive samples were additionally examined by means of SNAP® 4Dx® Plus EIA. Of these, 25 had a positive *A. phagocytophilum* antibody titre (one sample was negative and one had a borderline result of 1:50), but only three of them (antibody titres of 1:1600, 1:3200 and >1:3200) were confirmed positive by the specific peptide-based assay (12 %; 95 % CI: 2.5–31.2 %).

Discussion

High prevalence rates were documented for anti-*B. canis* antibodies, *D. immitis* antigen (16.2 % each), anti-*E. canis* (21 %) and anti-*A. phagocytophilum* antibodies (46.1 %), while *B. burgdorferi* seroprevalence was low (2.4 %). The high proportion of positive samples for at least one agent (108 of 167 tested samples; 64.7 %; 95 % CI: 56.9–71.9 %) and for dual infections (39.8 %; 43/108) underlines the importance of CVBDs in pet dogs in central-southern Bulgaria. Even multiple infections with up to four different organisms were detected.

The high *E. canis* seroprevalence confirmed the results of previous studies from that country (Tsachev et al. 2006a, b). Although co-infections with *E. canis* and *H. canis* have also been reported from Bulgaria (Tsachev et al. 2008a), it was not possible to establish data for dual infection of this type in the present study as no serological assay was available for *H. canis*.

The mean age of positive tested dogs in the present study (n = 108; 5.3 years) was comparable to those found for positive dogs in other studies on CVBDs (*Anaplasma* spp., *E. canis*, *B. burgdorferi*, *D. immitis*), for example 6.3 years (Volgina et al. 2013) or 5 years (Villeneuve et al. 2011). While in the study of Volgina et al. (2013) the age of negative tested dogs was lower (2.6 years), this was not observed for the present study (n = 59; 5.8 years).

In another study from Spain, even higher seropositivity for *E. canis* and *B. burgdorferi* was observed in younger dogs (<1 year) compared to adult dogs, while no differences in the rates recorded for *Anaplasma* spp., *D. immitis* or *L. infantum* were identified (Miró et al. 2013). Overall, different studies also failed to find any association between gender and CVBDs analysed (e.g. Villeneuve et al. 2011; Miró et al. 2013).

A. phagocytophilum was the most common single infection encountered, followed by *B. canis* and *D. immitis*. The most common dual infection was *A. phagocytophilum/E. canis* (n=25), which was significantly more prevalent than the next most frequent combination of *A. phagocytophilum/D. immitis* (n=8; p=0.001). A more in-depth discussion of this commonest co-infection is therefore considered advisable. There are various possible explanations for this result. It can be hypothesized that this represents infection by or exposure to *A. platys* and not *A. phagocytophilum*, because from the serological point of view both are cross-reactive, regardless of the test used (whole cell- or peptide-based; Gaunt et al. 2010; Dyachenko et al. 2012). In fact, a potential co-infection with *A. platys* and *E. canis* is even more probable considering that they use the same tick vector (*R. sanguineus*), and that, based on previous studies, this latter tick appears to be a common ectoparasite of dogs in Bulgaria (Kirkova et al. 2013). However, there are so far no documented reports of *A. platys* occurring in Bulgaria, and no EDTA blood was available for species-specific PCR tests or PCR with subsequent sequencing for reliable molecular differentiation in the present study, as described previously by Dyachenko et al. (2012). Another possible explanation for the above-mentioned common co-infection is a serological cross-reaction of *E. canis*-infected dogs with the *A. phagocytophilum* analyte on IFA slides. An assumed disadvantage of *A. phagocytophilum* whole cell-based IFA compared to tests utilising specific peptides (e.g. SNAP® 4Dx®, Chandrashekar et al. 2010) is indeed a possible cross-reactivity with anti-*E. canis* antibodies. The strength

of this cross-reactivity increases with the duration of the *E. canis* infection and the anti-*E. canis* antibody titre (Harrus et al. 2012). For example, in dogs infected experimentally with *E. canis*, no cross-reactivity with *A. phagocytophilum* antigens (IFA) was observed in the acute phase. However, cross-reactive antibodies were first detected on day 55 post infection (p.i.), were then found in all dogs by day 150 p.i. and even increased in two dogs until 283 days p.i. (Waner et al. 1998; Harrus and Waner 2011). This could be due to variation of antibody patterns during *E. canis* infection, with initial production of antibodies against low molecular weight proteins and subsequently, during the chronic phase of infection, against larger proteins above 36 kDa (the latter representing cross-reactive common antigens between the two species). Waner et al. (1998) proposed furthermore that the appearance of IgG antibodies against *A. phagocytophilum* antigens after *E. canis* infection may even represent an indicator of long-term persistence of infection and replication of *E. canis* in the canine host. In the present study, a set (n=27) of *E. canis*-positive samples was tested using the latest generation of the above-mentioned peptide-based assay (SNAP® 4Dx® Plus) that shows no cross-reactivity between the genera *Anaplasma* and *Ehrlichia*. Out of 25 *E. canis*-positive samples with an *A. phagocytophilum* IFA titre above the test cut-off, only three confirmed positive within the specific peptide-based assay. It was also evident that especially samples with a high anti-*Anaplasma* antibody titre ($\geq 1:3200$) were *Ehrlichia*-positive, indicating samples with very high *Ehrlichia* antibody levels and/or long-term, chronic *E. canis* infections. It is therefore difficult to predict the real seroprevalence of *A. phagocytophilum* with the method (IFA) used in the present study. A possible way of estimating it would be to consider only the *Anaplasma* IFA-positive but *Ehrlichia*-negative samples (n=47); furthermore to assume that only 12% (i.e. n=4) of *Anaplasma/Ehrlichia* co-infected samples are true positives (proportion of samples confirmed by peptide-based EIA). With this approach, the estimated *Anaplasma* seroprevalence

would be 30.5% (51/176; 95% CI: 23.7–38.1%). At any rate, if the borderline results are added to this (30 of 32 were indeed *Ehrlichia*-negative), then again a comparable high *Anaplasma* seroprevalence of 48.5% (95% CI: 40.7–56.3%) would be achieved. Nevertheless, at least three samples (*Ehrlichia*-positive in SNAP® 3Dx® and additionally *Anaplasma*-positive in SNAP® 4Dx® Plus) can be regarded as a co-infection or at least co-exposure to both organisms. Interestingly, in experimental canine models (*A. platys* and *E. canis*) it was shown that subsequent acute *E. canis* infection can trigger the antibody response of a previous *Anaplasma* infection (Gaunt et al. 2010).

Surprisingly, all four *Borrelia*-positive samples were detected as mixed infections with at least *E. canis*. This indicates another intriguing co-infection that points on the one hand to exposure of dogs to at least two different tick vectors, *I. ricinus* and *R. sanguineus*. On the other hand, it is known that mixed infection with Lyme borreliae and rickettsias in the form of *A. phagocytophilum* increases the potential of a dog to develop clinical signs, in contrast to infections with a single agent (Beall et al. 2008). Nevertheless, *Borrelia* seroprevalence in dogs in Bulgaria is relatively low compared with Germany (9.7%; Krupka et al. 2007) but comparable with Poland, for example, (3.8%; Krämer et al. 2014) and even higher than in France (1.1%; Pantchev et al. 2009), all results obtained with a comparable C6-based EIA.

A relatively high infection rate was found for *D. immitis* antigen detection compared with previous investigations (Geogieva et al. 2001; Kirkova et al. 2008). One reason could be that previous studies relied mainly on microfilariae detection, and it is well known that up to 70% of heartworm infections can be occult and are only detected by antigen methods (Pantchev et al. 2011). Mixed infection with *D. immitis* and *D. repens* as described by Pantchev et al. (2011) for dogs exported from Bulgaria to Germany could not be detected in the present study as no microfilariae-based tests (e.g. Knott's, filtration or PCR tests) were performed. To

date, no serological test is available for *D. repens*, and the heartworm antigen test used in the present study does not react with *D. repens* (Pantchev et al. 2011). Moreover, cross-reaction as shown for some *D. immitis* antigen tests with sera of *A. vasorum* infected dogs (Schnyder and Deplazes 2012) could be excluded. In the present study, no *A. vasorum* circulating antigen was detected. All positive reactions for *D. immitis* antigen in the present study can therefore be regarded as specific serological reactions. A single dog was *A. vasorum* antibody-positive, which could be interpreted as a false positive reaction. In areas with expected low prevalence like Bulgaria, both *A. vasorum* ELISAs should be performed because seropositivity in both tests has a considerable high positive predictive value (Schnyder et al. 2013a). These results do not necessarily exclude the presence of *A. vasorum* in Bulgaria (no *A. vasorum* cases were reported so far), as the sampling number was restricted (indicated also by the large confidence intervals) and as the dogs originated from a restricted area around Stara Zagora, which is located in the central-southern part of the country. Considering the large number of recent reports of *A. vasorum* in several European countries, including Poland (Schnyder et al. 2013b) or northern Greece (Papazahariadou et al. 2007), bordering to Bulgaria, the detection of cases of *A. vasorum* has actually to be expected.

The situation with *L. infantum* was similar. One low positive sample and two borderline results were interpreted as cross-reactions with *Babesia* as shown, for example, by Mettler et al. (2005) as these samples also had high anti-*Babesia* antibody levels. *Leishmania* IFA for these three samples was negative, a constellation reported to occur with comparable ELISA and IFA tests (Mettler et al. 2005). This corroborates previous data of a seroepidemiological study (using IFA) that was carried out with 220 healthy pet dogs from 11 different Bulgarian regions (5 in the south, including 20 samples from Stara Zagora and 20 samples from the Plovdiv area, and 6 northern regions; Tsachev et al. 2007b). All samples in this latter study also tested negative for

Leishmania antibodies. The results of these studies contrast with human data from Bulgaria where, despite a low annual incidence of 0.06 per 100,000, most cases of *Leishmania* were reported from the southern part of the country, including the area of Stara Zagora (Harizanov et al. 2013). As high percentages of *D. immitis* infections in dogs were detected in the present study, the application of repellents with efficacy against insects, particularly mosquitoes and sand flies, seems not to be the reason for the negative *Leishmania* results. One explanation could be that *P. papatasi* and *P. sergenti*, which are the main sand fly species in Bulgaria, have a higher tropism towards humans than towards pet dogs, in contrast to *P. neglectus*, which is the main sand fly species transmitting *L. infantum* encountered in Greece (Léger et al. 1988; Garifallou et al. 1989; Killick-Kendrick and Killick-Kendrick 1999; Tsachev et al. 2007b). Additionally, many asymptomatic dogs in endemic areas mount predominantly cellular immunity with a negative to low specific antibody titre and may therefore be missed by serological methods (Baneth et al. 2008). For example, of 73 clinically healthy hunting dogs in neighbouring Greece, 12.3% tested positive by serology, whereas 63% tested positive by PCR (Leontides et al. 2002). The latter authors concluded that most of the dogs residing in areas where leishmaniasis is endemic become infected but often remain seronegative. Serological screening of the general canine population in these areas may therefore result in an underestimation of the true prevalence.

Data in the present study represent the first serological evidence of canine babesiosis in Bulgaria. It can be concluded that exposure to *Babesia* spp. seems to be common in dogs in Bulgaria. The most probable species involved in canine infections in this country is *B. vogeli* due to suitable vector availability (*R. sanguineus*) and its common finding on dogs, though, the presence of *B. canis* cannot be excluded. Further studies are necessary (as in Croatia, for example; Beck et al. 2009) to reliably differentiate serologically cross-reactive *Babesia* species by means of species-specific real time PCRs or PCRs

with subsequent sequencing or RFLP analysis in order to exactly determine the species involved. In regard to *B. gibsoni*, there is also speculation about non-vector-borne direct dog-to-dog transmission via bite wounds, saliva or ingested blood (fighting dogs; Irwin 2009), but so far no *B. gibsoni* has been described in Bulgaria.

There is one limitation to this serological survey as a positive antibody result is not necessarily equivalent to the existence of the pathogen in the canine or vector population of a particular geographic region; it is only evidence of prior exposure to the corresponding pathogen at some point and some location in the dog's history. With respect to the latter limitation, a more differentiated view needs to be taken of the detection of antibodies against C6 peptide of *B. burgdorferi*. The SNAP[®] 4Dx[®] Plus test detected infections with *Borrelia* at the earliest on day 35 or day 49 p.i., depending on the individual dog (Wagner et al. 2012). On the other hand, antibodies to C6 have been detected in the late stages of infection (>12 months) with a C6 detecting device (Levy et al. 2008; Wagner et al. 2012) and have been found to decrease significantly after specific treatment, so that at least for *Borrelia* the detection of C6 peptide might represent a more or less robust marker of infection.

In conclusion, this study represents an overview of the prevalence of important canine, but also zoonotic, pathogens in a canine population in Bulgaria. Our data underline the importance of CVBDs and especially co-infections which could influence the clinical outcome in dogs. Veterinarians in this country should be aware that these major canine vector-borne pathogens may occur in their practice area. Therefore, clinicians should include these diseases in their differential diagnosis and recommend the use of repellents along with prophylactic measures to prevent disease transmission by arthropod vectors.

Ethical standards

All investigations comply with the current laws of the countries in which they were performed.

Competing interests

N. Pantchev and M. Globokar are employed by IDEXX Laboratories. There is no commercial con-

flict of interest as the information generated here is solely for scientific dissemination. The authors declare that they have no competing interests.

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