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Tick-borne Diseases (Borreliosis, Anaplasmosis, Babesiosis) in German and Austrian Dogs: Status quo and Review of Distribution, Transmission, Clinical Findings, Diagnostics and Prophylaxis

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Abstract

Tick-borne diseases (TBD) in dogs have gained in significance in German and Austrian veterinary practices. The widespread European tick species *Ixodes ricinus* represents an important vector for spirochaetes of the *Borrelia burgdorferi* sensu lato group and Rickettsiales such as *Anaplasma phagocytophilum*. The meadow or ornate dog tick (*Dermacentor reticulatus*) is an important vector for *Babesia canis,* as is the brown dog tick (*Rhipicephalus sanguineus*) for *Babesia vogeli* in the Mediterranean region. The present work covers pathogen transmission by tick vectors, including the mechanisms and the minimum intervals required, in conjunction with possible non-vector-borne transmission routes. It also addresses the incubation periods, pathogenicity and clinical findings associated with each pathogen and genospecies and presents case examples. Current data on prevalence, annual fluctuations and distribution in various pre-selected dog populations (symptomatic versus asymptomatic) in both countries are depicted in maps. Reasons for changes in prevalence (especially of *Borrelia*) are discussed. Criteria and algorithms for clinical diagnosis and monitoring in dogs, including case history, direct detection (blood smears, molecular detection by species-specific PCR and sequencing) and indirect methods (whole-cell and peptide-based antibody tests), are presented, together with laboratory abnormalities (haematology, clinical chemistry, urine). The role

of anti-C6 antibody concentration (ACAC) and its correlation with proteinuria and Lyme nephritis are assessed on the basis of new data. Consideration is also given to the importance of blood smears, PCR and serology in the case of anaplasmosis and babesiosis, and the diagnostic value of combining these methods. The relevance of molecular differentiation of *Anaplasma* species (*A. phagocytophilum* versus *A. platys*) and *Babesia* spp. (large versus small forms) in cases of serological cross-reaction is emphasized. A summary is given of methods for prophylaxis using acaricide products (collars, spot-on solutions and oral treatments in both countries), vaccination (*Borrelia* and *Babesia* vaccines) and imidocarb-based chemoprophylaxis for large *Babesia*.

Introduction

Tick-borne diseases (TBD) in dogs have gained in significance in German and Austrian veterinary practices. The widespread European tick species *Ixodes ricinus* is a major vector of spirochaetes from the *Borrelia burgdorferi* sensu lato complex (*Bb*sl) and Rickettsiales such as *Anaplasma phagocytophilum*. The meadow or ornate dog tick (*Dermacentor reticulatus*) is an important vector for *Babesia canis*, as is the brown dog tick (*Rhipicephalus sanguineus*) for *Babesia vogeli* in the Mediterranean region (Table 1).

B. burgdorferi, the pathogen responsible for Lyme borreliosis (LB), was named after Dr. Willy Burgdorfer, who first discovered the bacterium in ticks in 1981 (reviewed by Horst 2003; Skotarczak 2014). They represent relatively large, helical-shaped (spiral) bacteria belonging to the spirochaetes (order Spirochaetales). The *Bb*sl complex currently comprises 19 species, including at least five genospecies pathogenic to humans (*Borrelia burgdorferi* sensu stricto/*Bb*ss, *Borrelia afzelii*, *Borrelia bavariensis, Borrelia garinii* and *Borrelia spielmanii*; Herzberger et al. 2007; Krupka and Straubinger 2010; Margos et al. 2013; Tijsse-Klasen et al. 2013b). Based on current information, pathogenicity (in the course of experimental and natural infections) in the dog has been demonstrated conclusively only for *Bb*ss, especially in the light of Koch's postulates complying after experimental infections (Hovius et al. 2000; Straubinger 2000; Straubinger et al. 2000; Liebisch and Liebisch 2003a; Littman et al. 2006; Wagner et al. 2012; Skotarczak 2014).

A. phagocytophilum is an obligate intracellular gram-negative bacterium, belonging to the order of Rickettsiales. This bacterium replicates mainly in neutrophils and can cause disease in humans, horses, dogs, cats and ruminants (Huhn et al. 2014). Since 2001, the former species *Ehrlichia equi* and *Ehrlichia phagocytophila*, and the agent responsible for human granulocytic ehrlichiosis ("HGE agent"), have been reclassified as a new species *A. phagocytophilum* on the basis of molecular data (16S rRNA gene; Dumler et al. 2001). These bacteria bind to glycoproteins on the surface of neutrophils and are incorporated into the cells through caveolae-mediated endocytosis. Thereafter, they prevent endosome-lysosome fusion and reproduce in membrane-bound vesicles, forming microcolonies called morulae (Carrade et al. 2009). A recent study showed furthermore that *A. phagocytophilum* was able to invade endothelial cells *in vitro* and that transmission of bacteria from microvascular endothelial cells to granulocytes occurred under flow conditions (Wang et al. 2015).

Babesia are protozoan agents belonging to the piroplasms (family Babesiidae, order Piroplasmida), and are the most important blood parasites found in domestic mammals. Canine babesiosis occurs worldwide. Intraerythrocytic parasites in dogs with symptoms comparable with babesiosis (fever, anaemia, splenomegaly) were first described in South Africa in 1893; in Europe, they were first described in Italy in 1895, some years after bovine babesiosis was first described by the Romanian Victor Babes in 1888 (Babes 1888; Piana and Galli-Valerio 1895; Baneth 2013). Until a few years ago, canine babesiosis was induced by three species of a large *Babesia* (merozoite size 3–5 µm), *Babesia*

Table 1 Tick-borne infections: vectors, pathogens and occurrence (sources include Olmeda-Garcia et al. 1993; Dongus et al. 1996; de la Fuente et al. 2008; Brianti et al. 2012; Petney et al. 2012; Deplazes et al. 2013; Ionita et al. 2013; Krücken et al. 2013; Tijsse-Klasen et al. 2013a; Najm et al. 2014; Rizzoli et al. 2014); * There is evidence that other *Ixodes* spp. such as *I. hexagonus* may also act as vectors for Lyme borreliae or *Anaplasma phagocytophilum* (Petney et al. 2012); ** Relative humidity; ***Currently assumed to be a cryptic species complex under the name "*R. sanguineus* sensu lato" with several different species included, presumed different distribution, host specificity and vector competence (Dantas-Torres & Otranto 2014a).

Vectors	Appearance	Potential vector for…	Occurrence in Germany and Austria?; habitat
Ixodes ricinus*: the Castor bean tick		*Borrelia burgdorferi / Borrelia miyamotoi, Anaplasma phagocytophilum,* tick-borne encephalitis virus, louping ill virus / Eyach virus / Tribec virus, *Babesia divergens / B. microti / B. venatorum (B. capreoli, B. annae?), Rickettsia helvetica / R. monacensis, Candidatus* Neoehrlichia mikurensis, *Bartonella henselae*	yes; humid habitats (>75% RH**): especially forest borders, roadsides, extensively farmed pasture, parks, gardens
***Dermacentor reticulatus:* the Ornate dog tick**		*Babesia canis / B. caballi, Theileria equi, Francisella tularensis, Rickettsia raoultii / R. slovaca / R. helvetica*	yes; local foci: humid forest and meadow habitats, river banks
Rhipicephalus sanguineus*: the Brown dog tick**		*Babesia vogeli / B. gibsoni, Anaplasma platys, Ehrlichia / Hepatozoon canis, Dipetalonema dracunculoides, Cercopithifilaria* spp., *Rickettsia conorii / R. massiliae, Bartonella vinsonii* subsp. *berkhoffi*	only temporary as an outdoor tick; locally as an imported, indoor ("domestic") populations in yearround tempered buildings

canis, *Babesia vogeli* and *Babesia rossi (*formerly *Babesia canis* subsp. *canis*, *B. canis* subsp. *vogeli* and *B. canis* subsp. *rossi)*, and one small *Babesia* (1–3 µm), *B. gibsoni* (Uilenberg et al. 1989; Zahler et al. 1998; Carret et al. 1999; Irwin 2009; Ogo et al. 2011). Recently, however, molecular studies resulted in the addition of further species. There are now at least nine genetically different species recognized (Tables 2–3). The recent findings of *Babesia annae* in foxes in Germany, Austria and Hungary (see Table 3) are intriguing, as no concurrent infections in dogs were reported from the corresponding areas. The provisional assignment of this agent as "*Theileria annae*" is controversial, because the organism is phylogenetically closer to *Babesia microti*. Typical features of the genus *Theileria*, such as pre-erythrocytic stages or paucity of transovarial transmission in the tick vector, have not been proven for *B. annae* (Dixit et al. 2010; Simões et al. 2011). One current study assign this organism as *Babesia vulpes* sp. nov., after its natural host *Vulpes vulpes* (Baneth et al. 2015).

Prevalence/occurrence and distribution

The first large-scale, methodically and geographically comprehensive serological analysis in the field of canine TBD in Germany was conducted in the form of a countrywide study with 5,881 samples from dogs tested (Krupka et al. 2007). Serum samples from 3,005 dogs (group A; not pre-selected) and 2,876 dogs (group B; showing symptoms of borreliosis) were submitted to two different

Table 2 Current *Babesia* species in the dog (large forms): at least six genetically different species / isolates (according to Beck et al. 2009; Irwin 2009; Birkenheuer 2012).

Species	Synonym	Vector	Distribution	special features / clinical findings
Babesia vogeli*	*Babesia canis* subsp. *vogeli*	*Rhipicephalus sanguineus*	global throughout the tropics and subtropics, Mediterranean region	in colder zones, vector also adapted to indoor, year-round tempered climate of buildings
Babesia canis	*Babesia canis* subsp. *canis*	*Dermacentor reticulatus*	Europe	haemolytic anaemia, fever; moderate virulence
Babesia rossi	*Babesia canis* subsp. *rossi*	*Haemaphysalis elliptica*	Sub-Saharan Africa; South Africa	haemolysis, immune disease; high virulence
Babesia* sp.*	unnamed large *Babesia* sp. ("North Carolina"/ NC isolate)	at present unknown	North Carolina (USA)	thrombocytopenia, haemolytic anaemia, leukopenia, pigmenturia
***Babesia* sp.**	unnamed large *Babesia* sp. (UK isolate)	at present unknown	United Kingdom	thrombocytopenia, haemolytic anaemia, leukopenia, pigmenturia
Babesia caballi	–	unknown (*D. reticulatus?*)	Croatia	molecular detection only

*shows weak virulence for adult dogs but can also take a severe course in the presence of predisposing factors (young dogs, immunosuppression (Cushing's disease, corticosteroid administration or co-infections)); **apparently the *Babesia* species from NC ("*Babesia* sp. *coco*") affect mainly immunosuppressed dogs; *in vitro*, it shows few ultrastructural differences compared to other *Babesia* spp.; according to the phylogenetic results (18S rRNA) of a current study, this large piroplasm from NC could be related to the genus *Rangelia* (Eiras et al. 2014)

laboratories where they were investigated using a serological rapid test (SNAP® 4Dx®) for the presence of specific antibodies to *Borrelia* C6 antigen (not induced by vaccination, see below). In group A, 7.7 % of the dogs (232/3,005; 95 % confidence interval (CI): 6.8–8.7) and 11.8 % of the dogs in group B (340/2,876; 95 % CI: 10.7–13.1) tested positive (statistically significant difference of $p < 0.001$; chi-square (CS) test). Overall, regional seroprevalences of 1.9 % to 10.3 % were found for *B. burgdorferi* (group A). Similar seroprevalence was observed for humans in a recent study from Germany (9.4 %; 741/6,945), with significant correlation for seropositivity in southern Germany, male sex and residence in rural areas. Interestingly, having a dog/cat in the house was not associated with a higher risk for seropositivity (Wilking et al. 2015). Within the above mentioned canine group A 3 % of the dogs (91/3,005; 95 % CI: 2.4–3.7) also showed antibodies to *A. phagocytophilum* (Fig. 1). The seroprevalence for *A. phagocytophilum* (total samples tested 5,683; antibodies to MSP2 (P44) antigen) was 21.5 % (95 % CI: 20.5–22.6). It can therefore be assumed that at least one fifth of dogs in Germany had contact with this agent. Overall, regional seroprevalences of 17.6 % to 31.1 % were found for *Anaplasma* spp. (Krupka et al. 2007). In comparison, the seroprevalence with the same test (SNAP® 4Dx®) for *Borrelia* and *Anaplasma* in Poland (3,094 canine samples) was 3.75 % and 12.31 %, respectively (Krämer et al. 2014). Even lower rates (1.09 % for *Borrelia* and 2.72 % for *Anaplasma*) were found in in France (the same test; 919 dogs; Pantchev et al. 2009).

To document the presence of *A. phagocytophilum* and *Bb*sl in dogs in Austria, routine diagnostic data from the authors' laboratory were evaluated retrospectively (previously unpublished data; methods according to Dyachenko et al. 2012). In April/May 2011, *Anaplasma* spp. real-time PCR was used to

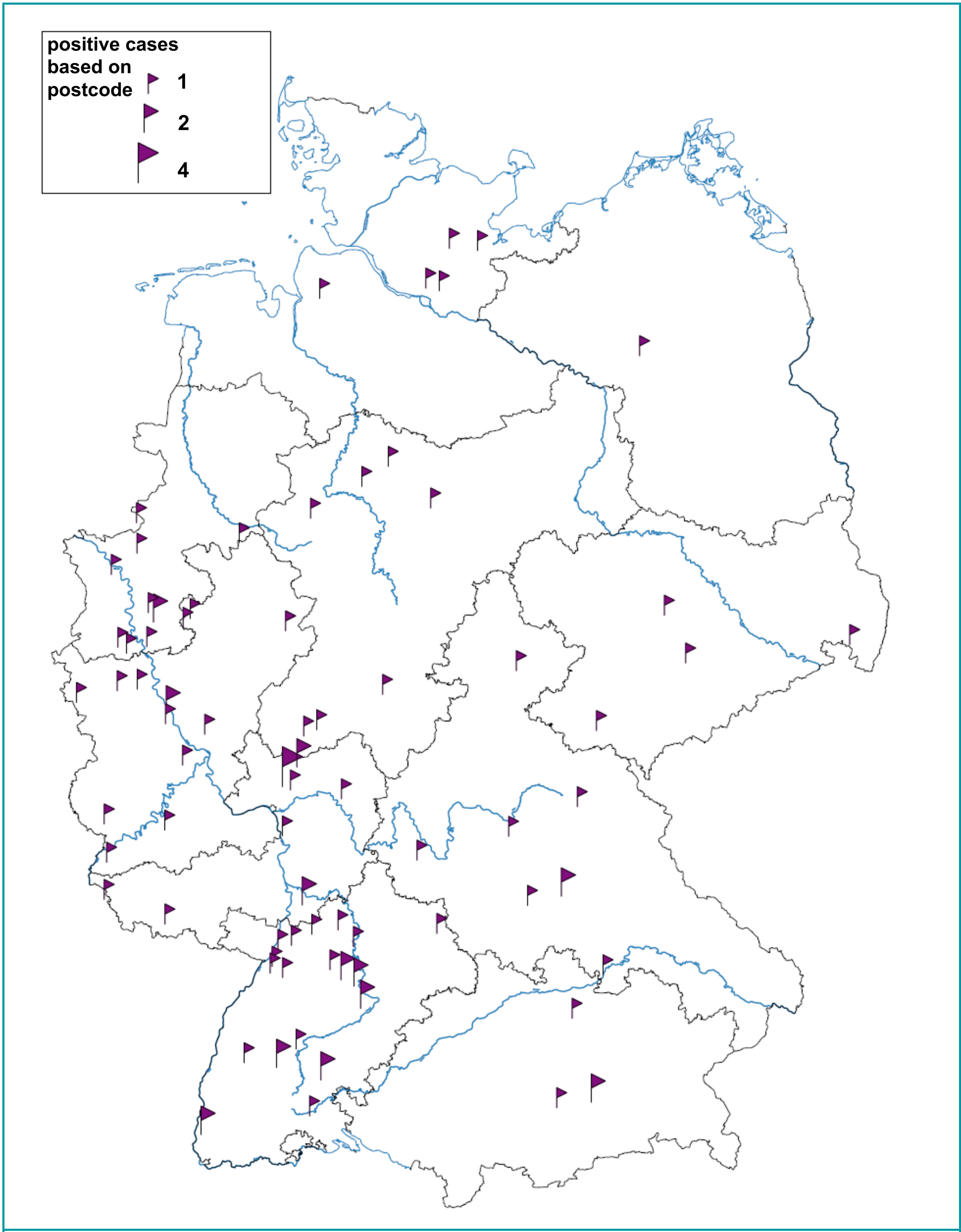

Fig. 1 *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in Germany: origin of simultaneously detected antibodies by postcode (map produced using EasyMap; positions correspond to submitters' postcodes).

Table 3 Current *Babesia* / *Theileria* species in the dog (small forms): at least five genetically different species (according to Zahler et al. 2000a,b; Beck et al. 2009; Irwin 2009; Birkenheuer 2012; Falkenö et al. 2013; Najm et al. 2014; Duscher et al. 2014; Gallusova et al. 2014; Rosa et al. 2014; Baneth et al. 2015; Farkas et al. 2015).

Species	Synonym	Vector	Distribution	special features / clinical findings
Babesia gibsoni	*Babesia gibsoni* Asia strain	*Haemaphysalis bispinosa, Haemaphysalis longicornis, (Rhipicephalus sanguineus)*	mainly in Asia, sporadically also in Africa, Australia, Europe, North and South America	outside Asia, infection often associated with pit bull terriers and other fighting dogs with non vector-borne transmission
Babesia conradae	"Small *Babesia* sp." California isolate	unknown (a wild animal reservoir is assumed)	California	haemolytic anaemia, vomitus
Babesia annae	*Babesia microti*-like, *Theileria annae*; *Babesia vulpes* sp. nov.; *Babesia* Spanish dog isolate	*Ixodes* spp. (assumed)	Northwest Spain, Portugal, Croatia, Sweden, USA; Germany / Austria / Hungary (only in fox)	severe haemolytic anaemia, eosinophilia, renal involvement
Theileria equi	*Babesia equi*	unknown	Spain, Croatia, France, Romania, Jordan; South Africa	molecular detection only; thrombocytopenia, anaemia
Theileria annulata	–	unknown	Spain	molecular detection only
***Theileria* sp.**	unnamed *Theileria* sp., South African *Theileria* sp.	unknown	South Africa	molecular detection only; thrombocytopenia, anaemia

test a total of 50 EDTA blood samples submitted by veterinarians in Austria (this test had been specifically requested, so the dogs were presumably showing clinical signs of infection). Eight out of 50 samples (16 %; 95 % CI: 7.1–29.1) tested positive. In comparison, a total of 30 samples were tested in the same period in 2010; five of these tested positive (16.7 %; 95 % CI: 5.5–34.7). One hundred and sixty-four dogs were tested serologically for antibodies to *Borrelia* spp. C6 antigen in April/May 2011 (serum samples; again submitted by Austrian veterinary surgeons specifically requesting this test), and seven were positive (4.3 %; 95 % CI: 1.7–8.6). In comparison, a total of 90 samples were tested in the same period in 2010; five of these tested positive (5.6 %; 95 % CI: 1.8–12.5). Strikingly, much larger numbers of samples were submitted for both tests in 2011 (increases of approx. 67 % and 83 % respectively compared with 2010). However, the differences in positive percentages for the two years were not statistically significant ($p = 0.644$ for *Borrelia* and $p = 0.938$ for *Anaplasma* spp.; CS test). Regarding the distribution of positive samples, some federal states showed an overlapping incidence of both organisms (Vienna, Lower/Upper Austria and Carinthia). In other regions, only *Anaplasma* (Vorarlberg, Styria and Burgenland) or only *Borrelia* (Tirol) was detected. Salzburg was the only state with no positive cases (Fig. 2).

Previously, canine babesiosis was typically associated with travel to Mediterranean countries. In a study conducted in 2005 and 2006, blood samples from 5,483 dogs living in Germany with a history of travel were tested for relevant pathogens. *Babesia* species (DNA detection by means of conventional PCR technique) were detected in 2.4 % of these samples (Hirsch and Pantchev 2008). However, canine babesiosis is increasingly endemic in both Germany (Barutzki et al. 2007) and Switzerland

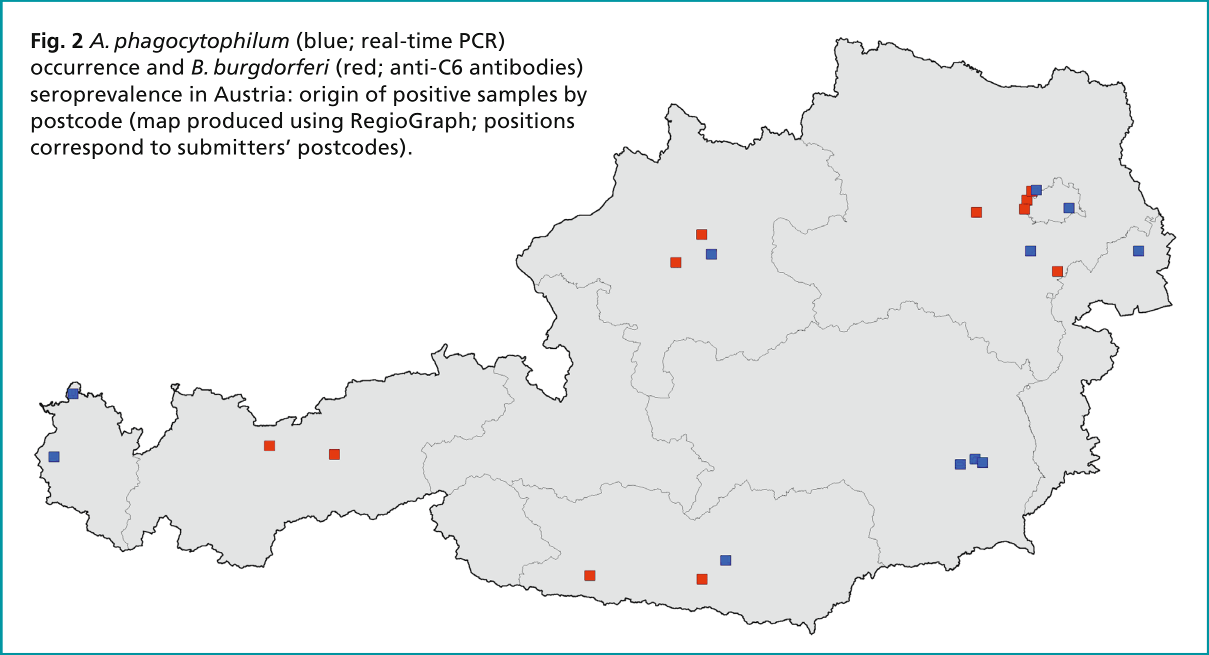

Fig. 2 *A. phagocytophilum* (blue; real-time PCR) occurrence and *B. burgdorferi* (red; anti-C6 antibodies) seroprevalence in Austria: origin of positive samples by postcode (map produced using RegioGraph; positions correspond to submitters' postcodes).

(Schaarschmidt et al. 2013). This is believed to be the result of dogs introducing infected ticks, which have subsequently found suitable biotopes (for example, due to re-naturalization of agricultural areas) and climatic conditions (Heile et al. 2006). In a recent study, a new *B. canis* ELISA was used to test 4,579 canine samples from Germany for *B. canis* antibodies as part of travel disease profiles (no immediate suspicion of babesiosis) and 937 samples from dogs with suspected babesiosis (Pantchev 2012a). Because a travel disease profile had been ordered, the dogs in the first group were assumed to have travelled outside Germany; in the second group, a history of travel could not be entirely ruled out. 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 authorized by the German Friedrich Löffler Institute for the detection of *B. canis*-specific antibodies in dogs. However, it shows cross-reactivity with other *Babesia* spp. (such as *B. vogeli*, Dyachenko et al. 2012) as well as *B. gibsoni* and *Rangelia vitalii* (own unpublished observations; see below). This whole-cell based assay can therefore be regarded as more piroplasma-specific. Three hundred and nineteen dogs (travel disease profile) within the above-mentioned study tested positive for antibodies (7 %; 95 % CI: 6.2 – 7.7) and a further 112 samples were borderline (2.4 %). In dogs with suspected babesiosis, higher proportions of samples were seropositive (12.7 %; 95 % CI: 10.6 – 15; significant difference of $p < 0.001$, CS test) and 3.5 % of samples were borderline. Regarding the distribution of positive samples in both groups within the German federal states, it can be observed, that only dogs with a history of travel tested positive in four federal states, and one state had no cases at all (Fig. 3). Whether this result is due to the absence of endemic canine babesiosis in these states, or to the simple fact that insufficient samples were submitted, should be a subject for further studies.

The occurrence and distribution of *B. canis* infection in dogs in Austria was established using the

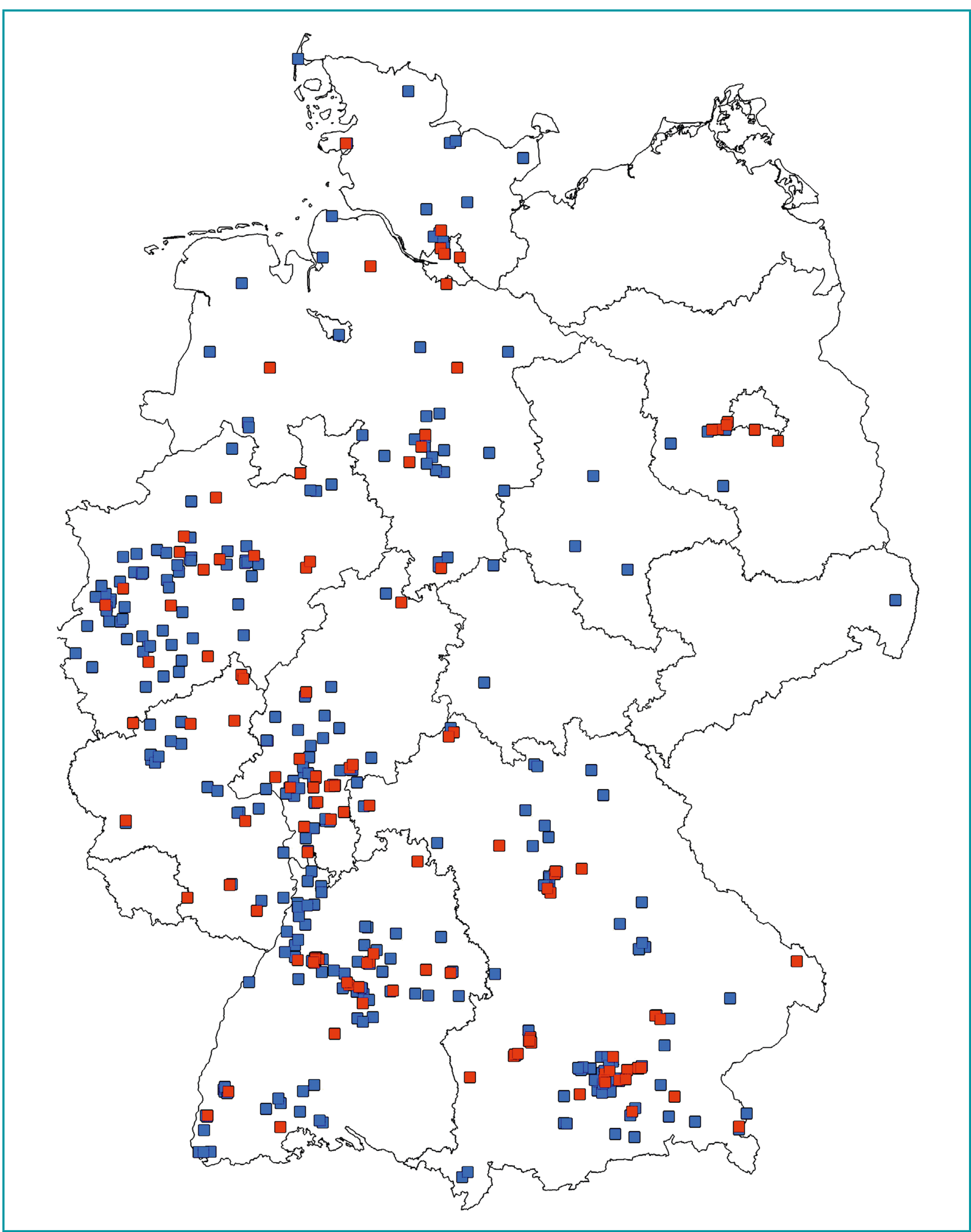

Fig. 3 *Babesia canis* in Germany: Origin of seropositive dogs with a history of travel (blue; n = 319) and suspected babesiosis (red; n = 119; according to Pantchev 2012a; map produced using RegioGraph; positions correspond to submitters' postcodes).

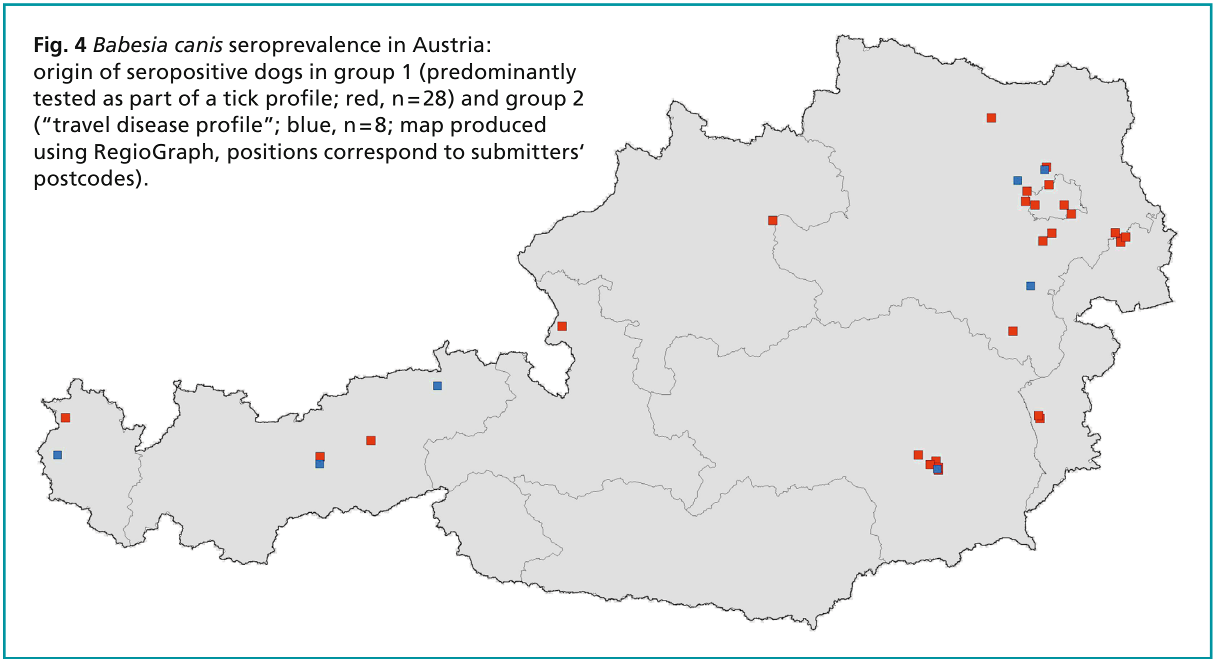

Fig. 4 *Babesia canis* seroprevalence in Austria: origin of seropositive dogs in group 1 (predominantly tested as part of a tick profile; red, n = 28) and group 2 ("travel disease profile"; blue, n = 8; map produced using RegioGraph, positions correspond to submitters' postcodes).

same *Babesia* ELISA (see above; previously unpublished data). The serum samples were submitted to the authors' laboratory by veterinarians in Austria from 2012 to 2013. Two hundred and fifty-nine samples in group 1 were tested either in response to a direct request for a *Babesia* spp. antibody test (n = 70; 27 %) or as a part of a serological tick profile (n = 189; 73 %). As a comparison, a second group was assessed over the same time period (group 2; n = 172). Animals in group 2 were assumed to have a travel history, because the serum samples were submitted by the veterinarian as part of a travel disease profile. In group 1 (mostly comprising "tick profiles") 28 samples tested positive (10.8 %; 95 % CI: 7.3 – 15.2; red in Fig. 4) and 6 samples were borderline (2.3 %). As part of the travel disease profile, however, only 8 dogs tested positive (4.7 %; 95 % CI: 2 – 9; blue in Fig. 4), which is significantly fewer than in group 1 ($p = 0.024$; CS test). Additionally, six samples in group 2 tested borderline (3.5 %). As can be deduced from Figure 4, there appears to be a cluster of seropositive dogs in the eastern part of Austria.

Pathogen transmission and incubation period

Various mechanisms are required to activate the relevant pathogen in the tick. In general, transmission of bacteria and parasites does not occur immediately. The shortest transmission intervals reported are 16 to 65 hours for *Borrelia* and 24 hours for *Anaplasma* (Crippa et al. 2002; Kahl et al. 1998; Diniz and Breitschwerdt 2012). *Borrelia* spp. produce various outer surface proteins ("Osp"), which enable the spirochaetes to adapt to a wide range of environmental conditions (Kenedy et al., 2012). *Borrelia* organisms in the tick's gut express mainly OspA, which allows the bacteria to adhere to the tick receptor for OspA ("TROSPA"; Tsao 2009). Contact with the host's skin causes a temperature rise in the tick, which triggers an activity signal in *Borrelia* organisms, enabling them to migrate from the tick's gut to its salivary glands. Following contact with blood, OspA (and OspB) are replaced within 36 to 48 hours by newly produced OspC, which binds to the tick salivary protein Salp15 (Kenedy et al. 2012). After 24 to 48 hours, *Borrelia* organisms are transmitted to the dog (Radolf and Caimano 2008; Straubinger and Pantchev 2010). In the mammalian host,

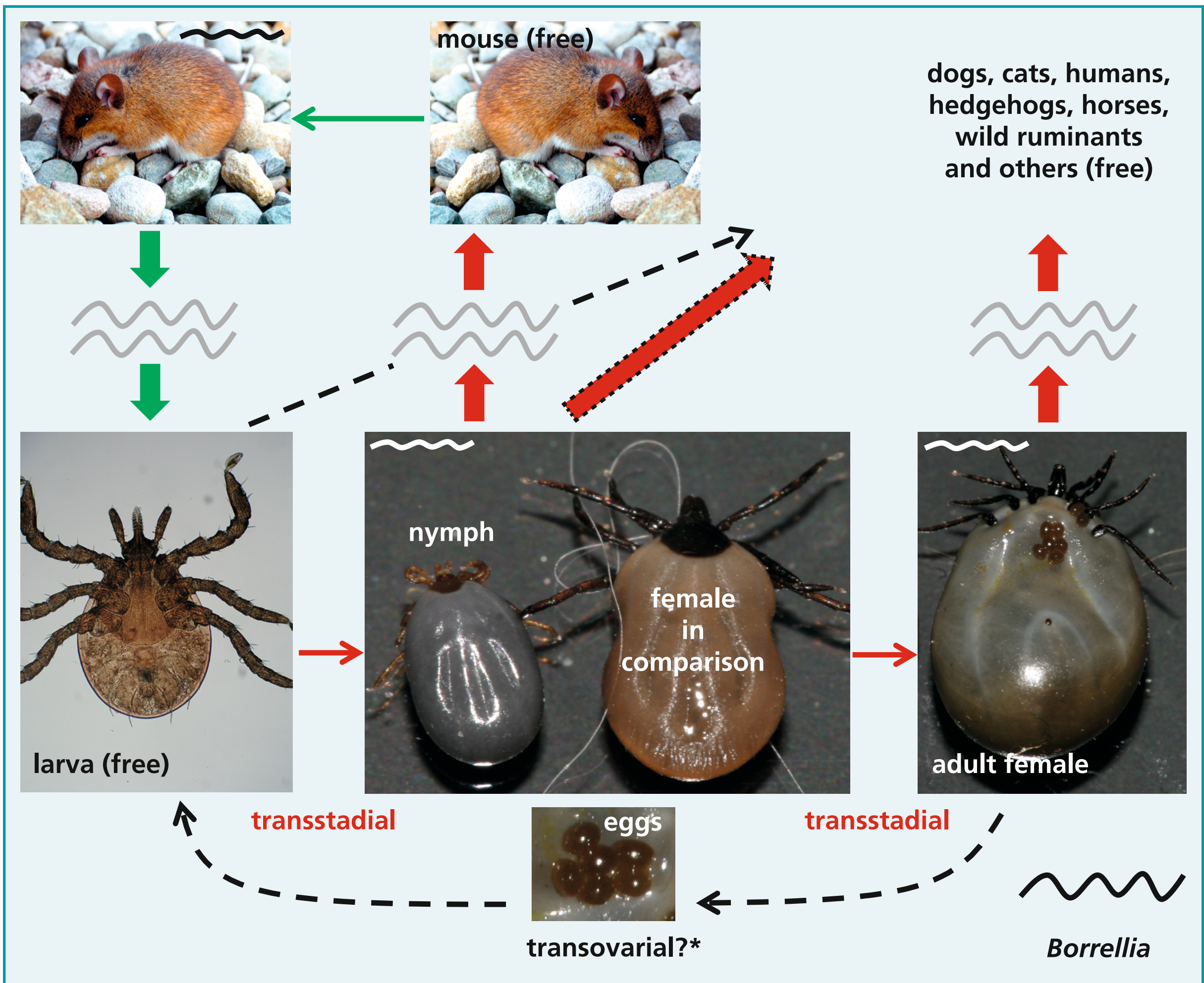

Fig. 5 Life cycle of *Ixodes ricinus* and transmission of rodent-associated *Borrelia* spp. (e.g. *B. afzelii* / *Bbss*; sources include Tsao 2009; Greene et al. 2012; Tappe et al. 2014); within the figure of *Ixodes* nymph (centre), the female is shown only for size comparison; * see text

spirochaetes express the protein VlsE (variable major protein-like sequence, expressed) on their surface. The variability of this protein allows *Borrelia* to evade the host's immune defences (Kenedy et al. 2012). A shift in the outer surface proteins seems to be crucial for infection (Appel et al. 1993; Radolf and Caimano 2008). In experimental studies it was shown that the incubation period in the dog is between two and five months (Appel et al. 1993; Straubinger 2000; Straubinger et al. 2000; Wagner et al. 2012). Therefore, no clinical signs were observed in dogs that had not previously seroconverted (Appel et al. 1993). In contrast, the incubation period for anaplasmosis following a tick bite or experimental (i.v.) infection is reported to be 1 to 2 weeks (Scorpio et al. 2011; Diniz and Breitschwerdt 2012), which can precede seroconversion (see below). Shorter transmission intervals may exceptionally occur. For example, occasional cases of systemic infection with *Borrelia* spp. in the tick were described, with *Borrelia* present in the salivary glands before tick attachement (also described for *A. phagocytophilum*; Crippa et al. 2002; Diniz and Breitschwerdt 2012). Earlier transmission is also possible, if previously attached (and thus "activated") ticks infest a new host. Moreover,

I. ricinus appears to transmit *B. afzelii* more rapidly than *Bb*ss (Crippa et al. 2002; Moehrle and Rassner 2002). It is generally assumed that transmission of *Bb*sl takes place via nymphs and adult ticks, and specifically that larvae become infected by feeding on infected reservoir hosts (e.g. rodents), followed by transstadial transmission of *Borrelia* (illustrated in Fig. 5; applies similarly to *A. phagocytophilum*). Whether a transovarial transmission of *Bb*sl can take place in the tick, as proposed following increased molecular detection in larvae in a recent study (Tappe et al. 2014), or the transovarially transmitted agent is actually *Borrelia miyamotoi* (Rollend et al. 2013), has to be evaluated in further studies. Even if larvae did contain *Bb*sl in sufficient amounts for transmission, the risk to dogs is put into perspective by the fact that these larvae live primarily at ground level, and their preferred hosts are small mammals and birds (Liebisch and Liebisch 2003b; Greene et al. 2012; Deplazes et al. 2013). This is supported by a recent study from Switzerland, in which only 0.5 % of collected ticks from dogs we identified as being larvae (Eichenberger et al. 2015). Non-vector-borne transmission of *A. phagocytophilum* in humans through blood transfusions (Annen et al. 2012) or perinatal infection (Horowitz et al. 1998; Dhand et al. 2007) has been described. Moreover, transplacental transmission as a result of experimental infection is possible in ruminants (Diniz and Breitschwerdt 2012) and was also confirmed after natural congenital infection in a calf in northern Germany (Henniger et al. 2013). Vertical transmission of *Bb*ss did not occur under experimental conditions in dogs (maternal antibodies declined to negative 4 weeks postpartum; Appel et al. 1993). Vertical transmission of *Borrelia* spp. and other non-vector-borne modes of transmission (via semen, urine, or blood) are unlikely in the dog under natural conditions (Appel et al. 1993; Greene et al. 2012). According to guidelines on the collection, storage, transport and administration of blood and blood products in veterinary medicine, issued by the German Federal Office of Consumer Protection and Food Safety, dogs used as blood donors in Germany should be tested by PCR for *Anaplasma* spp. (*A. phagocytophilum*) and *Babesia* spp. (*B. canis;* see below), depending on the region and the prevailing epidemiological situation http://www.bvl.bund.de/DE/05_Tierarzneimittel/05_Fachmeldungen/2011/leitlinien_blutprodukte.html?nn=1644492).

A striking feature of *Babesia* species is not only the transstadial but also the efficient transovarial transmission in the tick. By means of vertical transmission to the next 3 to 4 generations, tick populations in an endemic region can remain infected for several years, despite having no opportunity for reinfection (Deplazes et al. 2013). This ensures long-term survival in the tick population (distribution strategy; Chauvin et al. 2009). This process is limited to some extent because only 10 to 20 % of the tick eggs are infected, and infected females produce fewer eggs (Deplazes et al. 2013). The development of *Babesia* in the tick, specifically the infestation of the salivary glands accompanied by the production of sporozoites, does not happen immediately, but is triggered by nervous stimulation of various organs. When ticks attach to the host, developmental stages of *Babesia* known as kinetes are released. These enter the tick's salivary glands via the haemolymph. Dogs exposed experimentally to *D. reticulatus* containing *B. canis* tested positive for *Babesia* (PCR, blood smears) after a 72-hour infestation; males which had already had a blood meal were an exception in terms of immediate transmission (Heile and Schein 2007). The same study showed that nymphs already have the ability to transmit *B. canis*. However, because nymphs rarely infest dogs, this mode of transmission plays only a minor role from an epidemiological perspective. The incubation period of *Babesia* in the dog is reported to be 7 to 21 days p.i. (Belton 2003; Deplazes et al. 2006). Non-vector-borne transmission routes may include blood transfusion, as shown in the case of *B. canis* in an experimental design (Brandao et al. 2003) or in the case of *B. gibsoni* (Belton 2003; Boozer and Macintire 2005). In regard to *B. gibsoni,* there is also speculation about

direct dog-to-dog transmission via bite wounds, saliva or ingested blood (fighting dogs), and transplacental transmission (*B. canis*, *B. gibsoni* and *B. annae*) also appears to be possible (Belton 2003; Boozer and Macintire 2005; Irwin 2009; Ayoob et al. 2010; Ogo et al. 2011; Simões et al. 2011; Mierzejewska et al. 2014).

Diagnostics

Lyme borreliosis (LB)

The presumptive diagnosis of LB in dogs implies several criteria (according to Littman et al. 2006; Krupka and Straubinger 2010). These include a history with regard to tick exposure and living in endemic regions, diagnostic evidence of infection with the pathogen, exclusion of differential diagnoses, compatible clinical symptoms, and response to specific treatment including monitoring of treatment success. Detection by direct methods (PCR and/or culture) ante mortem is difficult and of little practical relevance. *Borrelia* organisms are rarely detected in body fluids such as blood (Fig. 6), urine (bladder involvement is also rare), synovial fluid or cerebrospinal fluid (Leschnik et al. 2010; Krimer et al. 2011; Susta et al. 2012). They are more commonly found in connective tissue, fascias, joint capsule (most promising in the affected joint), skin (near the tick bite), lymph nodes, muscle (including the heart), etc. (frequently in low numbers at the time of detection; Appel et al. 1993; Chang et al. 1996; Straubinger 2000; Chou et al. 2006). The majority of dogs first develop lameness in the joint closest to the site of tick attachment, which further supports the fact that *B. burgdoferi* does not disseminate throughout the body via the blood stream (Straubinger et al. 1998). Direct methods in many cases produce false-negative results, because tissue samples commonly contain few or no bacteria at the time of collection, so serology is widely used. However, the only reliable way to differentiate the genospecies is direct detection followed by molecular characterization (as is frequently performed

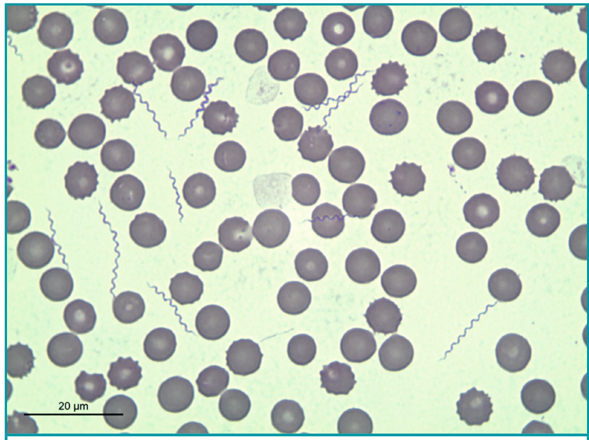

Fig. 6 *Borrelia hispanica* in the stained blood smear of a dog: in contrast to relapsing-fever (RF), Lyme borreliosis (LB) spirochetes are not detected in blood samples.

in tick studies; Skotarczak 2014), because the relevant antigens display serological cross-reactivity (Hovius et al. 2000). As a result, it is not currently possible to use different serological tests (enzyme immunoassays or immunoblots) based on individual genospecies or their antigens to perform reliable serological differentiation (Kurzova et al. 2014).

Four aspects should be considered when interpreting positive serological results. Serological tests detect circulating antibodies to an infectious agent, which may indicate active infection with that agent, previous infection or exposure, vaccination, or cross-reactivity with another (generally closely related) organism. These points should be considered, when evaluating serological tests for *Borrelia* spp.-specific antibodies. In human and veterinary medicine, antibody detection using specific peptides such as C6 is becoming increasingly popular (e.g. Liang et al. 1999a; Embers et al. 2007; Krupka and Straubinger 2010; Wagner et al. 2012), as they show advantages over other methods, taking into account the four points mentioned above. The C6 peptide is part of VlsE, a 35-kDa surface lipoprotein of *Borrelia burgdorferi* (Kenedy et al. 2012). IR6 (synthetic peptide = C6) is the most immunodominant of six invariable regions (IR_1-IR_6) within the central variable domain (six variable regions, VR_I-VR_{VI}, interlaced with the invariable regions;

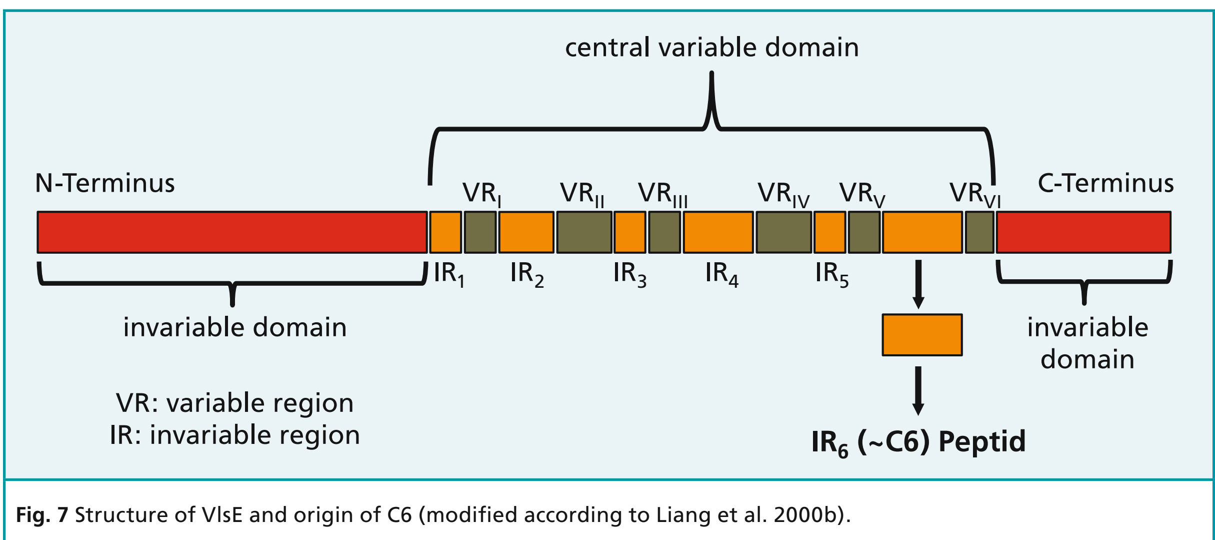

Fig. 7 Structure of VlsE and origin of C6 (modified according to Liang et al. 2000b).

Liang et al. 1999b; Embers et al. 2007; Fig. 7). In addition, C6 is conserved among different *Borrelia* species (Liang et al. 2000a). The VlsE gene locus is located on a plasmid (lp28–1; Brisson et al. 2012; Embers et al. 2012), its expression is suppressed in the tick (Bykowski et al. 2006; Tilly et al. 2013) and drastically upregulated in the host. Moreover, recombination takes place only in the host, not in the tick or in *in vitro* cultures (Lin et al. 2009). Antibodies to *Borrelia* C6 antigen are not induced by vaccination in the dog (O'Connor et al. 2004; Goldstein et al. 2007), possibly due to lost of VlsE expression after repeated serial passage of cultured spirochaetes (Liang et al. 1999a,b; O'Connor et al. 2004; Lin et al. 2009). According to current scientific knowledge, VlsE is also one of the most important virulence factors of Lyme borreliae, preventing their elimination by the immune system and triggering a persistent infection in the host. This is made possible by constant alternation in the six variable regions (Lin et al. 2009; Brisson et al. 2012; Kenedy et al. 2012).

The advantage of *Borrelia* C6-based tests over previous methods (IgM/IgG-ELISA or IFA) is the absence of a cross-reaction with vaccine-induced antibodies (O'Connor et al. 2004; Töpfer 2005; Goldstein et al. 2007) or with antibodies to other spirochaetes such as *Leptospira* (Liang et al. 2000b). Thus, C6 represents a diagnostic approach that facilitates DIVA (Differentiation of Infected from Vaccinated Animals). Whole-cell-based assays can cross-react with other spirochaetes as shown e.g. for *Leptospira* species (Štefančikova et al. 2008) or with vaccination (e.g. Gauthier and Mansfield 1999; Straubinger et al. 2002; Töpfer 2005). 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 concentrations (ACAC) may drop within 3 to 6 months (a decrease of more than 58.3 % was seen after 6 months in animals with an initial concentration over 29 U/ml; Levy et al. 2008), whereas the values in whole-cell-based tests and OspF-based tests do not fall to the same extent (Straubinger 2000; Straubinger et al. 2000; Littman 2013; Fig. 8). 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 infection in the dog. 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 and can be used to monitor treatment success. This has also been shown in experimental infection in monkeys (Embers et al. 2012).

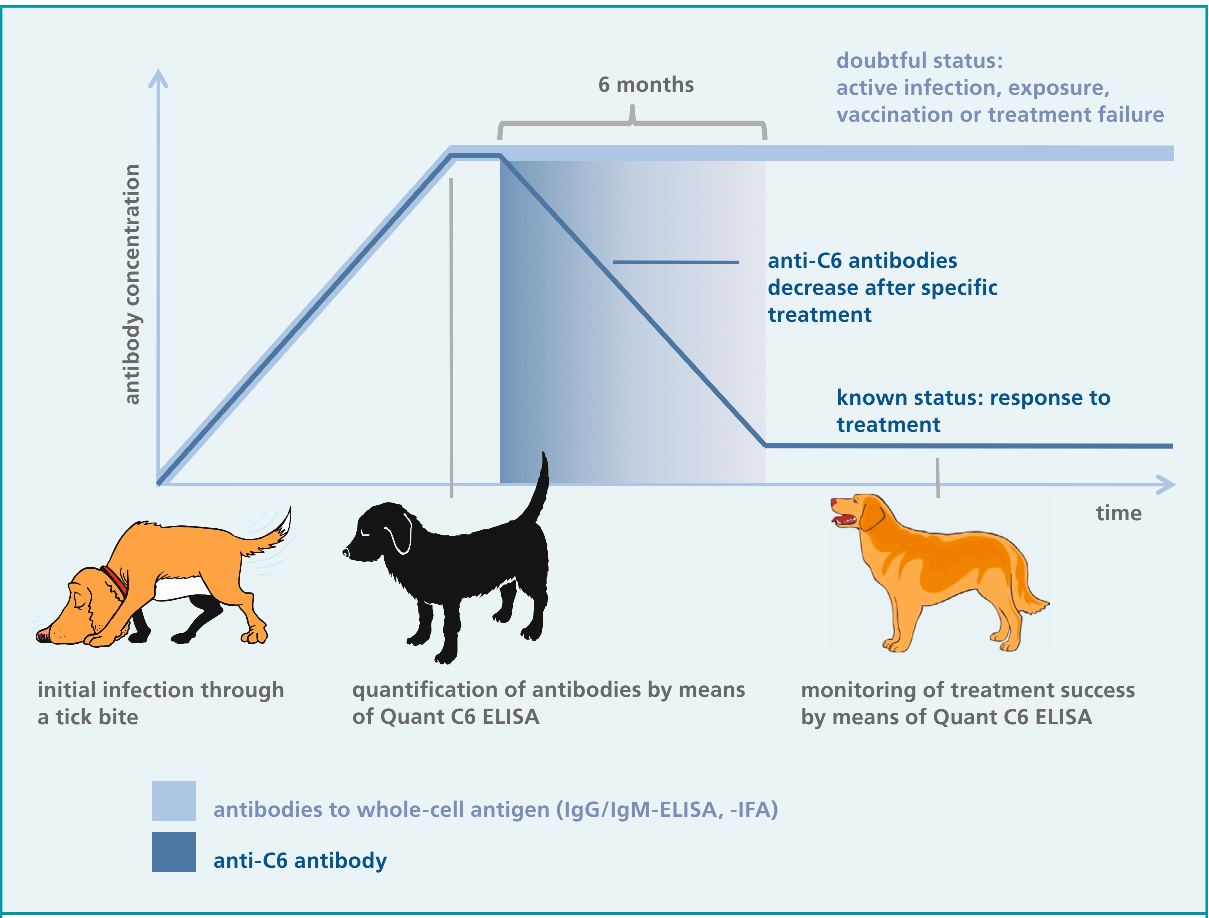

Fig. 8 Quant C6 ELISA for determination of anti-C6 antibody concentration applied to monitor treatment success (schematic display modified according to Straubinger 2000; Levy et al. 2008; Littman et al. 2013).

For correct diagnosis it is also important to rule out potential differential diagnoses, bearing in mind the travel history of the dog and potential infections from abroad such as *Leishmania infantum* or *Ehrlichia canis*. Useful tests in this regard are listed by Littman et al. (2006), Greene et al. (2012) and Pantchev (2012b). They include the following examinations: routine laboratory tests (haematology, clinical chemistry, urine analysis), tests for other infectious agents (serology, PCR and antigen detection if necessary) and immune-mediated diseases (rheumatoid factor, antinuclear antibodies or Coombs test), x-rays of one or more limbs, joint tap for cytology and culture (up to 76,000 cells/microlitre (normal <3000), up to 97% of cells neutrophils (Straubinger et al. 1998), increased protein concentration and turbidity are compatible with Lyme arthritis), tumour detection (thoracic x-ray, abdominal ultrasound, fine needle aspiration of lymph nodes and bone marrow cytology). The procedure for a *Borrelia*-positive dog is shown in Fig. 9. Additionally, Susta et al. (2012) found an inflamed or borderline synovial aspirate (100-cell differential count; inflamed if ≥20 % neutrophils, borderline at 8–19 % and non-inflamed when <8 % neutrophils) in 47 % (8/17) of experimentally infected dogs. Moreover, post-mortem histopathological scoring of the synovial membrane (elbows and stifles), using a proposed grading scheme, was a reliable method for discriminating infected and non-infected animals (88.2 % sensitivity and 100 % specificity).

As Fig. 9 shows, it is important to test *Borrelia*-positive dogs for proteinuria. An association is assumed between *Borrelia* infection and renal disease, termed "Lyme-associated protein-losing nephropathy" (PLN) or "Lyme nephritis" (LN). In single studies, breeds commonly affected included labrador retriever, golden retriever and sheltie. In one study, 17 out of 20 Bernese mountain dogs were seropositive (IgG-IFA), but, because the immunohistochemical studies were negative, a familial nephropathy was assumed (Minkus et al. 1994). Dogs with suspected LN were younger (53 % ≤5 years with an average of 5.6 years) than dogs with similar diseases of different genesis (7.1 years for glomerulonephritis and 7.8 years for amyloidosis). There was no gender predisposition, and the animals were usually presented in summer and autumn with acute or chronic kidney disease accompanied by anorexia, vomitus, thromboembolism, hypertension, oedema, oliguria or pigmenturia (Dambach et al. 1997; Littman et al. 2006; Chou et al. 2006; Goldstein et al. 2013; Littman 2013). Clinical signs associated with the central nervous system (CNS) can also occur in association with nephropathy, for example as a consequence of vasculitis, hypertension, thromboembolism and uraemic encephalopathy. Laboratory abnormalities include non-regenerative anaemia, thrombocytopenia, hypoalbuminaemia, azotaemia, hyperphosphataemia and proteinuria, with a urine specific gravity below 1.022. In contrast to leptospirosis, LN is not the consequence of direct renal invasion of spirochaetes, but it is regarded as an immune-mediated disease. It is described as an infection-related sterile immune-complex glomerulonephritis with deposition of *Borrelia*-specific antigen-antibody complexes. This renal form is frequently IHC-positive for antigen but PCR-negative for DNA, with a p.i. disease incidence of 1.85 % (Dambach et al. 1997; Chou et al. 2006; Hovius 2013; Littman 2013). In regard to histopathology, LN is presented as an immune-mediated membranoproliferative glomerulonephritis ("MPGN"), combined with lympho-plasmocytic interstitial infiltrates and tubulonephrosis. In one study, 27 out of 32 dogs (84 %) with glomerulonephritis diagnosed by histology showed positive IHC staining of renal tissues (rabbit-derived polyclonal antiserum directed against a whole-cell preparation of *B. burgdorferi* strain B31 was used; Chou et al. 2006). Currently, it is not well understood whether LN is under-recognized, whether mild or early forms exist, and whether it responds to early intervention. According to Littman (2013), it may be under-diagnosed, especially in the case of mild or early forms in *Borrelia*-positive but asymptomatic dogs, or in positive symptomatic dogs not tested for proteinuria. On the other hand, LN may be over-diagnosed where a proteinuria in *Borrelia*-positive dogs is merely coincidental, due to urinary tract disease such as pyelonephritis, leptospirosis or a PLN of different genesis (infectious, genetic, systemic lupus erythematosus, neoplasia or haemolytic uraemic syndrome). There is also a need for further clarification as to whether non-*Borrelia* spp.-specific immune complex deposition also occurs in cases of LN. One study found that 16 % of suspected LN cases did not show a positive IHC response to immune complexes (Chou et al. 2006). A further open question remains as to whether the specific immune complexes cause LN or are only passively deposited there (or not adequately eliminated) due to host-pathogen factors such as the *Borrelia* spp. strain, genetic podocytopathies or immunopathological changes (Littman 2013). The latter hypothesis is supported by a recent case in which the authors considered LN as the expression of a breed-related (soft-coated wheaten terrier / SCWT) PLN, in which the *Borrelia* spp. antigens may have triggered the development of the initial immune complexes (Horney and Stojanovic 2013). This is additionally corroborated by a recent human case of Lyme-associated glomerulonephritis with a probable underlying IgA nephropathy at baseline (chronic ethanolic hepatitis with polyclonal increase of IgA; Rolla et al. 2013).

There are currently no officially recognized tests able to predict which dogs might be susceptible to

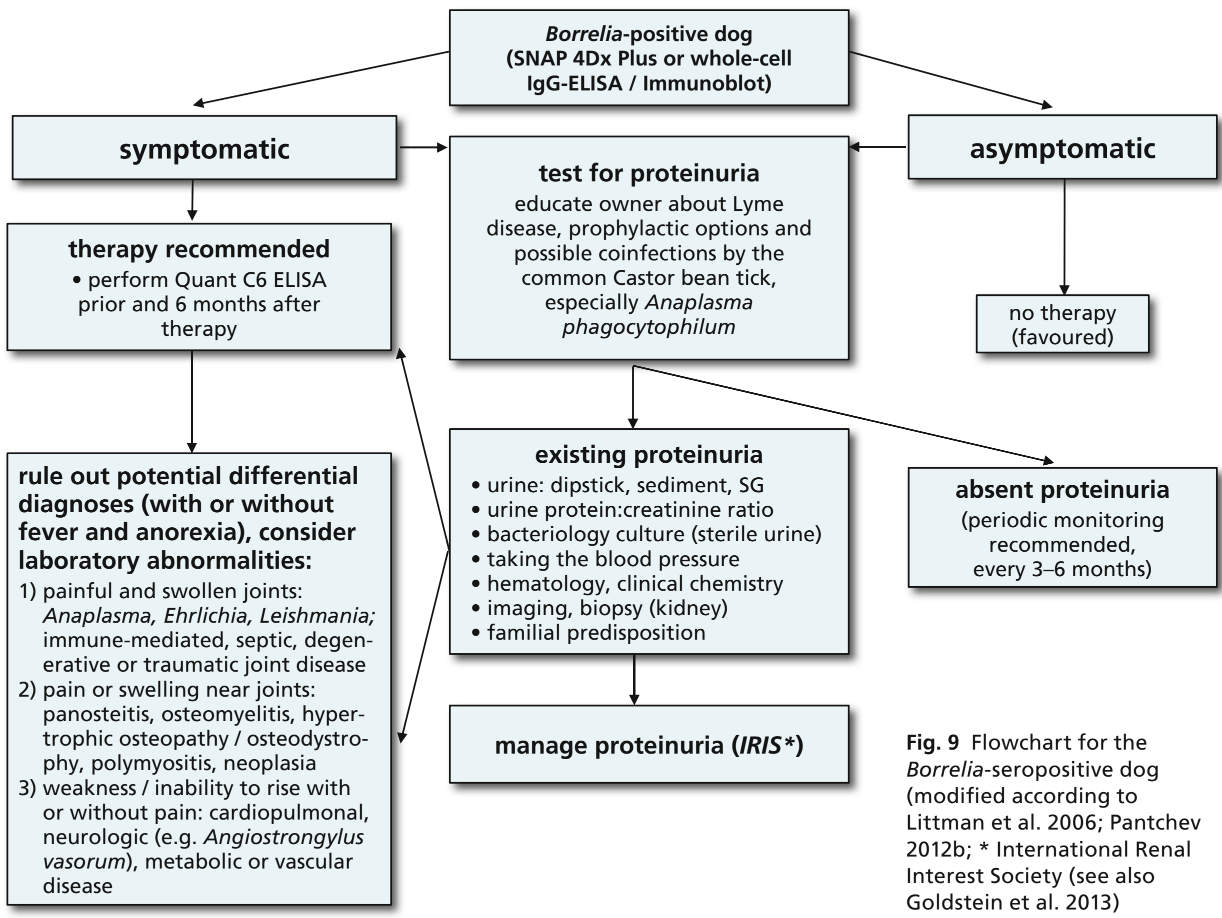

Fig. 9 Flowchart for the *Borrelia*-seropositive dog (modified according to Littman et al. 2006; Pantchev 2012b; * International Renal Interest Society (see also Goldstein et al. 2013)

LN, although some attempts to fill this gap were made in the past. Despite monthly tick prophylaxis, a study group of dogs showed 18.7 % seropositivity using a C6-antibody-based test (SNAP® 3Dx®), but no correlation with microalbuminuria (present in 6.1 %; E.R.D. HealthScreen Urine Test) was found (Goldstein et al. 2007). This group consisted of young, clinically normal labradors and golden retrievers (median age 1.5 years; n = 268), in a Lyme-endemic region in the USA. However, this study only compared qualitative C6 seropositivity with microalbuminuria, and then only in young dogs. In experimentally infected dogs, Susta et al. (2012) found an urine protein: creatinine ratio (UPC) 120 days p.i. that was elevated, compared with the ratio 90 days p.i. in 8 out of 17 animals. However, the difference was not statistically significant, the chosen cut-off of <1 appears to be too high (see below) and the method used to determine the UPC was not stated. In a naturally infected, 6-year-old SCWT in Canada, mentioned above, which was initially presented with lameness, peripheral lymphadenopathy, anorexia and weakness, ACAC of 176 U/ml and an elevated UPC (8.58) were found (Horney and Stojanovic 2013). In addition to supportive treatment for a suspected familial, breed-related PLN, doxycycline was administered (5 mg/kg b.i.d. for 4 weeks). ACAC subsequently decreased to 41 U/ml and the UPC to 1.73. Results of the latter study indicate that ACAC could represent a test able to predict which dogs are predisposed for development of LN or which could facilitate the diagnosis of LN. The correlation between (high) ACAC and proteinuria in dogs (without preselection based on age) has not been previously investigated.

Recently, we evaluated 103 canine samples: single samples of serum and urine provided by European veterinarians with the request for Quant C6 ELISA (serum; performed according Levy et al. 2008) und UPC (urine; for methods see Table 4) during 2009–2013 (Pantchev 2014). Submissions were received from Germany and other European countries (n = 71 from Germany, n = 7 from Austria, n = 1 from the Czech Republic, n = 4 from Denmark, n = 1 from France, n = 4 from Finland, n = 2 from Luxembourg, n = 6 from the Netherlands, n = 2 from Norway and n = 5 from Sweden). Data were obtained by testing serum and urine in parallel. Subsequently, samples were divided into 3 groups on the basis of their ACAC: <10, 10–30 and >30 U/ml and a statistical comparison (CS) with their UPC value (>= 0.6 as a cut-off) was conducted. In total, 55 samples had an UPC greater than or equal to 0.6 (53.4 %; 95 % CI: 43.3–63.3) and 46 dogs showed an ACAC above 30 U/ml (44.7 %; 95 % CI: 34.9–54.8). Dogs with ACAC in the moderate-to-high range (over 30 U/ml; group 3) also showed a statistically significantly higher probability of increased UPC (greater than or equal to 0.6 according to IRIS criteria) in urine samples compared to dogs with ACAC in the low (10–30 U/ml; group 2) and negative to very low range (under 10 U/ml; group 1; Table 4).

Table 4 Correlation of anti-C6 antibody concentration (Lyme Quant C6 ELISA in Units/ml) and proteinuria (urine protein:creatinine ratio/UPC) based on 103 canine serum and urine samples tested in parallel from the years 2009–2013.

Group	Quant C6 Units/ml*	anti-C6 antibody level	UPC >= 0.6**	% and 95% CI***
1	< 10	negative to very low positive	15/39	38.5; 23.3–55.4
2	10–30	low	5/18	27.8; 9.7–53.6
3	> 30	moderate to high	35/46	76.1; 61.2–87.5

* allocation according to Levy et al. 2008

** cutoff according to Goldstein et al. 2013 and www.iris-kidney.com; methods in brief:
creatinine determination in mg/dl by means of the Jaffe method (blank corrected, kinetic and photometric),
determination of protein in mg/dl, turbidimetric (505 nm) after the addition of benzethonium chloride

*** statistical significance of increased UPC with regard to anti-C6 antibody concentration (chi-square test; p < 0.05)
Group 1 versus 2: p = 0.432; not significant
Group 1 versus 3: p < 0.001 significant
Group 2 versus 3: p < 0.001 significant
Groups 1 and 2 (20/57; 35.1%, 95% CI: 22.9–48.9) versus 3 (35/46): p < 0.001 significant

Table 5 Total submissions for the Lyme Quant C6 ELISA from the years 2009–2013 (n = 15,757 canine serum samples) and fractions with an anti-C6 antibody concentration over 30 U/ml.

Year	Total samples	Samples over 30 U/ml	percentage	95 % CI	significance*
2009	2944	564	19.2	17.8–20.6	↓; p < 0.001
2010	2882	605	21.0	19.5–22.5	↓; p < 0.001
2011	3288	753	22.9	21.5–24.4	ns; p = 0.111
2012	3292	911	27.7	26.2–29.2	↑; p < 0.001
2013	3351	981	29.3	27.7–30.8	↑; p < 0.001
2009–2013	**15757**	**3814**	**24.2**	**23.5–24.9**	–

*** compared to overall result 2009–2013 (chi-square test; p < 0.05); ns = not significant; –: not applicable**

On the other hand, groups 1 and 2 showed no difference with regard to increased UPC (Table 4). Dogs from group 3 with ACAC over 30 U/ml (median 137 U/ml) and elevated UPC (median 6.5) were also significantly younger (average age of 5.5 years, median 5 years) compared to dogs from groups 1 and 2 with an elevated UPC (median 1.4), but ACAC was less than or equal to 30 U/ml (average age of 7.9, median 9 years). This corroborates the above mentioned results by Dambach et al. (1997) for LN based on 49 canine cases with renal lesions putatively associated with *B. burgdorferi* infection. This might indicate that proteinuria in dogs with C6 antibody levels lower than or equal to 30 U/ml occurs coincidentally and might have another genesis as proposed by Dambach et al. (1997). Within Group 3 (>30 U/ml) 11 of 46 dogs (23.9 %; 95% CI: 12.5–38.8) with UPC below 0.6 also showed a lower median ACAC (94 versus 137 U/ml); only 4 of these 11 dogs (36.4 %; 95% CI: 9.8–69.9) showed ACAC >100 U/ml, compared with 21 of 35 dogs (60 %; 95% CI: 42.1–76.2) with ACAC >100 U/ml and increased UPC; however, this difference was not significant ($p = 0.435$). Another study in dogs showed a median UPC of 5.7 (0.47 to 43.4) for glomerulonephritis, 22.5 (11.2 to 46.6) for amyloidosis and 2.9 (1.5 to 10.5) for chronic interstitial nephritis (CIN; Center et al. 1985). So it is suggested that UPC in dogs is usually less than 5 with CIN, whereas primary glomerular disease is associated with UPC above 5. This corresponds well to the median value of 6.5 for UPC found in our study for dogs in group 3 (see above).

Our data suggest that ACAC, in particular a concentration over 30 U/ml, in combination with a corresponding history can serve as a marker of LN in dogs. Further studies with a larger number of samples are needed to show whether an ACAC above 100 U/ml can be regarded as high and may indicate an early form of LN in dogs without proteinuria. The correlation between high antibody levels and disease has generally been disputed (e.g. Littman 2013). A study in which cases of high ACAC in asymptomatic dogs were found (Levy et al. 2008), might further support this hypothesis. However, no haematology, clinical chemistry or urine analyses were performed by Levy et al. (2008). While dogs were clearly described as asymptomatic, they were not defined as healthy as suggested by Dantas-Torres and Otranto (2014b). For example, it is not known whether these dogs with initial ACAC in the moderate-to-high groups (Levy et al. 2008) may have had proteinuria. Whether ACAC also correlates with the level of specific immune complexes (which were higher in dogs with clinical signs of infection), as proposed by Goldstein and Atwater (2006), should be a topic for further studies.

In addition to the above retrospective study comparing ACAC with UPC in a dog collective (Table 4), we evaluated data from 15,757 canine serum samples submitted by veterinarians for a Lyme Quant C6 ELISA only (for methods see above) in 2009 to 2013 (previously unpublished data). The largest fraction of animals was the group under 10 U/ml (n = 9,669; 61.4 %; 95 % CI: 60.6–62.1). The next group with 10–30 U/ml contained 14.4 % of the cases (n = 2,274; 95 % CI: 13.9–15) and the third group with ACAC >30 U/ml contained 24.2 % (n = 3,814; 95 % CI: 23.5–24.9). The differences in group 1 and 3 with ACAC under 10 U/ml and over 30 U/ml were statistically significant compared with the dogs with suspected LN (parallel testing for ACAC in serum and UPC in urine; Table 4) ($p = 0.003$ and $p < 0.001$, respectively). Interestingly, the percentage of dogs with a request for Lyme Quant C6 ELISA and with ACAC over 30 U/ml has increased continuously and significantly over the years, from 19.2 % (95 % CI: 17.8–20.6) in 2009 to 29.3 % (95 % CI: 27.7–30.8) in 2013 (Table 5). It is not clear yet what effects might be responsible for this significant increase in the proportion of samples over 30 U/ml. Possible reasons include better preselection by the attending veterinarian of dogs matching the clinical picture in terms of age, breed, etc. Other reasons might be geographical (region-specific) influences or vector-related influences (other *Borrelia* spp. strains with different plasmid content and virulence in the field). New

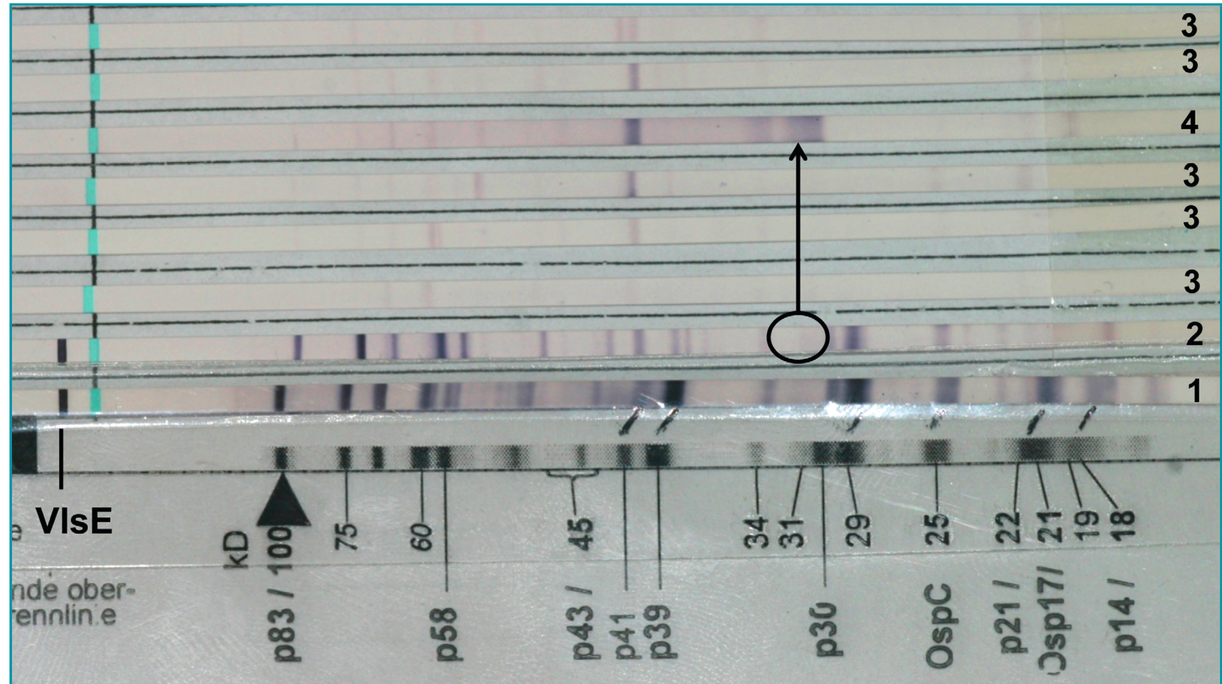

Fig. 10 Immunoblot of a dog with suspected Lyme nephritis (LN); 1: positive control serum with (below) a protein marker with molecular weight of the bands in kDa; 2: suspected LN case: typical band pattern of an infected, unvaccinated dog (e.g. VlsE, p83/100, p58, p39, p29/OspD and OspC protein bands present, but OspA (31 kDa; predomimant signal associated with vaccination) absent (oval); 3: negative samples (some only reactive for the 41-kDa flagellin band); 4: prominent OspA band of a vaccinated, non-infected dog (arrow) (*Borrelia* "MiQ" + VlsE ViraBlot/Viramed; secondary antibodies: AP-conjugated goat anti-dog IgG (H+L) – KPL).

studies differentiating *Borrelia* spp. by means of multilocus sequence typing (MLST) indicate that different *Bb*ss strains have different dissemination potentials within the organism, pointing to different pathogenic properties (Hanincova et al., 2013). Other relevant effects might include co-infections or co-diseases and a shift in prophylactic methods used against ticks and in the frequency or type of vaccinations (see below).

In dogs with suspected LN, the importance of testing the band pattern in immunoblot assays is also highlighted (Littman 2013). In one suspected case tested in our laboratory (hunting crossbreed dog, 5 years old; previously unpublished data), the immunoblot test showed a field infection with almost all specific bands reacting positively, including VlsE, but without the classic vaccine band OspA (Fig. 10). The dog also showed severe proteinuria (UPC of 17.8), hypoalbuminaemia, azotaemia, anaemia, and a high ACAC of 430 U/ml. In comparison, the total IgG level, measured using a non-C6/VlsE-based whole-cell ELISA (*B. burgdorferi* veterinary ELISA, Virotech), was relatively low at 35.6 units (positive cut-off: 12 units). X-ray of the kidneys revealed no abnormalities; a biopsy was not performed. No other causes were found for the renal damage, following negative tests for *Leptospira*-specific antibodies (microagglutination test; MAT), *L. infantum*-specific antibodies (ELISA; Wolf et al. 2014), *A. phagocytophilum*-specific and *E. canis*-specific antibodies (IFA and SNAP® 4Dx®; Dyachenko et al. 2012) and antinuclear antibody (IFA, Kallestad™ HEp-2 cells, Biorad). A familial predisposition was ruled out. The dog was presented at the veterinary practice in August, showing fever, fatigue, vomitus and polydipsia.

Table 6 Three cases of canine granulocytic anaplasmosis in dogs presented to veterinarians in April (modified according to Pantchev 2010a; methods according to Dyachenko et al. 2012); ALKP: Alkaline phosphatase, IFA (indirect immunofluorescence assay), ALT: alanine aminotransferase, CRP: C-reactive protein, Ct value (= cycle threshold: lower values represent higher amount of pathogen DNA).

	Dog from Germany	Dog from the Netherlands	Dog from Austria
Age	6 years	10 years	11 years
Clinical findings	41.3–41.8 °C, peracute disease accompanied by reluctance to move, lethargy, anorexia, pale mucous membranes	40.9 °C, lethargy, upper abdominal pain	lethargy, anorexia, pale mucous membranes, severe diarrhoea
	Laboratory		
CRP (0–9.7 mg/l)	↑48.8	↑57.0	↑63.8
Thrombocytes (150–500 G/l)	↓50	unevaluable	↓118
Lymphocytes (1000–4000 /ul)	↓656	↓748	↓284
Monocytes (0–500 / ul)	↑1031	↑534	↑663
Albumin (32–47 g/l)	↓31	↓30	↓23.8
ALKP (<81 U/l)	↑436	→65	↑115
ALT (5–125 U/l)	→65.5	↑277	→50.4
Blood smear			
Real-time PCR	positive (Ct value: 16)	positive (Ct value: 26)	positive (Ct value: 14)
Serology (IFA)	negative (<1:50)	1:100 (2 weeks later 1:3200)	negative (<1:50)

Canine granulocytic anaplasmosis (CAG)

It is essential to take into consideration co-infections with *Borrelia* in the diagnostic workflow of CAG. Studies show that dogs co-infected with *A. phagocytophilum* and *Borrelia* spp. run twice the risk of developing disease with clinical signs such as lameness, fever, lethargy, joint pain (swelling) and anorexia compared to with single infections with either pathogen (Beall et al. 2008). The concurrent presence of an intracellular (*Anaplasma* spp.) and extracellular (*Borrelia* spp.) infection may lead to an adverse immunological interaction during the infection course (Krupka et al. 2007). In the majority of dogs, the clinical signs of CAG are non-specific and confined to the acute phase of the infection (Diniz and Breitschwerdt 2012). Thus, "CAG" pose a diagnostic challenge. The criteria for diagnosing this disease according to the Centers for Disease Control and Prevention (modified according to Kohn et al. 2008) include tick contact or blood transfusion with clinical symptoms or laboratory abnormalities, positive PCR and/or morulae in neutrophils and a four-fold increase in antibody levels within four weeks. There appears to be no breed-related predisposition, although there may be a predisposition based on age. For example, in the USA 35.3 % of affected dogs were between 8 and 10 years old, 58.8 % were at least 6 years old and

Table 7 Two ways to detect a pathogen (serology versus PCR): what are the differences?

Serology	PCR
Detects the immunological antibody response of the infected host	Detects nucleic acid of the infectious agent
Antibodies persist over time – suitable for screening	The infectious agent (or its DNA) must be present in the sample – suitable in the acute phase of the disease
The immune response takes time, so clinical symptoms may appear before a measurable antibody response	A positive result indicates the presence of the agent, but a negative result does not necessarily rule out infection

only 11.7 % were under oder equal the age of 1 year (Greig et al. 1996). In a Swedish study, 28.6% of the dogs were over the age of 9 and 0% under the age of 1 (Egenvall et al. 1997). The pattern is similar in humans, as patients are commonly in the 50- to 60-year age range, with children only rarely affected (Diniz and Breitschwerdt 2012). A possible explanation might be that repeated reinfection is necessary (hence older dogs), or a fully developed immune system is required, because *Anaplasma* spp. impact on various immunological processes in order to survive (Egenwall et al. 1997; Carrade et al. 2009; Woldehiwet 2010). There appears to be a degree of seasonality, in parallel with the emergence of ticks (approximately from April to September; Kohn et al. 2008; Carrade et al. 2009). The characteristic clinicopathological abnormalities and the options for specific diagnosis are illustrated in the form of a diagram in Table 6 on the basis of three case examples (one each from Germany, the Netherlands and Austria).

Among the clinicopathological abnormalities, thrombocytopenia is the most important alteration in more than 80 % of cases (Greig et al. 1996; Kohn et al. 2008; Carrade et al. 2009; Diniz and Breitschwerdt 2012). In one study, over half of the dogs reacted positively in a platelet-bound antibody test (Kohn et al. 2008). Other important abnormalities are lymphopenia (approx. 50 %), anaemia (approx. 60 %; mild to moderate, non-regenerative and normochromic), occasionally hypoalbuminaemia (mild to moderate) and an increase in alkaline phosphatase and possibly other liver enzymes such as alanine aminotransferase (Table 6). Proteinuria has been also described. A relatively new but definitely additional promising test appears to be the measurement of C-reactive protein (CRP), which can increase by up to tenfold in the acute phase of the disease (Pantchev 2010a). CRP levels might also be useful in monitoring the success of treatment, as they are not influenced by the administration of glucocorticoids, for example (Kjelgaard-Hansen et al. 2006). Because of the seronegativity of some clinical cases (see Table 6), a confirmed diagnosis of CAG, from a single diagnostic test, may not be sufficient. Following experimental infection (with various isolates; i.v. with autologous infected neutrophils, not with ticks), dogs tested positive by PCR (blood) from 2 days p.i., seroconversion occurred after 10–14 days p.i. using IFA, and morulae were visible in blood smears between days 10 and 11 p.i. (Scorpio et al. 2011). This shows that the results of diagnostic tests will vary according to the time point post-infection (Fig. 11) and that concurrent use of serology and PCR will therefore increase the likelihood of an accurate diagnosis (Table 7).

The options for specific diagnosis in veterinary practice are limited to the detection of intracytoplasmatic inclusions (morulae) in neutrophils (in stained blood smears) during the acute infection phase and to indirect serological procedures using the SNAP® 4Dx® rapid test (currently available as SNAP® 4Dx® Plus for the additional detection of antibodies to *Ehrlichia ewingii*). This assay detects antibodies (qualitatively; 17–30 days p.i.; Scorpio

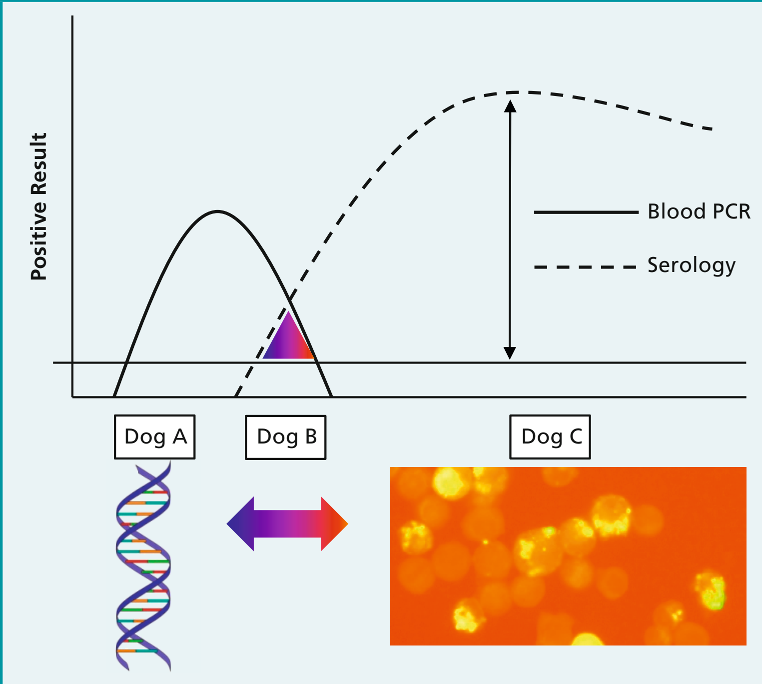

Fig. 11 Results of diagnostic tests according to time p.i.; **dog A:** shortly after infection (the dog may already show symptoms): pathogen DNA is present in blood samples and can be detected by PCR; no immune response occurred so far, antibody detection is not possible; **dog B:** shows clinical signs: PCR result is positive although the number of pathogens in the blood is already decreasing; serology is also positive due to a sufficient immune response, but the antibody level is still low (rising; color triangle represents overlap zone of both tests), **dog C:** weeks or months elapsed since infection; the dog may be asymptomatic: PCR is negative as pathogens are no longer circulating in the blood; serology is positive with a potentially high IFA titre; schematic display (modified according to Pantchev 2010b).

et al. 2011) to a major surface protein (p44/MSP2) of *A. phagocytophilum*. Antibodies to *Anaplasma platys* are also detected, as was shown in a experimental study (Gaunt et al. 2010). An advantage of this test is the option of simultaneously detecting antibodies to the C6 peptide of *Bb*sl, and for dogs with a history of travel in endemic regions, antibodies to *E. canis* and heartworm antigen. Further diagnostic options in reference laboratories include IFA for serology and specific real-time PCR tests for molecular detection. IFA allows semi-quantitative determination of antibody levels in blood (titre determination). This is useful in order to observe fluctuations (increase or decrease) in antibody response. Seroconversion occurs two to five days after the initial appearance of morulae in peripheral blood (see above; Scorpio et al. 2011). Titres then increase within 2 to 3 weeks (see also Table 6; Scorpio et al. 2011) and decrease between 4 to 8 months after the detection of inclusions within a blood smear and treatment with doxycycline (Egenwall et al. 1997). An increase in titres in some dogs in the Swedish study after the next (tick) season can most probably be explained by asymptomatic re-infections. A disadvantage of the *A. phagocytophilum* IFA compared with tests using specific peptides (e.g. SNAP® 4Dx®; Chandrashekar et al. 2010) is possible cross-reactivity with *E. canis* antibodies. The strength of the cross-reactivity of such sera when tested with *A. phagocytophilum* antigen increases with the duration of the *E. canis* infection and the *E. canis* antibody titre (Harrus et al. 2012). For example, in dogs infected experimentally with *E. canis*, no cross-reactivity with *A. phagocytophilum* antigens was observed in the acute phase; however, cross-reactive antibodies were first detected on day 55 p.i. and were then found in all dogs by day 150 p.i. (Harrus and Waner 2011). The PCR for direct detection of pathogen DNA in blood is more sensitive than the microscopic scanning of stained blood smears, demonstrated with experimentally infected dogs. The dogs tested positive for *Anaplasma* spp. DNA, 6 to 8 days before morulae could be detected in peripheral blood (Scorpio et al. 2011). The authors also showed that in the canine model the bacterial load (quantitative real-time PCR) correlated with platelet decline, observed until the end of the study period (60 days p.i.). However, there

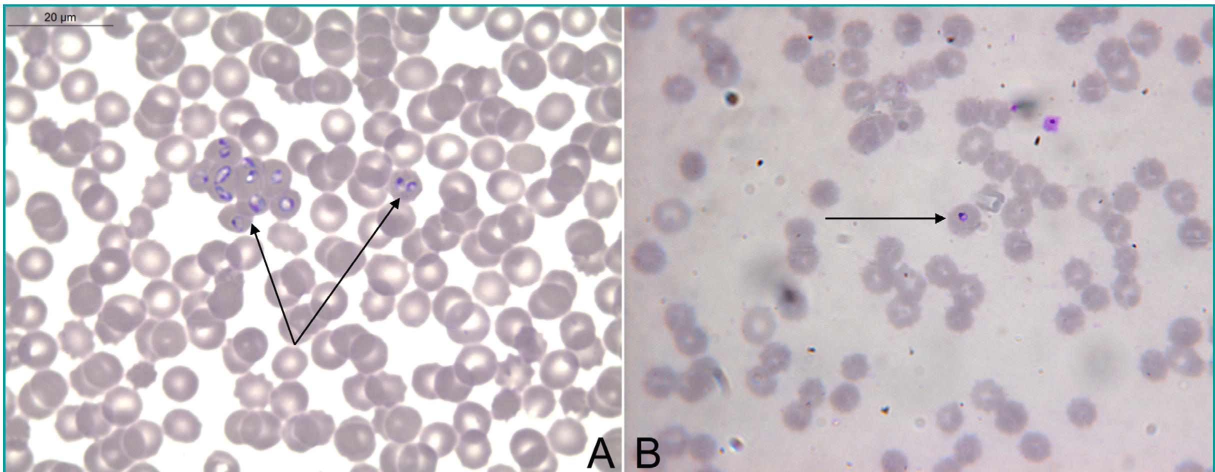

Fig. 12 A: large *Babesia* (arrows); in this case characterized as *Babesia rossi* by SSU rDNA amplification with subsequent sequencing according to Carret et al. (1999) in a stained blood smear from a dog imported from South Africa
B: atypical, smaller, rounded form of *Babesia canis* (arrow) characterized by SSU rDNA amplification and sequencing (see above); scale bar = 20 µm

were some differences based on the *A. phagocytophilum* strain and cell type used for i.v. infection. PCR allows the differentiation of *A. phagocytophilum* and *A. platys* (the latter is important in dogs with a history of travel to endemic regions; Dyachenko et al. 2012), which again can cross-react serologically (Gaunt et al. 2010). There have been recent reports of PCR-positive findings in skin biopsies from dogs with skin lesions of unknown origin and a histological picture of vasculitis (Berzina et al. 2014). The involvement of *A. phagocytophilum* in such cases and the interpretation of DNA detection in skin tissue require further clarification.

Canine babesiosis

For the diagnosis of canine babesiosis the following points should be considered (according to Pantchev 2012a): anamnesis including history of travel in endemic areas and tick infestation, blood transfusion or other possible non-vector-borne transmission routes (see above), clinical findings and laboratory abnormalities, direct pathogen detection in blood smears stained using Giemsa, Wright or Diff-Quik or DNA detection by PCR, and indirect pathogen detection by serology (ELISA, IFA). Typical laboratory findings, which differ depending on the *Babesia* species (see Tables 2 and 3), are haemolytic anaemia (initially non-regenerative, normocytic and normochromic, later regenerative, macrocytic and hypochromic), secondary immunohaemolytic anaemia with positive Coombs test, spherocytes, leukocytosis with a left shift in many cases, and thrombocytopenia and neutropenia. Further clinicopathological abnormalities are haemoglobinuria, bilirubinuria, bilirubinaemia and proteinuria (due to intravascular haemolysis), an increase in aspartate aminotransferase, alanine aminotransferase (hepatopathy) and azotaemia, and chronic hypoalbuminaemia (hepatopathy and glomerulopathy) (Ayoob et al. 2010; Birkenheuer 2012). The specific diagnostic options in veterinary practice during the acute phase are limited to stained blood smears, which allow differentiation between large (Fig. 12A) and small *Babesia* species. Capillary blood or buffy coat preparations are more suitable in many cases, because infected erythrocytes sequestrate in capillaries and *Babesia* tend to invade reticulocytes rather than mature erythrocytes (Irwin 2009; Ayoob et al. 2010; Ogo et al. 2011). Differentiation of large versus small forms is usually possible, except in rare cases of atypical

B. canis stages which unexpectedly can take on a small, rounded form (Fig. 12B). In these cases, PCR is indicated to ensure reliable differentiation (see also Demeter et al. 2011). Small and large *Babesia* species should be clearly differentiated at diagnosis, because they require different treatment protocols (see below).

Further testing options in a reference laboratory include quantitative serology and PCR. Large *Babesia* (*B. canis, B. vogeli* and *B. rossi*) are known to cross-react with each other's antigen using IFA, even though they produce stronger IFA reactions in homologous systems (e.g. *B. canis* antigen with *B. canis*-infected dog; Uilenberg et al. 1989; Jongejan et al. 2011). Beyond that, *Babesia* can also cross-react at genus level (e.g. *B. canis* and *B. gibsoni*), irrespective of the test used (IFA or ELISA), if the test uses whole-cell antigen (Belton 2003; Birkenheuer et al. 2003; Ogo et al. 2011; own observations). Within the above-mentioned whole-cell *B. canis* ELISA, cross-reaction with *B. vogeli* has already been shown (Dyachenko et al. 2012). Furthermore, seven out of nine molecularly confirmed *B. gibsoni* canine samples from Hungary, Sri Lanka, Germany and Czech Republic (seven of them were fighting dogs; real-time PCR according to Dyachenko et al. 2012) also showed clearly positive reactions within the same *B. canis* ELISA (own unpublished observations). Additionally, three canine samples from mixed-breed dogs from Argentina (provided by Diego Fernando Eiras, Universidad Nacional de La Plata) suspected of having *R. vitalii* infections (merozoite stages in erythrocytes and leukocytes; see Eiras et al. 2014) reacted clearly positively as well (so far unpublished data). *R. vitalii* represents a large piroplasm occurring in Brazil and Argentina, and is proposed as the causative agent of canine rangeliosis (known as "bloody ears" disease; Eiras et al. 2014; see also Table 2). In general, serology cannot distinguish between animals with an acute or chronic infection (Irwin 2009; Ogo et al. 2011). Specific antibodies were detected on day 14 p.i. in 5 out of 7 dogs experimentally infested with *D. reticulatus* and infected with *B. canis*, and all animals seroconverted on days 21 and 28 p.i. (with titres from 1:160 to ≥1:2560; Jongejan et al. 2011). Similar results were achieved after sporozoite-induced subcutaneously infection with a French *B. canis* isolate (seroconversion at 13 to 20 days p.i.; Uilenberg et al. 1981). Following experimental parenteral infection with *B. vogeli* in dogs, seroconversion occurred seven days p.i., with increasing titres from 14 to 21 days p.i. and maximum titres of 1:1280 to 1:5120 on 48–55 days p.i.. The antibody titres then decreased slightly until day 160 (approximately 2 dilution levels) to 1:640–1:1280 (Brandao et al. 2003). The serology results for the treated group in the latter study (treatment with imidocarb, 7 mg/kg on days 15 and 27 p.i.) were significantly different, as titres were lower and decreased faster (from day 34 p.i. onwards). Capillary parasitaemia accompanied by fever was observed from 2 days p.i. until 41 days p.i. (intermittently during this time; Brandao et al. 2003). The disappearance of *Babesia* from the blood approximately correlated with the maximum antibody titres. Interestingly, antibody titres ≥1:320 showed a protective effect. In the latter experimental study parasitaemia had been observed 2 days p.i., which is earlier compared to the typical case in natural (tick-borne) infections of 6 to 20 days p.i. (Brandao et al. 2003). This time lag may be due to the fact that, in the study cited, the dogs were inoculated with erythrocytes containing merozoites, while in natural settings the sporozoites are the initial stages introduced by the ticks. After sporozoite-induced infection (subcutaneously with a French *B. canis* isolate) the prepatent period (blood smear from ear veins) was 5–7 days, and it was slightly shorter than the incubation period for fever (6–8 days; Uilenberg et al. 1981). The advantages of serology are the diagnosis of cases with low-level or intermittent parasitaemia (Uilenberg et al. 1981; Brandao et al. 2003) and of chronic infections (Irwin 2009). The limitations are cross-reactions (especially between different *Babesia* species, see above) and false-negative findings in young or immunosuppressed dogs, or early in

the course of infection before seroconversion has occurred (Ayoob et al. 2010). This final limitation requires the testing of paired sera, with the second taken after an interval of 2 to 3 weeks. It should also be considered that dogs in *B. canis*-endemic regions may show high titres without showing clinical signs (Ayoob et al. 2010). Specific treatment with imidocarb and diminazene early in the course of infection (approx. 1 week p.i. after direct detection of *B. canis* in blood) prevented seroconversion in one out of eight dogs, as shown with an experimental model (Beugnet et al. 2014).

Another important aspect of *Babesia* diagnostics in dogs is the use of PCR tests. The detection limit of PCR protocols is given variously as 50 organisms per ml in one protocol and 9 per µl in another (reviewed by Irwin 2009). In one study, it was 1,300 times lower (better) than the detection limit of light microscopy (Birkenheuer et al. 2003). A single microscopic blood smear test, compared with PCR as the established gold standard, had a relative sensitivity of 38 % and a relative specificity of over 99 %; there was moderate agreement between the results of the two methods (*kappa* = 0.54; Globokar Vrhovec 2013). In the patient cohort tested by Globokar Vrhovec (2013; dogs living in Germany), microscopy detected *Babesia* significantly more frequently in animals that had stayed within Germany compared to animals with a history of travel or imported animals. These data agree with the serological data obtained for Germany (see above). It is possible 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. Another major advantage of PCR is that, following a positive *Babesia* PCR result, differentiation of *Babesia* species can subsequently be performed by either species-specific real-time PCR (Dyachenko et al. 2012), or SSU rDNA amplification with subsequent sequencing (Beck et al. 2009). This is important in order to select an appropriate treatment, as large and small *Babesia* require different therapeutic approaches. The treatment of choice is imidocarb for large forms and a combination of atovaquone and azithromycin for *B. gibsoni* (Pantchev 2012a; Birkenheuer 2012). PCR itself has limitations, especially when no organisms are present in the blood (as in chronically infected animals), or if the parasitaemia is intermittent (Brandao et al. 2003; Irwin 2009). In these cases it is helpful to repeat PCR tests, or combine PCR with serology. In the above-mentioned study, which evaluated data from three different diagnostic procedures for *B. canis* (dogs living in Germany, 2004 to 2006), antibodies were detected (by IFA) in 11.5 % (n = 2,653) of dogs, parasitic stages were found in Giemsa-stained blood smears in 2.1 % of samples (n = 9,966) and DNA was detected (by conventional PCR) in 3.3 % of submitted blood samples (n = 15,155; Globokar Vrhovec 2013).

Another currently available tool used for detection of *B. canis* protein fractions in canine serum is the mass spectrometry (MALDI-TOF; Adaszek et al. 2014). Latter authors identified a protein fraction of 51 – 52 kDa in samples of infected but not in control dogs. Further studies are necessary to estimate the sensitivity and specificity of this new technique for routine diagnostics with canine blood samples in comparison to currently applied species-specific real-time PCRs.

Prophylaxis

Prophylaxis is based on a range of different actions aimed at preventing infection or the development of disease following infection. There are four basic cornerstones of prophylaxis for TBD: vector prophylaxis with acaricides / repellents (directed against the ticks which carry the pathogens), chemoprophylaxis (generally targeted directly against the pathogen), vaccination (by pathogen-specific vaccination) and behavioural prophylaxis (reducing vector exposure by avoiding risk areas during the vector's periods of activity, for example; Pantchev 2013).

Acaricides / repellents

According to the product information for certain acaricide-based products, some ticks already present at the time of treatment are not killed within the first 48 hours and remain attached and visible. It is advisable to remove such ticks mechanically before starting prophylaxis. In one study, rotating devices (in order of success: tick-pliers (tick remover/extractor), lasso, tick twister) proved better than pulling devices (tweezers, tick card). However, in regard to the tick mouthparts, there was no difference between the two groups (Robisch 2010). With the above-mentioned in mind, it is important to begin prophylaxis before the first ectoparasite exposure, e.g. efficacy against ticks develops within two days after applying a collar (using the example of imidacloprid/flumethrin in Seresto®). Ticks infesting dogs in Europe do not tend to harm their hosts directly, with the main risk being that pathogen transmission can occur and disease potentially results from the infection. The applied compounds show different effects on ticks (repellency, antifeeding effect, disruption of attachment, expellency, killing effect), so it is important to use suitable acaricides to kill the ticks as quickly as possible before pathogens are released. It is even better to prevent ticks from attaching (tick repellency *sensu stricto*; Halos et al. 2012). As one study showed, compounds with a repellent effect can prevent TBD (*Anaplasma*/*Borrelia*) more efficiently than active substances, which only show a killing effect (Blagburn et al. 2005). It is therefore important for veterinarians to take note of the relevant product information.

Various acaricidal dog collars effective against ticks are licensed for use in Germany and Austria. They contain flumethrin/imidacloprid (Seresto®; duration of efficacy/DOE 7 to 8 months) or deltamethrin (Scalibor®; DOE 5 to 6 months) as active ingredients. Furthermore, a range of spot-on products is also available. It should be noted that products containing permethrin are to be avoided in cats because of their toxic effect on feline patients (e.g. Boland and Angles 2010). Permethrin-based products for dogs include: permethrin/imidacloprid (Advantix®; DOE for *I. ricinus* / *R. sanguineus* 4 weeks, for *D. reticulatus* 3 weeks), permethrin only (Exspot®; DOE *I. ricinus* / *R. sanguineus* up to 4 weeks), permethrin/indoxacarb (Activyl Tick Plus®; DOE up to 5 weeks for *I. ricinus*, up to 3 weeks for *R. sanguineus*), permethrin/fipronil (Frontect®; DOE 4 weeks), or permethrin/dinotefuran/pyriproxyfen (Vectra 3D®; DOE for *I. ricinus* / *R. sanguineus* for 1 month, *D. reticulatus* up to 3 weeks). Non-permethrin-based products include: fipronil (Frontline®/Frontline Combo®; DOE up to 4 weeks), fipronil/amitraz/(S)-methoprene (Certifect®; DOE 5 weeks), or pyriprol (Practic®; DOE 4 weeks). Collars should be adjusted to the right size, thus not too tight or too loose, with two finger-widths between the collar and the animal's neck. Spot-on products should be applied correctly, thus the spot-on solution should be applied to the skin, and not the hair of the dog, and licking off by the animal itself or companion animals needs to be prevented. Oral products using the new active substance class of isoxazolines are currently also available as fluralaner (Bravecto®; DOE for *I. ricinus, D. reticulatus, D. variabilis* 12 weeks, for *R. sanguineus* 8 weeks), and afoxolaner (NexGard®; DOE for *D. reticulatus, I. ricinus, R. sanguineus* up to 1 month) in the form of chewable tablets for dogs. These are systemic acaricides, thus ticks have to attach to the host and start to feed in order to come into contact with the active ingredients. One current study showed that afoxolaner was able to prevent infection with *B. canis* in dogs experimentally infested with *D. reticulatus* ticks (Beugnet et al., 2014).

Vaccination

In controlled approval studies in dogs, ectoparasiticides are usually tested using 50 adult ticks (EMEA/CVMP/005/00 Rev. 2). Consequently, 100 % protection cannot be guaranteed in every case, especially in conditions of high tick exposure (numbers exceeding 50 ticks, e.g. hunting dogs). Seropositivity against *A. phagocytophilum* can also be used as an indicator of inadequate tick

prophylaxis. Vaccination may therefore be considered in such individual cases, if available. However, it should not be regarded as a replacement for tick prophylaxis.

Borrelia vaccination

Borrelia vaccination has been a topic of controversy in recent years (Littman et al., 2006; Straubinger and Pantchev 2010; Greene et al. 2012; Pantchev 2013). One reason for this is that clinical borreliosis is rare in dogs. Koch's postulates have only been fulfilled experimentally for a clinical picture of transient fever, anorexia and clinical arthritis with lameness as well as reluctance to move, which was detected in puppies but not in dogs ≥6 months (e.g. Appel et al. 1993; Straubinger 2000; Straubinger et al. 2000; Summers et al. 2005; Susta et al. 2012; Wagner et al. 2012). In contrast to humans, no erythema migrans has been observed in dogs (Appel et al. 1993), but synovial lesions were significant in infected dogs in all canine experimental studies (Appel et al. 1993; Summers et al. 2005; Susta et al. 2012; Wagner et al. 2012). After natural infection, lameness has been described in <5% of dogs (Levy and Magnarelli 1992; McKenna et al. 1995; Hovius et al. 2000; Chou et al. 2006; Littman et al. 2006), less than 1–2% show kidney disease (Dambach et al. 1997; Chou et al. 2006; Littman 2013), and the very rare cases with cardiac (Chou et al. 2006; Agudelo et al. 2011; Janus et al. 2014) and neurological (McKenna et al. 1995) involvement are assumed rather than documented. No association was found between exposure to either *Borrelia* species (or *A. phagocytophilum)* and neurological signs or inflammatory CNS disease in retrospective and prospective studies (Jäderlund et al. 2007, 2009), which have been subsequently confirmed by experimental infection (Krimer et al. 2011). The different clinical picture of what is seen in veterinary practice and after experimental infection in dogs is still not well understood. It has been suggested that clinical illness might result from the host's own inflammatory response (Straubinger et al. 1998; Summers et al. 2005; Greene et al. 2012). Experimental infections are performed usually only with a single breed (beagle, e.g. Summers et al. 2005), but for LN, a breed predisposition has been proposed. It might indicate that other breed-related factors are necessary for disease development, as proposed by Appel et al. (1993) and Horney and Stojanovic (2013). Moreover, multiple exposures in nature (reinfections) or other *Borrelia* spp. strains (e.g. also *B. miyamotoi*; Schreiber et al. 2014) with a different plasmid content and virulence (Hanincova et al. 2013) are additional preconditions. For example, the risk of infection with *Borrelia* spp. for a single dog has been calculated to be up to 23% per year in rural areas of eastern Austria (Leschnik 2014). Experimentally, disease could be induced after repeated exposure (3 times with 2-week intervals; tick nymphs with 16% *Bb*ss infection rate), but single exposure to 100 nymphs (same infection rate) did not result in clinical signs (Appel et al. 1993). This was supported later on by another study, where two cycles of tick challenge increased the frequency of clinical arthritis compared to a single challenge (Straubinger et al. 1998). Furthermore, co-infections transmitted by the common tick vector *I. ricinus*, especially *A. phagocytophilum* (Summers et al. 2005; Beall et al. 2008), but also other little-studied organisms in regard to their pathogenicity in the dog such as *Bartonella* spp. (Diniz et al. 2009), *Rickettsia* spp., (small) *Babesia* spp. (Appel et al. 1993; Skotarczak 2014) or *Candidatus* Neoehrlichia mikurensis (Diniz et al. 2011; Schreiber et al. 2014; see Table 1), might play a role. In particular, co-infections (by different strains or species) are probably one of the least experimentally investigated areas to date, as is the (immunological) influence of repeated infections over a longer period of time.

A further reason for controversy regarding vaccination in Europe in recent years is the fact, that different vaccines have been commercially available. For example, one vaccine contains only *Bb*ss (isolated in France from an infected *I. ricinus*; Wiedemann and Milward 1999) and another contains two isolates (*B. garinii* and *B. afzelii)*. One

point of controversy is that, although *B. garinii* and *B. afzelii* are indeed commonly found in ticks in various parts of Europe (e.g. Piesman and Gern 2004), their clinical relevance to dogs has not been conclusively demonstrated (see above). A new product available in Germany and Austria is Merilym 3 (inactivated *Bb*sl vaccine), which contains all three genospecies (*Bb*ss, *B. garinii* and *B. afzelii*). A debatable question is whether this trivalent vaccine has advantages over purely *Bb*ss-based products. Naturally infected dogs showing clinical signs of borreliosis have been found to have co-infections with *Bb*ss and other genospecies (especially *B. garinii*; Hovius et al. 2000). Although the effects of co-infections in the dog have not yet been studied in an experimental model, co-infection with *Bb*ss and *B. garinii* in a murine model showed a more severe progression of symptoms than an infection with *Bb*ss alone (Hovius et al. 2007). Because it is assumed that OspA antibodies (the basis of the vaccine) are not cross-protective among *Borrelia* species (Straubinger et al. 2002; Töpfer 2005), it would be worth considering whether the vaccination regime should include other species (*B. garinii*/*B. afzelii*) in addition to the canine pathogen *Bb*ss recognized to date. Based on its principle of action (mainly induction of antibodies to OspA; see above), vaccination prevents future infection, because anti-OspA antibodies ingested during feeding inactivate the *Borrelia* directly within the tick's gut (Straubinger and Pantchev 2010). Before vaccinating dogs, therefore, it is important to test adult dogs with an unknown infectious status and dogs that are already potentially infected. One reason is, that vaccination has no effect on an existing *Borrelia* infection. Another reason is that dogs with high ACAC following vaccination with a lyophilized product show very high levels of circulating immune complexes (Greene et al. 2012). Hebert and Eschner (2010), for example, included dogs in their vaccination programe only if they showed ACAC below 30 U/ml, together with normal blood and urine values. On the other hand, dogs with ACAC over 30 U/ml were treated (doxycycline 10 mg/kg q.d. for 28 days) and followed up after 30 days (laboratory tests and clinical examination). Dogs with no abnormal findings were admitted to the vaccination programe, with the Lyme Quant C6 ELISA being repeated six months later (see above). Dogs with glomerulonephritis and suspected LN may require a longer period of doxycycline administration. In such cases (UPC above 0.5), specific recommendations regarding treatment and monitoring can be found in Goldstein et al. (2013).

Babesia vaccination

The homologous (strain-dependent) protection provided by vaccination with Pirodog® (not licensed in Germany) is around 80 to 90 % in endemic regions of France but lower in other regions. This vaccine contains soluble parasite antigen (SPA) of *B. canis,* and a positive antibody titre (>1:160) should develop in around 75 % of animals following vaccination (source: product information). Broader protection, including protection against heterologous strains of *B. canis*, has been offered by the vaccine Nobivac Piro® (contains SPA of *B. canis* and *B. rossi* and was licensed for some years in the EU). This broader heterologous vaccine protection was achieved by the addition of *B. rossi* antigen (Schetters et al. 1995; Schetters 2005; Irwin 2009; Ayoob et al. 2010). However, this vaccine is not currently available on the European market.

Babesia chemoprophylaxis

The compound imidocarb, which has been used for chemoprophylaxis of infection with large *Babesia,* may induce potentially serious adverse effects, mainly due to cholinesterase inhibition, including also anaphylactoid reactions and renal or liver impairment (Ayoob et al. 2010; Dyachenko et al. 2012). Moreover, its prophylactic effect is of variable duration (reported to range from 2 to 6 weeks at a dose of 3–6 mg/kg BW; Deplazes et al. 2006) or even not present in sporozoite-induced experimental infections with a French *B. canis* isolate (6 mg/kg BW 2 to 5 weeks before infection; Uilenberg et al. 1981). In combination, it is either not recommended

for use as chemoprophylaxis (Ayoob et al. 2010) or only for dogs under one year of age (with the recommendation that older animals should be treated if symptomatic; Tenter and Deplazes 2006). Finally, imidocarb does indeed eliminate *B. canis,* but it also prevents the development of immunity, leaving dogs susceptible to reinfection (Brandao et al. 2003).

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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