Retrospective Analysis of Canine Vector-borne Diseases (CVBD) in Germany with Emphasis on the Endemicity and Risk Factors of Leishmaniosis

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Abstract

A cross-sectional study was designed to provide data on the detection of canine vector-borne diseases (CVBDs) with special emphasis on leishmaniosis in Germany. For this purpose, results of blood or serum samples sent by local veterinarians to a veterinary diagnostic laboratory were retrospectively analysed. Samples were examined for *Leishmania* spp., *Babesia* spp., *Ehrlichia canis*, *Dirofilaria immitis* and *Anaplasma phagocytophilum* during the years 2004–2006 and 2014–2016 (*Leishmania* only). Erythrocyte stages of large *Babesia* spp. or *Babesia* DNA were found in 1.7 % of 9,966 blood smears and 3.3 % of 15,555 samples examined by PCR, respectively. Large *Babesia*

merozoites were found more frequently in Giemsa-stained smears from dogs born in Germany when compared to blood samples of dogs originating from south or south-east European countries. A total of 15 blood samples of German dogs which have never been abroad were positive for *Babesia* DNA. Antibodies titres (≥ 80) against *Babesia canis* were detected by IFAT in 11.5 % of 2,653 serum samples. Out of 570 samples 3.2 % were positive for *E. canis* using PCR. Antibodies against *E. canis* and *A. phagocytophilum* (both at titres ≥ 50) were detected by indirect IFAT in 15.1 % and 41.9 % of 18,652 and 794 serum samples, respectively. Using Knott's test 4.5 % of 440 blood samples were positive for microfilariae, and *Dirofilaria immitis* antigen was found by ELISA in 1.4 % of

9,381 serum samples. *Leishmania* spp. DNA was detected in 11 % of 301 whole blood or tissue samples examined by PCR. Antibodies against *Leishmania* were found in 23.5 % (23,665 samples) and 22.7 % (54,103 samples) of blood samples by IFAT (titres ≥ 50) and ELISA (≥ 7 test units), respectively (2004–2006 versus 2014–2016). Antibodies against *Leishmania* (IFAT) were detected in 80.6 % (399/495) of dogs imported from endemic areas, in 57.6 % (34/59) of German dogs travelling outside Germany and in 4 (n=8) German dogs without any history of travelling. Potential endemicity of leishmaniosis in Southern Germany was prospectively evaluated. For some of these infectious agents, sex or age of dogs and season were identified as risk factors.

Introduction

Dogs are in close contact with humans and other animals and are exposed to a wide range of pathogens. They can act as a reservoir of pathogens of zoonotic and veterinary importance (Otranto et al. 2009b). Canine vector-borne diseases (CVBDs) are caused by parasites, bacteria or viruses transmitted by the bite of hematophagous arthropods (mainly ticks and mosquitoes). The occurrence of some vector-borne diseases of dogs and cats is increasing in Europe, i.e., canine leishmaniosis and filariasis (Otranto et al. 2009a, Pantchev et al. 2011, Wolf et al. 2014) or babesiosis (Pantchev et al. 2015a). CVBDs show an impact on animal and human health and a spread to non-endemic areas (Hirsch and Pantchev 2008). Some changes, such as travelling with pets, changes in human habitats, social and hobby activities, but also climate changes have a direct impact on arthropod vectors (abundance, geographical distribution and vectorial capacity) as well as development of vector-borne diseases within the vectors (Gothe 1991, Gothe et al. 1997). Arthropod-borne diseases are gaining importance in Germany as emerging endemic parasites and have been increasingly diagnosed in veterinary

practices. This is reflected by a high number of dogs examined in this survey. The objective of this study was to characterize the occurrence of vector-borne pathogens in native German dogs (without history of travelling), in German dogs travelling to potentially endemic areas and dogs from endemic areas imported to Germany with special emphasis on endemicity and risk factors for leishmaniosis.

Material and methods

The retrospective study involved results of examinations of 30,970 and 54,103 canine blood or serum samples, routinely submitted by German veterinarians to a veterinary diagnostic laboratory (IDEXX Laboratories, Ludwigsburg) from 2004 to 2006 and from 2014 to 2016, respectively. These samples had been tested for *Babesia* spp., *E. canis*, *A. phagocytophilum*, and *D. immitis* (in 2004–2006 only) and *Leishmania* spp. (in both examination periods). The samples had not been randomly collected because a large proportion of them had been submitted either as a part of a “travel disease profile” or with clinical suspicion. Information on the age, gender, origin or travel history of the dogs were available for a part of the samples. Additionally, in 2004–2006, telephone interviews with the respective veterinarians were performed for dogs being *Babesia*-positive using blood smears or PCR, being *Leishmania*-positive using PCR or having *Leishmania* antibodies (titer ≥ 200) as well as being positive for *D. immitis* antigen to receive data on the place of birth and residence as well as travelling of these dogs. Furthermore, 45 dogs were prospectively tested by *Leishmania* PCR of conjunctival swabs (left and right eyes were sampled), presented 2007 at two veterinary clinics in Southern Germany (Urbach and Schwanstetten) for various reasons.

Giemsa-stained blood smears (n=9,966) were performed as usual and examined for *Babesia* spp. stages.

Whole blood samples (n=440) were examined for microfilariae using a modified Knott's test according to Pantchev et al. (2009a, 2011). Serum samples (n=9,381) were tested for circulated *D. immitis* antigen by a commercial rapid assay test system (IDEXX SNAP[®] canine heartworm[®]) following the manufacturer's instructions (see also Pantchev et al. (2009b)).

For molecular diagnosis of *Babesia* spp. (15,155 samples) and *E. canis* (570 samples), DNA was extracted from whole blood samples according to Dyachenko et al. (2012), and a conventional PCR was performed according to Carret et al. (1999), targeting the SSU rDNA gene (*Babesia* spp.) and to McBride et al. (1996), targeting the 16S ribosomal RNA gene (*E. canis*). For molecular diagnosis of *Leishmania* spp., DNA was extracted from different samples (lymph nodes, skin, spleen, liver, nasal discharge, joint puncture, urine, bone marrow or whole blood; n=301), and a conventional PCR targeting the SSU rDNA was implemented according to van Eys et al. (1992). In presumably autochthonous *Leishmania*-positive samples (n=2) a second conventional PCR targeting the kinetoplast DNA (kDNA) minicircles was performed as described by Le Fichoux et al. (1999) to confirm the results. The latter PCR was also implemented to prospectively test dogs (n=45) by means of ocular swabs. In the present study no determination on species level for *Babesia* and *Leishmania* was performed.

The presence of IgG antibodies against *Babesia* spp. had been tested in 2,653 serum samples by a commercial indirect IFAT (MegaScreen FLUOBABESIA, Diagnostik MegaCor GmbH, Hörbranz, Austria) following the manufacturer's instructions. The test cutoff was set at dilutions of $\geq 1:80$. Titre of 80 was considered low (borderline) and titres ≥ 160 positive.

For IgG antibodies against *E. canis*, 18,652 samples had been examined by a commercial indirect IFAT (MegaScreen FLUOEHRlichia canis, Diagnostik MegaCor GmbH, Hörbranz, Austria).

The test cutoff was set at dilutions of $\geq 1:50$. Titres of 50 and 100 were considered low (borderline) and titres ≥ 200 positive. As a modification with regard to kit contents, another FITC-conjugated secondary antibody was used at a dilution of 1:1000 (Fluorescein-labeled affinity purified antibody to dog IgG produced in goat from Kirkegaard & Perry Laboratories), with Evans Blue (2.5 ml, Bio-Rad) as counter stain and Biognost[®] mounting medium (Bios) to cover the wells prior to microscopy.

The presence of IgG antibodies against *A. phagocytophilum* had been tested in 794 samples by a commercial indirect IFAT (MegaScreen FLUOAN-APLASMA phagocytophilum, Diagnostik MegaCor GmbH, Hörbranz, Austria) according to Pantchev et al. (2015b). The test cutoff was set at dilutions of $\geq 1:50$. Titres of 50 and 100 were considered low (borderline) and titres ≥ 200 positive.

In 2004–2006, 23,665 serum samples had been tested for the presence of IgG antibodies against *Leishmania* spp. using a commercial indirect IFAT (MegaScreen[®] FLUOLEISH, Diagnostik MegaCor GmbH, Hörbranz, Austria) according to Wolf et al. (2014). The test cutoff was set at dilutions of $\geq 1:50$. Titres of 50 and 100 were considered low (borderline) and titres ≥ 200 positive. In 2014–2016, 54,103 samples were examined using an ELISA (*Leishmania*-ELISA-Dog, Afosa, Luckenwalde, Germany) according to the manufacturer's instructions (see also Wolf et al. 2014). This ELISA converts the colorimetric data into test units classed in the following test ranges: negative (<7), borderline (7–12), and positive (>12).

The statistical analysis was performed using the BMDP/Dynamic, Release 7.0 (Dixon 1993). The observed prevalence and 95% confidence intervals (CI) were calculated for each pathogen. Results were analysed using chi-square test. To identify risk factors for parasite infestation, a multivariable logistic regression approach was used. Differences with P values <0.05 were considered as significant. Statistical analysis of serological examinations included low (borderline) and positive titres.

Results

Leishmania

Leishmania spp. DNA was detected in 11 % (CI: 7.7–15.1 %; n=33) of 301 samples examined by PCR. Regarding the sample type, *Leishmania* DNA was detected in 16 out of 245 blood samples (6.5 %; CI: 3.8–10.4 %) and 10 out of 15 (66.7 %; CI: 38.4–88.2 %) skin scrapings. All four joint punctures, both blood smears as well as one of two tested bone marrow samples were also tested positive. The origins of positively tested dogs in the retrospective analysis were as follows: 13 dogs were imported (nine originated from Spain/Portugal, one each from Italy and Greece and two from other countries), two PCR positive dogs had a travel history to Spain/Portugal. Risk factors were not identified by multivariate logistic regression. All 90 conjunctival swabs (from 45 dogs), examined during the prospective study at two sites in Southern Germany, revealed negative results.

In 2004–2006, antibodies against *Leishmania* were found in 23.5 % (CI: 23.0–24.1 %; n=5,572) of the samples using IFAT. Positive titres were detected in 10.5 % (CI: 10.1–10.9 %; n=2,477) of the samples; additional 13.1 % (CI: 12.7–13.5 %; n=3,095) of the sera showed low (borderline) results (Table 1). On multivariate logistic regression, the

prevalence of *Leishmania* antibodies was higher in older (>12 months) than in younger (<12 months) dogs ($p < 0.001$). Male dogs were significantly more often seropositive than female dogs ($p < 0.05$). From 67 dogs born in Germany, 59 travelled outside the country (34 dogs were seropositive and 25 were seronegative for *Leishmania*) and 8 dogs have never left Germany (Table 2). One of those eight dogs had a low (borderline) titre, and another three showed positive titres of 200 (n=2) and 400 (n=1). All three dogs originated from the southern part of Germany, two from Baden Württemberg (Urbach and Welzheim) and one from Franconia (Schwanstetten near Nürnberg). Furthermore, in two of those dogs with positive titres and no travel history (from Urbach and Schwanstetten), *Leishmania* DNA was detected.

In 2014–2016, antibodies against *L. infantum* were detected by ELISA in 22.7 % (CI: 22.4–23.1 %; n=12,320) of the samples. Positive results (>12 test units) were detected in 18.6 % (CI: 18.3–18.9 %; n=10,056) of the sera, while another 4.2 % (CI: 4.0–4.4 %; n=2,264) of the samples showed borderline results. The proportion of *Leishmania* seropositive samples was significantly higher ($p < 0.001$) in 2014–2016 (18.6 %) than in 2004–2006 (10.5 %) using ELISA and IFAT, respectively. However, both examination periods did not differ significantly

Table 1 IFAT results for antibodies against *L. infantum* in 23,665 canine serum samples from 2004–2006

	Titre	%	95 % –CI ¹
negative	< 50	76.5	75.9–77.0
low (borderline)	50	9.6	9.2–10.0
	100	3.5	3.3–3.7
positive	200	2.3	2.1–2.5
	400	0.9	0.7–1.0
	800	2.2	2.0–2.4
	1,600	0.5	0.5–0.7
	3,200	3.3	3.1–3.5
	> 3,200	1.2	1.1–1.4

¹95 % confidence interval

Table 2 Relation between origin/travelling of dogs and detection of *L. infantum* antibodies (n=562)

group	travelling (A) or origin (B)	n	% seropositive
A) dogs from Germany (totally) no travelling travelling (totally)		67	56.7
		8	50.0
		59	57.6
	Spain/Portugal	15	73.3
	Italy	4	100.0
	Greece ¹	10	70.0
	Hungary	7	0
	Southern Europe	4	50.0
	other countries	13	46.1
	unknown	6	66.6
B) dogs not from Germany (totally)		495	80.6
	Spain/Portugal	240	84.6
	Italy	52	92.3
	Greece ¹	75	81.3
	Turkey	4	75.0
	Hungary	12	33.3
	Southern Europe	30	70.0
	other countries	23	47.8
	unknown	59	81.3

¹ incl. former Yugoslavia**Table 3** Relation between origin/travelling of dogs and detection of *Babesia* spp. (by Giemsa-stained blood smear, n=165 and PCR, n=332)

		Giemsa-stained blood smear		PCR	
group	travelling (A) or origin (B)	n	% sample positive	n	% sample positive
A) dogs from Germany (totally) no travelling travelling (totally)		40	52.2	75	76.0
		8	87.5	15	100.0
		32	43.8	60	70.0
	Spain/Portugal	8	12.5	13	46.1
	Italy	1	0	2	50.0
	Greece ¹	4	50.0	6	50.0
	Hungary	6	83.3	12	100.0
	Southern Europe	2	50.0	3	66.7
	other countries	10	50.0	17	88.2
	unknown	1	0	7	42.8
B) dogs not from Germany (totally)		125	9.6	257	42.4
	Spain/Portugal	57	1.7	114	38.6
	Italy	8	12.5	22	27.3
	Greece ¹	19	0	35	37.1
	Turkey	0	0	1	0
	Hungary	4	25.0	9	77.8
	Southern Europe	9	0	19	52.6
	other countries	13	53.8	21	81.0
	unknown	15	13.3	36	33.3

¹ incl. former Yugoslavia

when borderline results were considered as positive (22.7% vs. 23.5%).

Babesia

Erythrocyte stages of large *Babesia* spp. were detected in 1.7% (CI=1.5–2.0%; n=170) of the Giemsa-stained blood smears. Considering the multivariable logistic regression, male dogs were more often infected with *Babesia* spp. than bitches ($p<0.05$), and positive blood smears were more often found in spring and autumn than in winter ($p<0.001$). Additionally, *Babesia* stages were more frequently detected in samples of dogs born in Germany than in those originating from other countries. *Babesia* DNA was detected in 3.3% (CI=3.0–3.6%; n=502) of the samples examined by PCR. The proportion of DNA-positive results was significantly higher ($p<0.05$) for younger (<1 year) than for older dogs as well as for male dogs than for bitches. *Babesia* DNA was more often detected from samples collected in spring and autumn than in winter ($p<0.001$). A significant association between detection of *Babesia* DNA and dog's origin ($p<0.001$) or travelling ($p<0.01$) could be shown (Table 3).

The results of the Giemsa-stained blood smears moderately correlated with those of the PCR (kappa value: 0.54). The relative sensitivity and relative specificity of the blood smear was 38% (CI: 32.4–44.2%) and 99% (CI: 99.8–99.9%), respectively, employing the PCR as the gold standard. Antibodies against *B. canis* were detected in 11.5% (CI: 10.3–12.8%; n=306) of the serum samples. 3.8% (CI: 3.1–4.6%; n=100) of dogs showed low (borderline) titres, while positive titres were found in 7.8% of the samples (CI: 6.8–8.8%; n=206). Male dogs were significantly more often seropositive than bitches ($p<0.01$). No other risk factors could be identified using the multivariate logistic regression.

Ehrlichia canis

E. canis DNA was isolated from 3.2% (CI: 1.9–4.9%; n=18) blood samples using PCR. Risk factors were not identified by a multivariable analysis.

Antibodies against *E. canis* were found in 15.1% (CI: 14.6–15.65%; n=2,816) of the serum samples. 8.5% (CI: 9.1–8.9%; n=1,585) of canine sera showed positive titres, while low (borderline) titres were found in 6.6% of the samples (CI: 6.2–7.0%; n=1,231). Female dogs had 1.2-fold higher chance to be *E. canis* seropositive than male dogs ($p<0.05$); the age of dogs was not found as a risk factor. *E. canis* antibodies were more often detected from samples collected in autumn (18.8%) compared to winter (15.5%), spring (10.8%) and summer (14.6%) ($p<0.001$).

Anaplasma phagocytophilum

Antibodies against *A. phagocytophilum* were detected in 41.9% (CI: 37.5–44.4%; n=325) of the serum samples. Positive titres were detected in 36.2% (CI: 32.8–39.6%; n=287) of the sera, while 5.7% (CI: 4.2–7.5%; n=45) showed low (borderline) titres. Antibodies were more likely to be detected in dogs older than 12 months than in dogs younger than 12 months ($p<0.001$).

Filariae

Microfilariae and *D. immitis* antigens were found in 4.5% (CI: 2.8–6.9%; n=440) and 1.4% (CI: 1.2–1.7%; n=9,381) of samples, respectively. Dogs aged >12 months were found to be more often seropositive for heartworm antigen than younger dogs ($p<0.01$). All 10 antigen positive dogs with known origin dogs were imported to Germany. No significant association was found between *D. immitis* and the origin of the dogs (data not shown).

Co-infections

Co-infections of vector-borne diseases were also observed (Table 4). Antibodies against *Leishmania* and *Ehrlichia* were detected most frequently (in 617 of 15,955 samples).

Table 4 Number of dogs with mixed infections

	<i>Leishmania</i> IFAT positive (n examined)	<i>Babesia</i> IFAT positive (n examined)	<i>Ehrlichia</i> IFAT positive (n examined)
<i>Babesia</i> Giemsa-stained blood smear	8 (6,915)*	3 (156) ^{ns}	10 (6,975) ^{ns}
<i>Babesia</i> PCR	88 (14,194) ^{ns}	16 (197)**	64 (14,229) ^{ns}
Microfilariae Knott's test	3 (108) ^{ns}	1 (16) ^{ns}	–
<i>Ehrlichia</i> PCR	4 (104) ^{ns}	1 (23) ^{ns}	8 (133) ^{ns}
<i>Babesia</i> IFAT	50 (777)**	–	42 (1,252)**
<i>Ehrlichia</i> IFAT	617 (15,955)**	–	–
<i>Anaplasma</i> IFAT	5 (96)*	6 (157) ^{ns}	21 (293) ^{ns}

*significant ($p < 0.05$) ** high significant ($p < 0.001$) ^{ns} not significant ($p > 0.05$)

Discussion

This retrospective study evaluated a large number of canine samples, routinely submitted by German veterinarians to a diagnostic veterinary laboratory. Occurrence and risk factors for *Leishmania*, *Babesia*, *E. canis*, *D. immitis* and *A. phagocytophilum* were shown. Additionally, possible autochthonous cases of leishmaniosis were assumed and potential endemicity of leishmaniosis was prospectively evaluated. Finally, seroprevalence of antibodies to *Leishmania* spp. were compared for the periods of 2004–2006 and 2014–2016.

Leishmania

Leishmania spp. DNA was detected in 11 % ($n = 33$) of 301 samples examined by PCR targeting the SSU rDNA. For 15 positive samples, information regarding the origin of dogs could be acquired. They were all either imported or traveled to endemic countries (mainly Spain or Portugal). Using PCR technique, sensitivity depends on the parasite numbers within the biological material obtained (Hernandez et al. 2015) and the copy numbers of target gene. Parasite numbers within a certain material could be further influenced by time point of infection (Hernandez et al. 2015) or treatment (Manna et al. 2015). Sensitivity of 87 % for blood

samples and 100 % for bone marrow was shown by Steuber et al. (1998). Using other samples like spleen or lymph node aspirates a sensitivity of 86 % was demonstrated by Maia et al. (2009). Strauss-Ayali et al. (2004) reported about slightly higher sensitivity (92 %) for conjunctival swabs (sampled from both eyes). In contrast, another study found lower amounts of parasites in conjunctival swabs on days 120 and 360 p.i. compared to vulvar or oral swabs as well as to blood or bone marrow samples (Hernandez et al. 2015). This could be due to the different PCR protocols (nested/conventional/real-time PCR; gene target) of respective studies, or because swabs do not contain a uniform cell number, depending on the technique of sampling. The most sensitive PCR assay is the method using kinetoplast DNA (kDNA), detecting the copy number of the target regions between 50- and 250-fold higher than splice leader mini-exon (SLME) and the SSU rRNA (ITS1) (Bensoussan et al. 2006). In the present study, *Leishmania* DNA was found in blood samples, skin scrapings, joint punctures and in bone marrow. All four joint samples were positive, which is particularly interesting. Agut et al. (2003) and Soubasia et al. (2013) reported about leishmanial arthritis in dogs. Leishmaniosis associated with a joint disease shows an erosive or none-erosive mono- or polyarthritis with

lymphoplastic to granulomatous inflammation. *Leishmania* amastigotes damage synovial membranes and cause neutrophilic inflammation. Additionally, immune complexes accumulate and lead to orthopaedic disorders (Saridomichelakis 2009). Typical clinical sign is lameness, but neuralgia, foot pad ulceration, polymyositis and osteomyelitis can also occur. This leads to severe destructive osteolytic and osteoproliferative injuries of long bones.

Positive *Leishmania* antibody titres observed in 2004–2006 (10.4%; IFAT) were significantly lower than the proportion of positive results in 2014–2016 (18.6%; ELISA). Nevertheless, if borderline results are allocated as positive, there was no statistically significant difference of *Leishmania* seroprevalence from 2004–2006 (23.5%) to 2014–2016 (22.8%). IFAT and ELISA technique, based on whole-cell *L. infantum* MON1 antigen, are the most used methods for detecting *Leishmania* antibodies in dogs (Wolf et al. 2014). Both tests have a high sensitivity and specificity and provide quantitative results (Solano-Gallego et al. 2014), but, they are not able to discriminate antileishmanial antibodies in *Leishmania*-infected or vaccinated dogs (Paltinieri et al. 2016). Overall the two assays (IFAT and ELISA) utilized in the present study showed high accordance regarding the percentage of identical results (83.7%; $rs=0.90$, $p<0.0001$) (Wolf et al. 2014). Within the period 2004–2006 antibodies were found in 92.3% dogs originating from Italy, 84.6% from Spain/Portugal, 81.3% from Greece/former Yugoslavia and 33.3% from Hungary. Moreover, 73.3% of German dogs with travel history in Spain/Portugal, 70% in Greece/former Yugoslavia and 100% in Italy, had also contact to *Leishmania* (Table 2). The detection of *Leishmania* antibodies in the present study confirmed serological data for dogs tested positive in the Netherlands, imported from Southern Europe (Teske et al. 2002). Meanwhile many imported dogs with CVBD, introduced to Central Europe, originate rather from eastern than from the southern European countries (Willi et al. 2015). Nevertheless, detection of antibodies

between 2014–2016 did not significantly decrease in contrast to the previous time period. This might either reflect a long seropositivity state or increasing occurrence of leishmanial infections also in eastern European countries (e.g. Romania, Dumitrache et al. 2016).

A significant association was found between risk factors like gender and age, and detection of *Leishmania* antibodies. *Leishmania* antibodies were detected more frequently in male than in female dogs. These findings corresponded with results of Zaffaroni et al. (1999), Cringoli et al. (2002), Keck and Dereure (2003), Mettler et al. (2005), Zivcinkjak et al. (2005) and Solano-Gallego et al. (2006). This can be explained by an increased outdoor activity in males. According to our findings, the risk for older dogs was higher than for younger. Cringoli et al. (2002), Mettler et al. (2005), Miro et al. (2007), Alonso et al. (2010), Cortes et al. (2012) and Carvalho et al. (2015) observed more frequently *L. infantum* antibodies in dogs with increased age. Old dogs may be more affected due to longer exposure to vectors in many transmission seasons. In contrast, Leontides et al. (2002) and Cardoso et al. (2004) did not consider age as a risk factor for leishmaniosis.

Four dogs with antibodies to *Leishmania* have never left Germany regarding the statement of their owners. Three of those dogs with titres of 200 or higher originated from southern part of Germany, two from Baden Württemberg and one from Franconia; in two dogs, leishmaniosis was additionally confirmed by two different PCR protocols. Autochthonous infections due to sand fly bites are unlikely as competent vectors for human and canine leishmaniasis as e.g. *Phebotomus perniciosus* has not been detected in Southern Germany since first finding (Beran 2010, Haeberlein et al. 2013). So far, Naucke and Schmitt (2004) observed *P. perniciosus* only once in Southern Germany. Another sand fly species, *Phlebotomus mascittii*, was reported recently in Germany (German state of Hesse) by Melaun et al. (2014). In Eastern Austria a stable population for *P. mascittii* seems to

exist (Obwaller et al. 2014). Despite isolated findings of *P. mascittii* in Germany, the vector competence of this sand fly species for *Leishmania* has not been verified yet. A recent study from Austria found *L. infantum* DNA in one *P. mascittii*, but did not provide evidence of metacyclic promastigotes or prove of transmission through experimental infections (Obwaller et al. 2016). Prospective examinations were conducted in Urbach and Schwannstetten (location of the two DNA positive dogs presumably infected in Germany) by analyzing conjunctival swabs from 16 and 29 dogs, respectively, presented in the same clinics for various reasons. All swabs were negative for *Leishmania* DNA pointing toward either a non-autochthonous infection (not reported or unknown to current dog owners) or a non-vector transmission in these two dogs. Possible mechanisms include breeding, vertical transmission, blood transfusion, and possible biting (e.g. Karkamo et al. 2014). Vectors, such as sand flies, could also be introduced accidentally to non-endemic areas via travel equipment.

Babesia

The prevalence of babesiosis depends on tick population and occurrence of *Babesia* in ticks. Babesiosis in Germany caused by *B. canis* is no longer a “travel disease”. The pathogen occurs in some foci in different regions. Various factors (global warming, ecological habitat changes, increased host and vector population, and increased mobility of owners with their dogs) probably caused spreading of ticks and this piroplasm (Zahler et al. 2000, Barutzki et al. 2007). In the present study, data from three different diagnostic procedures for *Babesia* were evaluated. In dogs living in Germany, antibodies (IFAT) were detected in 11.5%, parasitic stages (Giemsa-stained blood smears) in 1.7% and DNA (PCR) in 3.3% of submitted samples. Detection of large *Babesia* merozoites in Giemsa-stained blood and *Babesia* DNA in blood samples were observed significantly often in native dogs from Germany (without travel history) than in imported dogs (Table 3). These data

agree with recent serological data (*B. canis* ELISA) obtained for Germany (Pantchev et al. 2015a). A possible explanation is that a larger proportion of the animals with a history of travel were tested preventively irrespective of symptoms, whereas the animals that had stayed within Germany were tested on the basis of clinical suspicion. Dogs travelling with their owners to Hungary were frequently more often infected with *Babesia* than dogs staying in other countries. Regarding results of the present study, it can be concluded that the highest risk for canine babesiosis exists in Germany and in Hungary. Furthermore, *Babesia* (using Giemsa-stained blood smears and PCR) were found more frequently in male than in female dogs. Moreover, higher occurrence of *Babesia* DNA was detected in younger than in older dogs. Puppies and young dogs seem to be more susceptible for this protozoan than adult dogs; the outcome can be lethal if not diagnosed and treated properly (Farwell et al. 1982). Our results confirmed the surveys from Gothe et al. (1989), Porchet et al. (2007) and Zygner et al. (2009) regarding the seasonal distribution of *Babesia* with a peak in spring and autumn. This is associated with a seasonal activity of vector, *Dermacentor reticulatus* in March/April and October/November. As the present study demonstrated, a single microscopic blood smear test, compared with PCR as the established gold standard, had a relative sensitivity of only 38% and a relative specificity of over 99%. Another major advantage of the PCR technique is that, following a positive *Babesia* result, differentiation of species can subsequently be performed by either species-specific real-time PCRs or SSU rDNA amplification with subsequent sequencing (Pantchev et al. 2015a).

Filariae

Microfilariae were detected more often in blood samples (4.5%) than heartworm antigen using ELISA (1.4%). One explanation is that microfilariae originated from other filarial species (*Dirofilaria repens*, *Acanthocheiloneuma reconditum* or *Dipetalonema dracunculoides*) being also endemic

in Europe and occurring in subcutaneous tissue or peritoneal cavity (Pantchev et al. 2011). Cases of heartworm infections with a negative antigen result but with microfilaria circulating in the blood were also observed, but more rarely (Pantchev et al. 2011). An adulticid treatment, only a few adults, delayed antigenemia or immune complexes are possible reasons for this constellation (Weil et al. 1984, Pantchev et al. 2011). With regard to diagnostic procedures for imported or travel-accompanying dogs within Europe, other filarial species have to be considered in addition to *D. immitis*, but the awareness of veterinarians in Germany to search for microfilariae based on concentration assays (filtration, Knott) was particularly low in the study period 2004–2006 (e.g. only 440 Knott's tests were asked for by veterinarians). Thus, other filarial species than *D. immitis* were probably underdiagnosed. Currently, many laboratories offer PCR tests for microfilariae in blood samples (also within travel disease profiles), so the risk to miss such infections in dogs appears to be lower. Although the sensitivity of PCRs is generally high, the limit of detection is different and depends on target gene copies, PCR protocol (i.e. single or multiplex reactions), as well as mixed or single filarial infections among others (Pantchev et al. 2011, Cabanova et al. 2015, Ionica et al. 2015, Manzocchi et al. 2017). In the present study, *D. immitis* was detected only in dogs originating from endemic areas like Spain/Portugal and Greece. In our findings increased age was a risk factor for dirofilariosis. Elder dogs are usually longer exposed to blood-sucking mosquitoes and therefore also frequently infected with microfilariae (Peres-Sanchez et al. 1989, Poglayen et al. 1996, Miterpakova et al. 2008).

Ehrlichia canis

In this study a higher seroprevalence was shown compared to DNA detection (15.1% versus 3.2%). A persistence of antibodies after disappearance of bacteria from blood can lead to a poor correlation between both methods. Furthermore, in a chronic stage of disease dogs can show a low bacteremia

(Iqbal and Rikihisa 1994, Neer et al. 1999, Breitschwerdt et al. 2002). When comparing different samples using PCR method, a spleen aspirate is more suited sample compared to blood regarding sensitivity (Harrus et al. 1998, Baneth et al. 2009). In the present study female dogs harbored the infection more often than male dogs. The difference in hormone level and inherent immune response could be the reason for the gender susceptibility (Klein 2004). In contrast Sainz et al. (1996), Harrus et al. (1997) and Hernandez et al. (2004) could not find any sex predilection. Harrus et al. (1997) observed a season predilection for ehrlichiosis between May and October. In our study the majority of the dogs had antibodies against *Ehrlichia* in autumn, presumably associated with the seasonal activity of the vector (*Rhipicephalus sanguineus*). No information was available about the origin of positively tested dogs, but as most of serology was run within travel-disease-profiles, it can be assumed that these dogs were imported to Germany. Another study found 2 out of 60 seropositive dogs living in Germany to be presumable autochthonous infections, while most of imported infections came from Italy (Kuffer-Frank et al. 1999). One of those two dogs revealed a *R. sanguineus* infestation, presumably introduced from Italy through the partner dog. Krupka et al. (2007) found a seroprevalence of 1.1% (CI: 0.7–1.5) in randomly sampled German dogs (n=3,005) using a rapid test based on ELISA technology (peptides from p30 and p30 – 1 outer membrane proteins of *E. canis*; SNAP® 4Dx®).

Anaplasma phagocytophilum

Detection of *A. phagocytophilum* antibodies in 41.9% of samples was in accordance with results from other studies. E.g. Jensen et al. (2007) found similar seroprevalence in German dogs without history of staying abroad. Krupka et al. (2007) found a seroprevalence of 21.5% (CI: 20.5–22.6) in German dogs (n=5,683) using a rapid test based on ELISA technology (p44/msp2 antigen; SNAP® 4Dx®). Overall, a regional seroprevalences of 17.6%

to 31.1% were found in the above mentioned study, with lower seroprevalence in the western part of Germany. In the present study, dogs older than 12 months had more often antibodies against *A. phagocytophilum*. Age correlation and anaplasmosis in dogs is supported by studies from Sweden and USA (reviewed by Pantchev et al. 2015a). Possible explanation might be that repeated reinfection is necessary (hence older dogs), or a fully developed immune system is required, because *Anaplasma* impact on various immunological processes in order to survive. In contrast, Jensen et al. (2007) did not reveal any significant difference between age of dogs and anaplasmosis.

Co-infection

Most of the co-infected dogs in this study had antibodies against *E. canis* and *L. infantum* (Table 4). This represents at the same time the most important co-infection from clinical point of view. *E. canis* infection seems to predispose the dogs for *Leishmania* infection (Mekuzas et al. 2009). Moreover, co-infected dogs develop more frequently clinical signs (Ciaramella et al. 1997, Mekuzas et al. 2009); they show poor platelet aggregation and poor response to treatment as well as more antiplatelet antibodies (Cortese et al. 2009, 2011).

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Ethical standards

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

Competing interests

The authors declare that they have no competing interests. There is no commercial conflict of interest since the information generated here is solely for scientific dissemination.

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