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Recent study on canine vector-borne zoonoses in southern Slovakia – serologic survey

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Abstract

Over the last decade a significant spread of Canine Vector Borne Diseases has been recorded in Central Europe. The aim of the study described here, was to collect current data on the occurrence and distribution of three major canine vector-borne pathogens in the veterinary clinical practice by a newly-developed commercial ELISA test for the detection of *Dirofilaria immitis* antigen as well as specific circulating antibodies to *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato. Circulating *D. immitis* antigen was detected in five of 180 investigated sera samples. Two of *D. immitis* seropositive dogs revealed also microfilariae of *D. repens* in the blood and three of them were negative for the presence of microfilariae in the Knott´s test. From the practical point of view, the finding of *D. immitis* occult infections might influence existing knowledge about distribution of this species among dogs in Central European countries. In 11.7% of the tested dogs the presence of specific antibodies against *A. phagocytophilum* was confirmed. Antibodies against *B. burgdorferi* s.l. were detected in 2.8% of tested sera samples. Co-infection with *A. phagocytophilum* and *B. burgdorferi* s.l. was observed in two dogs from Košice district in south-eastern Slovakia. Our data point toward the presence of Canine Vector Borne Diseases in the studied area. Therefore, veterinarians should include these diseases in their differential diagnosis and higher awareness should be focused also on prophylactic measures to prevent the pathogens transmission by arthropod vectors.

Keywords

Canine Vector Borne Diseases, *Dirofilaria immitis*, Lyme borreliosis, canine granulocytic anaplasmosis, dogs, Slovakia

Introduction

Over the last decade a significant spread of Canine Vector Borne Diseases (CVBD) has been recorded in Europe. Many of them are zoonotic in nature and pose a serious risk for humans. Epidemiologists consider the expansion of climate change to be the main factor that significantly affects species composition, abundance and biology of flora and fauna, including vectors, and also extension of transmission season (Semenza and Menne 2009). Due to these changes, many of non-endemic diseases have spread rapidly and occur nowadays in Central Europe, including Slovakia (Genchi *et al*. 2011; Semenza and Zeller 2014). Other factors affecting the vector populations, their ecology and ethology are deliberate and uncontrolled human intervention in the environment such as landscape deforestation, urbanization and agriculture. An indispensable role in the spread of infections and vectors plays the increasing volume of domestic and international trade, tourism development, improved farming and traveling with animals.

Probably the most attention is in recent years devoted to the spread of mosquito-borne filarial infections represented mainly by species *Dirofilaria immitis* and *Dirofilaria repens*. In Slovakia, *D. repens* in a dog has been recorded for the first time in 2005, and following large scale epidemiological investigation revealed the existence of high-endemic areas in the southern regions, with the prevalence of the parasite in dogs reaching about 30% (Miterpáková *et al*. 2008, 2010; Iglódyová *et al.* 2012). The dominance of *D. repens* in the territory of Central Europe is also confirmed by studies in neighbouring countries, whereas autochthonous diseases caused by *D. immitis* are reported rather sporadically. The cases of heartworm disease were unequivocally diagnosed only in dogs in Hungary (Fók *et al*. 2007; Jacsó *et al*. 2009; Zittra *et al*. 2015) and Ukraine (Hamel *et al.* 2013), while in the Czech Republic and Poland only cases of *D. immitis* diagnosed by serolog-

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ical approaches without confirmation of the presence of microfilariae microscopically or by molecular methods were published (Svobodová *et al.* 2006; Świątalska and Demiaszkiewicz 2012). To date, no cases of autochthonous heartworm diseases in dogs were recorded in Austria. Regarding Slovakia, examination of more than 3,600 dogs revealed the presence of *D. immitis* in the blood of only 10 individuals, 9 times in mixed infection with *D. repens* and solely once as mono-infection, however the origin of infection is not unambiguously confirmed. All of the autochthonous cases of heartworm disease were diagnosed in dogs coming from regions of southern Slovakia (Iglódyová and Miterpáková 2014).

Besides mosquitoes, ticks represent another group of vectors involved in the spread of epidemiologically important pathogens of dogs and humans. *Ixodes ricinus* is the most abundant tick in the central Europe (Anderson 1991; Mihalca and Sándor 2013) and contributes to the spread of canine (and also human) granulocytic anaplasmosis (CAG) and Lyme borreliosis (LB). The agents of both infections were detected in all the countries of Central Europe, either in definitive hosts, tick-vectors or humans. In Slovakia, *A. phagocytophilum* was detected in *I. ricinus* and several species of domestic and wild animals (Štefanidesová *et al*. 2008; Víchová *et al*. 2014a). Only one case of human granulocytic anaplasmosis has been confirmed in Slovakia until now (Nováková *et al*. 2010). However, human LB has been registered in Slovakia since 1985 with the incidence 13.6/100,000 (Švihrová *et al*. 2011). The information about epidemiological situation in dogs is scarce. The only data have been published by Štefančíková *et al*. (2008), with discussing the issues of serological diagnosis of LB in animals. Latter study utilized whole-cell based tests with potential cross-reactions with vaccination (especially in dogs) and other spirochetes (e.g. *Leptospira* in cattle, horses and dogs).

The aim of the study described here was to collect current data on the occurrence and distribution of three major canine vector-borne pathogens in the veterinary clinical practice by a newly-developed commercial ELISA test (SNAP® 4Dx® Plus, IDEXX Laboratories, Inc., Westbrook, ME, USA) for the detection of *Dirofilaria immitis* antigen as well as specific circulating antibodies to *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato (*Bb*sl).

Materials and Methods

Study area

Slovakia is located in geographical centre of Europe and covers an area of 49,036 km^2. It is situated in the moderate Atlantic-continental climatic zone with four clearly distinguished seasons (Landscape Atlas of the Slovak Republic 2002).

The present study was carried out in two geographically outlying regions: south-western Slovakia bordering Austria and Hungary in the south; and south-eastern Slovakia bounded to Ukraine in the east and Hungary in the south (Fig.1). These two southern regions are the warmest parts of Slovakia with mean annual temperature over 10°C and 50–70 summer days per year (Landscape Atlas of the Slovak Republic 2002). Both localities under study are predominantly characterised by agricultural landscape. Two largest Slovak lowlands, Danubian Lowland and Eastern Slovak Lowland, with the important rivers Danube (in the south-west) and Bodrog (in the south-east) are situated in the study areas. Profound rainstorms, periodic floods in the river systems and associated recurring mosquito plague are typical for these regions.

Samples collection and testing

Both, the blood and sera samples of 180 dogs living in southern regions of Slovakia were collected in cooperation with veterinary practitioners. 116 dogs originated in south-western Slovakia and 64 animals came from south-eastern part of the country. Veterinarians were asked to choose dogs randomly; the samples were taken from clinically healthy animals and also from some dogs showing various clinical signs. Out of 180 dogs examined, complete anamnestic data from 117 individuals were acquired. In 64 animals no clinical signs were listed and 53 dogs showed various health problems. In most of them (18 patients) lethargy and lack of appetite was observed. In 10 dogs pyrexia was recorded and 10 patients suffered from lameness or joint pain. Otitis externa was diagnosed in 7 dogs and also dermatitis in 7 patients. The keepers of 6 dogs reported different gastrointestinal problems. 4 dogs suffered from keratokonjunctivitis sicca (KCS) and 3 dogs from rhinitis.

From medical records of 117 patients also deworming history was obtained. Most frequently (in 47 dogs) broad spectrum antihelminthics containing febantel (or fenbendazole), pyrantel and praziquantel were used. Oxantel/pyrantel/praziquantel combination was administered to 14 dogs. Antihelminthic preparation containing praziquantel and pyrantel was used in 12 individuals; combination of praziquantel/fenbendazole was applied in 7 cases and emodepside/praziquantel formula in 2 cases. One-component preparations of fenbendazole, resp. pyrantel were administered to 2 dogs. Only in 6 dogs prophylactic heartworm medications were applied: to 3 of them preparation containing imidacloprid/moxidectin was administered, in 2 cases selamectin and in 1 case combination of milbemycin oxime and praziquantel was used. None of dogs' keepers listed using special prevention against ectoparasites or mosquito repellents.

Citrate-treated blood samples were examined for dirofilariosis using modified Knott´s test (Knott 1939): 1 ml of blood and 9 ml of formaldehyde were mixed and centrifuged at 1,500 rpm at 5 min. The supernatant was decanted and sediment stained with a few drops of 0.5% methylene blue was examined under the microscope at 400x magnification for the presence of microfilariae.

Microfilariae positive blood samples were processed for molecular determination of filarial species. Briefly, DNA was extracted from 200 µl of blood using DNeasy Blood and Tis-

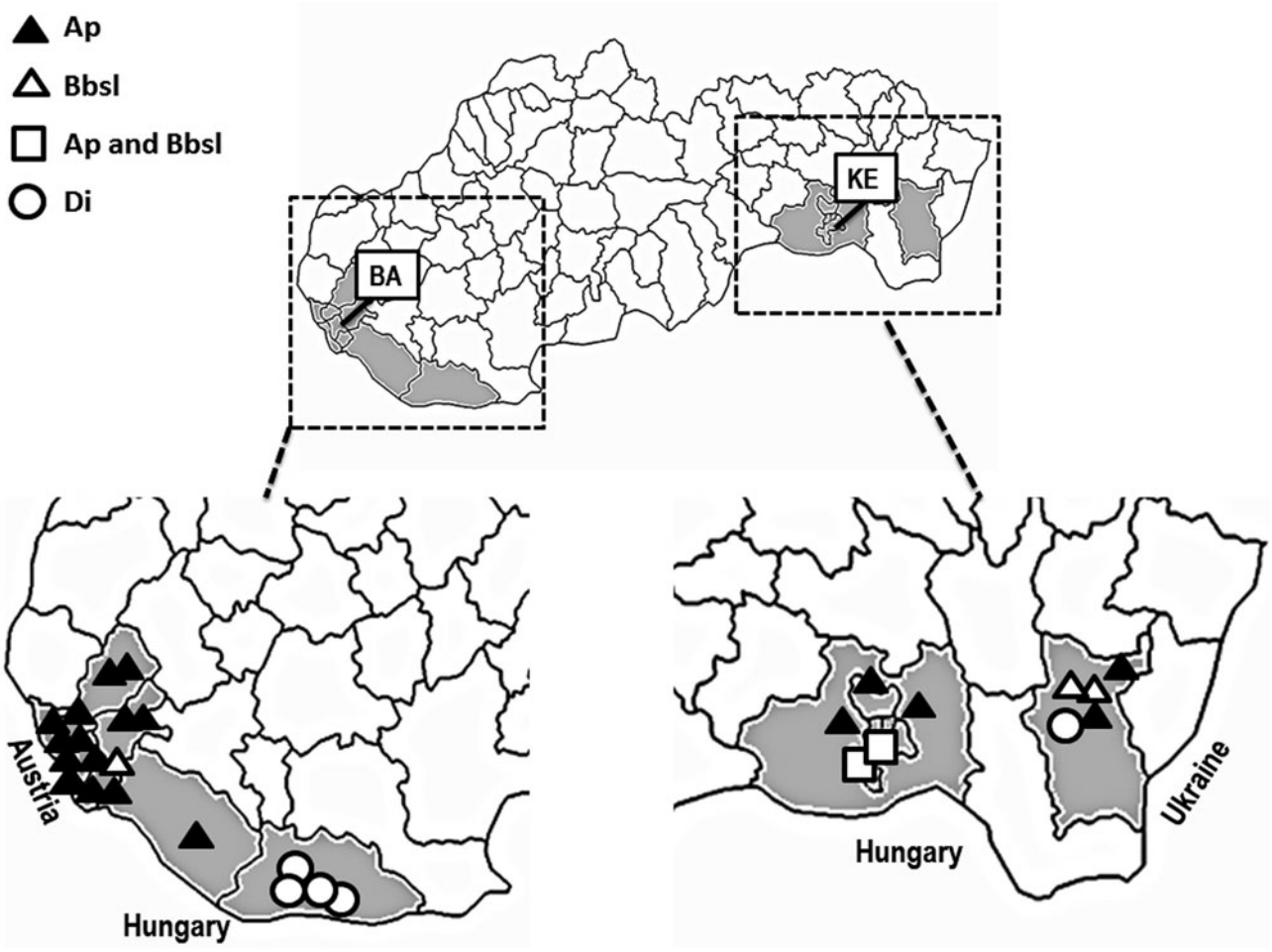

Fig. 1. Geographic distribution of dogs tested seropositive for *Anaplasma phagocytophylum* (Ap), *Borrelia burgdorferi* sensu lato (Bbsl) and *Dirofilaria immitis* (Di) (BA – Bratislava, KE – Košice)

sue Kit (Qiagen, Germany). Each DNA sample was tested using PCR approach which amplifies a 203-bp fragment of the *cytochrome c oxidase* subunit 1 (COI) gene of *D. immitis*, a 208-bp portion of the COI gene of *Acanthocheilonema reconditum* and also 209-bp portion of *D. repens* COI gene according to Rishniw *et al*. (2006). Previously positive samples, confirmed by sequencing, were used as positive controls in each PCR assay. Nuclease-free water was used as negative control.

All collected sera were tested using a newly-developed rapid test system based on enzyme immunoassay technique (SNAP® 4Dx® Plus, IDEXX Laboratories, Inc., Westbrook, ME, USA) following 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-colored spots. The test has been validated for dogs (Stillman *et al.* 2014) and is registered for the detection of *D. immitis* antigen (analyse is derived from monoclonal and polyclonal antibodies specific to heartworm antigens, which are primarily produced by adult females), and specific antibodies to synthetic peptides of *A. phagocytophilum*/*Anaplasma platys* (peptide from the major surface protein p44/MSP2), *Bb*sl (VlsE derived C6 peptide), *Ehrlichia canis* (peptides from p30 and p30-1 outer membrane proteins), and *Ehrlichia ewingii* (peptide derived from p28 outer surface protein family) in canine serum, plasma or anticoagulated whole blood. According to Stillman *et al*. (2014), the sensitivity and specificity of the performed test were 93.2% and 99.2% for *A. phagocytophilum*, 89.2% and 99.2% for *A. platys*, 96.7% and 98.8% for *Bb*sl, 97.8% and 92.3% for *E. canis*, as well as 98.9% and 99.3% for *D. immitis*, respectively. Latter study showed furthermore a cross-reactivity of *E. canis* antigens with anti–*E. chaffeensis* antibodies. Although some heartworm antigen tests can cross-react with *Angiostrongylus vasorum*, this was not observed with the SNAP® 4Dx® Plus (Schnyder and Deplazes 2012).

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).

Table I. Dogs' sera from southern Slovakia (n = 180) tested for the presence of circulating *Dirofilaria immitis* antigen (Di) and of specific antibodies against *Borrelia burgdorferi* s.l. (Bbsl) and *Anaplasma phagocytophylum* (Ap)

Region (number of examined)	Di		Bbsl alone		Ap alone		Bbsl and Ap		Ap total		Bbsl total	
	positive	%	positive	%	positive	%	positive	%	positive	%	positive	%
south-eastern Slovakia (n = 64)	1	1.6	2	3.1	5	7.8	2	3.1	7	10.9	4	6.3
south-western Slovakia (n = 116)	4	3.4	1	0.8	14	12.1	0	0	14	12.1	1	0.9
Total (n = 180)	**5**	**2.8**	**3**	**1.7**	**19**	**10.6**	**2**	**1.1**	**21**	**11.7**	**5**	**2.8**

Results

Filarial infections in sampled dogs

Using Knott´s test, microfilariae were observed in blood of 12 out of 180 examined dogs that represents the overall prevalence of 6.7% (95% confidence intervals (CI): 3.5–11.4).

DNA analyses confirmed *D. repens* in all samples. Five (4.3%; 95% CI: 1.4–9.8) out the microfilariaemic animals came from south-western part (n = 116) and seven (10.9%; 95% CI: 4.5–21.2) from south-eastern part of Slovakia (n = 64), though this difference was not statistically significant (p = 0.088). Neither *D. immitis* nor *A. reconditum* infections were found in examined dogs by DNA analyses (Table I).

Using rapid test system, circulating *D. immitis* antigen was detected in five (2.8%; 95% CI: 0.9–6.4) of investigated sera samples (Table I, II). One of these positive dogs came from eastern Slovakia (Michalovce district) (1.6%; 95% CI: 0.2–8.4) and the remaining four animals originated in Komárno district in south-western part of the country bordering Hungary (3.4%; 95% CI: 1.0–8.6). This difference was not statistically significant (p = 0.461). Two of *D. immitis* seropositive dogs revealed also microfilariae of *D. repens* in the blood, three of them were negative for the presence of microfilariae in the Knott´s test (Table II). *D. immitis* seropositive dogs showed no clinical signs.

Seropositivity of tick-borne pathogens

The sero-prevalence of tick-borne patogens is shown in Table I. None of the dogs was serologically positive for *E. canis*. In 21 of the tested dogs the presence of specific antibodies against *Anaplasma phagocytophilum* was confirmed (mean positivity 11.7%; 95% CI: 7.4–17.3). 14 (12.1%; 95% CI: 6.8–19.4) of the seropositive dogs were from south-western region and 7 (10.9%; 95% CI: 4.5–21.2) from south-eastern Slovakia (Table I). This difference was not statistically significant (p = 0.821).

Antibodies against *Borrellia burgdorferi* s.l. were detected in 5 (2.8%; 95% CI: 0.9–6.4) sera samples: in four (6.3%; 95% CI: 1.7–15.2) animals living in south-eastern sampling area and one dog (0.9%; 95% CI: 0.1–4.7) from Bratislava city in the south-west (Table I). This difference was statistically significant (p = 0.035).

Co-infection with *A. phagocytophilum* and *B. burgdorferi* s.l. was observed in two dogs from Košice district in south-eastern Slovakia.

Anamnestic data of dogs seropositive for CAG and LB are listed in Table III. In 5 from 21 *Anaplasma* – seropositive dogs no health problems have been noticed. In other dogs with CAG veterinarians and owners described mostly general symptoms as fever, lethargy and lack of appetite. In 3 dogs rhinitis was recognized and another 3 animals showed lameness. Haematuria, cystitis, renal insufficiency and gastroenteritis were observed in one *Anaplasma* – seropositive dog which suffered also from babesiosis due to *Babesia canis* diagnosed as described by Víchová *et al*. (2014b). KCS, polyarthritis, and spondylosis, respectively, were diagnosed in 2 dogs in which co-infection with *A. phagocytophilum* and *Bb*sl was confirmed. In other 3 *Borrelia* – seropositive dogs fever, lethargy and vasculitis were recognized, and for one dog with a co-infection of *Borrelia* and *B. cani*s also rhinitis and otitis externa were described.

Discussion

Heartworm disease in dogs in Slovakia

The study provides the first comprehensive survey of CVBD – pathogens in Slovakia using serological approaches.

Above all, the detection of *D. immitis* (heartworm) antigen should be discussed separately, as this allows a new prospect into the distribution of filarial infections in Slovakia.

Microfilariae were observed in blood of 12 dogs examined using Knott´s test. In all microfilaraemic animals the presence of *D. repens* DNA was confirmed by PCR using species-specific primers (Rishniw *et al*. 2006) for three filarial parasites widespread in Europe: *A. reconditum*, *D. repens* and *D. immitis.*

Two of these microfilaraemic dogs were positive also for the presence of *D. immitis* antigen while PCR confirmed only *D. repens*. Other three samples were positive only for *D. immitis* antigen but negative for the presence of microfilariae by Knott´s test (Table II).

The reason for this might be a small number of *D. immitis* microfilariae in the peripheral blood not detectable by PCR method used in the study. E.g., Pantchev *et al*. (2011) state detection limit being 5 microfilariae per 1 ml blood and Latrofa *et al*. (2012) 26 microfilariae per 1 ml of blood. The possibility of mixed *D. repens* and occult *D. immitis* infection (infection with adult female in absence of circulating microfilariae) should be also taken into account. In general, heartworm antigen tests accurately detect the antigens in serum from dogs infected with *D. immitis* if one or more mature female heartworms (6.5 – 8.5 months old) are present, but do not detect infections in the incubation time (under 5 months), and rarely if only males and/or immature and/or dead worms are present due to the lower amount of circulating antigens (Weil *et al*. 1984; Weil 1987; Courtney and Zeng 2001; Nelson *et al*. 2005; Stillman *et al*. 2014). Genchi *et al*. (1995) hypothesized that *D. repens* may act as potential inhibitor of *D. immitis* microfilariae production, based on a protective cross-immunity at individual level, as inferred by the experimental infection of dogs initially infected with *D. repens*, in which the ability of *D. immitis* to develop was reduced. Though, further investigations on this particular topic are needed. Furthermore, in the case of occult infection also drug-induced sterility of adult *D. immitis* should be taken into consideration (Rawlings *et al*. 1982). In addition, with regard to periodicity of microfilarieamia the blood should be collected in appropriate time (Pantchev *et al*. 2011). The fluctuation of microfilariae during the day shows remarkable geographical difference. For example, in the case of experimental infection with *D. immitis* in dogs in England with a Chinese strain Webber and Hawking (1955) found the highest number of microfilariae in blood of dogs at 6 p.m. and Grieve and Lauria (1983) in the USA between noon and 4 p.m. On the other hand, the maximum count of microfilariae in peripheral circulation of naturally infected dogs in Tanzania (Eastern Afrika) was found at 11 a.m. (Matola 1991), in Australia at 4 p.m. (Angus 1981) and in Korea at 9 p.m. (Rhee *et al*. 1998).

Much less is known about periodicity of *D. repens*. Webber and Hawking (1955) indicate that after an experimental infection with a Sardinian strain the highest number of microfilariae occurs in peripheral blood between 10 p.m. and 3 a.m. and the minimum at noon. In previous epidemiological study we have observed periodicity of *D. repens* microfilariaemia in two naturally infected dogs from Košice region, eastern Slovakia (not published yet) and our results partially correspond to those of Webber and Hawking (1955) from England. Both dogs showed the same microfilariae fluctuation pattern within 24 hour period. In the both cases significantly higher microfilariaemia was registered between midnight and 6 a.m. with the maximum count of microfilariae at 4 a.m. By contrast, minimum number of microfilariae was observed in peripheral blood between noon and 4 p.m. On the contrary, Di Cesare *et al*.

Table II. Dirofilarial infections diagnosed in 15 of 180 tested dogs´ blood and sera samples by three various diagnostic approaches

Sample	*Dog's residence	Detection of microfilariae by KNOTT test	Results of DNA analysis by PCR	** Detection of circulating *Dirofilaria immitis* antigen by rapid test system	Final diagnosis
1	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
2	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
3	SE	+	*Dirofilaria repens*	+	*Dirofilaria repens* *Dirofilaria immitis*
4	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
5	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
6	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
7	SE	+	*Dirofilaria repens*	–	*Dirofilaria repens*
8	SW	+	*Dirofilaria repens*	+	*Dirofilaria repens* *Dirofilaria immitis*
9	SW	–	–	+	*Dirofilaria immitis*
10	SW	–	–	+	*Dirofilaria immitis*
11	SW	–	–	+	*Dirofilaria immitis*
12	SW	+	*Dirofilaria repens*	–	*Dirofilaria repens*
13	SW	+	*Dirofilaria repens*	–	*Dirofilaria repens*
14	SW	+	*Dirofilaria repens*	–	*Dirofilaria repens*
15	SW	+	*Dirofilaria repens*	–	*Dirofilaria repens*

* SE: south-eastern Slovakia, SW: south-western Slovakia
** SNAP 4Dx Plus; IDEXX Laboratories, Inc., Westbrook, ME, USA

(2013) observed in *D. repens* naturally infested dog from Abruzzo region (Central Italy) the tendency of higher mean microfilariaemia between noon and 8 p.m. in comparison to the other sampling times. Relatively great differences between observations set above could be related to the wide variety of biotic factors inclusive activity and feeding behaviours of mosquito species occurred in different regions.

All above mentioned adverts to *D. immitis* occult infections can occur in dogs fairly often and absent (or low) microfilariaemia may thus lead to false negative results. Also Pantchev *et al*. (2011) found out that only 38 out of 127 *D. immitis* antigen – positive dogs imported to the Germany were microfilariaemic and therefore up to 70.0% of cases might represent an occult infection. A potential high percentage of occult *D. immitis* infections were reported also by Rawling *et al*. (1982), Grieve *at al*. (1986) and Labarthe *et al*. (1997).

This finding might influence existing knowledge about distribution of *D. immitis* species among dogs in Europe including Slovakia. The most studies and monitoring are based exclusively on testing for microfilariae presence and the species confirmation by molecular methods and not also on heartworm antigen detection. That probably has led to the underestimation of *D. immitis* prevalence in several European countries. Indeed, the opposite condition can also occur, as reviewed by Pantchev *et al*. (2011), when screening is only based on heartworm antigen assays. Concretely, in Slovakia within the 8 year's survey we have found, based on microfilariae detection techniques, only 10 dogs with heartworm disease – in 9 cases mixed infection with *D. repens* and in one dog only *D. immitis* was confirmed by PCR (Iglódyová and Miterpáková 2014). Herein presented 5 heartworm antigen – positive animals represent additional cases. All dogs with confirmed *D. immitis* infection came from southern Slovakia: two of them are living in Košice region, south-eastern Slovakia and 13 in south-western part of the country with cumulating in Komárno district lying on Slovak-Hungarian border. These results may refer to potentially higher risk of heartworm disease for dogs in southern Slovakia as it has been assumed. From epidemiological standpoint it is important, that high numbers of dogs from Bratislava and Košice shelters are exported abroad, especially to Germany and Austria (personal information from managers of the shelters) and the lack of appropriate diagnostic approaches can markedly contribute to the spread of the parasite into non-endemic areas.

From a practical point of view, to minimize risk of false negative results related to the occurrence of occult heartworm infections, all dogs particularly in endemic regions should be screened at once by mean of microfilariae concentration tests, molecular methods for species differentiation and a serological test for heartworm antigen presence. On the other hand, even though occult *D. immitis* infections may seriously threaten health of dogs and veterinarians should take this in the account, animals with microfilariaemia represent the main risk for the disease spreading. However, in endemic areas much greater notice should be focused also on prophylactic measures seeing that according to our knowledge (and also anamnestic data of dogs examined in this study) mosquito repellents and products for chemoprophylaxis to prevent *Dirofilaria* infections are used really seldom in Slovakia.

Anaplasma phagocytophylum and *Borrellia burgdorferi* s.l. infections in Slovakia

Seropositivity to *Bb*sl was overall low, but significantly higher in the south-eastern sampling area, including also two dogs co-infected with *A. phagocytophilum*. The lower seropositivity rate in the present study compared to previous investigations (e.g. 35% seropositivity recorded by Štefančíková *et al*. 2008) can be most probably explained by the nature of the applied test. The advantage of *Borrelia* C6-based tests over the whole-cell based methods used in the above mentioned study (e.g. IgG-ELISA) is the absent cross-reaction with vaccine-induced antibodies (e.g. O'Connor *et al*. 2004) or with antibodies to other spirochaetes such as *Leptospira* (Liang *et al*. 2000). Thus, C6 represents a diagnostic approach that facilitates DIVA (Differentiation of Infected from Vaccinated Animals). Whole-cell based assays can cross-react with other spirochetes as shown e.g. for *Leptospira* species (Štefančíková *et al*. 2008) or with vaccination (e.g. Gauthier and Mansfield 1999; Straubinger *et al*. 2002). Anti-C6 antibodies also represent an early marker of infection from 21 to 35 days p.i. (Wagner *et al*. 2012) and persist for at least 12 months in untreated dogs (Levy *et al*. 2008). After treatment, anti-C6 antibody concentration drops within 3 to 6 months – a decrease to more than 58.3% was seen after 6 months in animals with initial concentration over 29 U/ml (Levy *et al*. 2008), whereas the values in whole-cell based tests do not fall to the same extent (Straubinger 2000; Straubinger *et al*. 2000; Littman 2013). Additionally, Goldstein *et al*. (2007) showed a 93% correlation between a commercially available C6 test and an immunoblot test with regard to the diagnosis of natural infected dogs. Thus the last two criteria (antibody persistence and decrease in concentration after treatment) also emphasize that C6 can be viewed as a marker of active infection.

The seroprevalence of *Borrelia* and *Anaplasma* in the present study was comparable to the overall country-wide prevalence of a recently published large scale study from Poland (Krämer *et al*. 2014) and lower compared to overall data from Germany (Krupka *et al*. 2007), both performed with a similar test. Nevertheless both latter studies found significant regional differences of the seroprevalence. There are some limitations in regard to detected antibodies to tick-borne pathogens in the present study. Dogs testing positive in a specific area may have been exposed elsewhere. Furthermore, a positive antibody test is not always an equivalent to the existence of the pathogen in the canine 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. Anyway, *Bb*sl seropositivity of 6.3% found in dogs from south-eastern Slovakia correlates with infection rate of

Table III. Anamnestic data of dogs seropositive for canine granulocytic anaplasmosis (CAG) and Lyme borreliosis (LB)

Sample no.	*Dog's residence	Age (year)	Gender	Kept	**Serological result	***Co-infection	Clinical symptoms
1	Bratislava (SW)	4	female	indoors	CAG	–	–
2	Bratislava (SW)	2	female	outdoors	CAG	–	fever, lethargy, lack of appetite
3	Pezinok (SW)	3	female	outdoors	CAG	–	–
4	Pezinok (SW)	3	male	indoors	CAG	–	epileptic seizures
5	Michalovce (SE)	2	male	outdoors	CAG	*B. canis* *D. repens*	lethargy, rhinitis
6	Michalovce (SE)	4	male	outdoors	LB	*B. canis* *D. repens*	fever, lack of appetite, rhinitis, otitis externa
7	Michalovce (SE)	5	male	outdoors	LB	*B. canis*	vasculitis
8	Bratislava (SW)	2	female	indoors	CAG	–	–
9	Bratislava (SW)	9	female	outdoors	CAG	*B. canis*	lack of appetite, haematuria, cystitis, renal insufficiency, gastroenteritis
10	Bratislava (SW)	5	male	outdoors	CAG	–	–
11	Bratislava (SW)	9	female	outdoors	CAG	–	lameness
12	Bratislava (SW)	3	male	outdoors	CAG	–	fever, lethargy
13	Bratislava (SW)	9	female	outdoors	CAG	–	fever, lethargy
14	Bratislava (SW)	7	male	outdoors	LB	–	fever, lethargy
15	Dunajská Streda (SW)	1	male	outdoors	CAG	–	–
16	Senec (SW)	2	male	indoors	CAG	–	lameness, panosteitis
17	Bratislava (SW)	2	female	indoors	CAG	–	dermatophytosis, demodicosis
18	Bratislava (SW)	5	male	indoors	CAG	–	fever, lethargy, rhinitis, lack of appetite
19	Košice (SE)	3	male	indoors	CAG	–	lethargy
20	Košice (SE)	7	male	outdoors	CAG, LB	–	KCS, polyartritis
21	Košice (SE)	6	male	outdoors	CAG, LB	–	KCS, spondylosis
22	Košice (SE)	13	female	outdoors	CAG	–	lethargy, vomitus
23	Košice (SE)	11	female	outdoors	CAG	–	lethargy, vomitus
24	Košice (SE)	8	male	outdoors	CAG	–	lethargy, diarrhoea, lameness

* SE: south-eastern Slovakia, SW: south-western Slovakia;
** SNAP 4Dx Plus; IDEXX Laboratories, Inc., Westbrook, ME, USA;
*** confirmed by DNA Analysis

12.22% detected in *Ixodes ricinus* ticks sampled in Košice area. RFLP analysis of the positive samples revealed the presence of *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* sensu stricto and one case of mixed infection with *B. garinii* and *B. valaisiana* (Pangrácová *et al*. 2013). The incidence of human LB cases in Košice region during the last 5 years moves within the range of 6 to 12/100,000 (EPIS, www.epis.sk).

Difference in geographical distribution of CAG was not significant; seropositivity among dogs in south-eastern Slovakia reached 10.9%, in south-western part of the country 12.1% of tested dogs were positive for specific antibodies. Previous studies based on means of molecular analyses showed 3.27% prevalence of CAG in *D. repens* infected dogs (Víchová *et al*. 2014b). On the other hand, only one of 137 dogs suspected or having non-specific febrile disease was CAG positive (Víchová *et al*. 2014a). The screenings of *I. ricinus* ticks revealed the overall prevalence of *A. phagocytophilum* in eastern Slovakia between 1.4% and more than 5.5% (Pangrácová *et al*. 2013; Víchová *et al*. 2014a); in northern part of the country 7.8% of collected ticks were infected (Derdáková *et al*. 2011).

The anamnestic data provided for dogs with CAG and LB suspicion (Table III) are in principle in line with those published in a literature (e.g. Littman *et al*. 2006; Carrade *et al*. 2009; Scorpio *et al*. 2011; Wagner *et al*. 2012), but is should be noted, that in some dogs, a co-infection with other pathogens might have had contributed to the clinical picture (e.g. *Babesia canis*). Co-infections of *A. phagocytophilum* and *Bb*sl are of special interest as observed in two dogs in the present study showing clinical signs. It is essential to take co-infections with *Borrelia* into consideration in the diagnostic workup of CAG. Studies show that dogs co-infected with *A. phagocytophilum* and *Borrelia* run twice the risk of developing disease with symptoms as lameness, fever, lethargy, joint pain (swelling) and anorexia, than following a single infection with either pathogen (Beall *et al*. 2008). Concurrent presence of an intracellular (*Anaplasma*) and extracellular (*Borrelia*) infection may lead to an adverse immunological interaction during the infection course (Krupka *et al*. 2007).

Nevertheless, our data point toward the presence of *Borrelia* and *Anaplasma* in diseased dogs in the studied area. Therefore, veterinarians should include these two diseases in their differential diagnosis and recommend the use of repellents along with other prophylactic measures to prevent disease transmission by arthropod vectors.

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