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Review article

CANINE BABESIOSIS: WHERE DO WE STAND?

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Canine babesiosis is a tick-borne disease caused by protozoal haemoparasites of different *Babesia* species. Babesiosis is one of the most important globally extended and quickly spreading tick-borne infections of dogs. This comprehensive review gives an in-depth overview of *Babesia* species currently identified in dogs together with relevant vector tick species and their geographical distribution, life cycle and transmission of parasite. The main mechanisms in the pathogenesis of babesiosis are described and elucidated by recent literature overview. As *Babesia* infection causes a disease with very variable clinical manifestations, special attention is given to clinical signs, laboratory features and clinicopathological findings. The diagnosis of canine babesiosis by microscopy, serological and molecular methods is reviewed, together with recent advances in mass spectrometry based assays. Accurate detection and species recognition are important for the selection of the appropriate therapy, monitoring and prediction of the outcome of the disease. Finally, guidelines for the treatment and prevention of canine babesiosis are given.

Key words: babesiosis, dog, pathogenesis, tick-borne disease

INTRODUCTION

Babesiosis is one of the most important tick-borne diseases in dogs worldwide. It is a widespread hemoprotozoan disease that can infect various vertebrate hosts, including humans, and has a considerable global economic, human health and veterinary impact [1]. Babesiosis is recognized since ancient times, namely in the biblical Book of Exodus 9:3, a plague of the cattle of the Egyptian Pharach Ramses II was described that could have been red water fever of cattle based on hemoglobinuria as a prevalent sign [1]. At the end of 19th Century, in 1888, a Romanian bacteriologist Victor Babes discovered micro-organisms in erythrocytes of cattle [2] and later similar organisms in erythrocytes of sheep. These microorganisms were named in 1893 *Babesia bovis* and *Babesia ovis* [3]. Two years later the first case of *Babesia* spp. infection in dogs was described in

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Italy [4]. Interestingly, except the correct name of the genus (*Babesia*), several other names have been proposed since then, with the best known being *˝Piroplasm˝*. The name *˝Piroplasm˝* was given to these parasites due to their pear-shaped appearance after multiplication seen under light microscopy. Babesiosis and theileriosis are commonly grouped together under the designation ˝piroplasmoses˝. It is generally accepted that they are all synonyms of *Babesia* [5]. In addition to the above, *Babesia* and *Theileria* are related structurally, functionally and phylogenetically to *Plasmodium* species which cause malaria [6]. Namely, babesiosis and malaria, diseases caused by two genera of intra-erythrocytic protozoan parasites, share many common processes [7]. Also, since inflammatory mechanisms in these diseases are similar, as in other septic conditions, babesiosis is a form of ˝protozoal sepsis˝ [8,9]. All listed similarities between *Babesia* and *Plasmodium* have earned animal babesiosis the moniker of ˝animal malaria˝ [7,10]. Canine babesiosis, as one of the vector-borne diseases, is globally extended and quickly spreading owing to the expansion of tick habitats and increased mobility of animals. Today, there are over 100 species of protozoans on the basis of their exclusive invasion of erythrocytes in their mammalian hosts [11]. Parasites of the genus *Babesia* are preferably transmitted through tick bites and can infect different domestic and wild animals as well as humans [12-14]. At the same time, *Babesia* species are considered to be very specific in terms of infesting a small number of hosts [11]. Members of the *Babesia* genus parasitize erythrocytes of the definitive host, resulting in progressive anaemia and different clinical syndromes associated with *Babesia* infection.

TAXONOMY AND GEOGRAPHIC DISTRIBUTION

Species of the *Babesia* genus, as well as closely related *Theileria* genus, belong to the order Piroplasmida within the phylum Apicomplexa. Traditionally, *Babesia* species infecting dogs have been distinguished on the basis of morphologic appearance of the intra-erythrocytic stages of the parasite, host/vector specificity and susceptibility to drugs, and pragmatically are classified either as large forms (*Babesia canis* 2.5-5.0 μm) or small forms (*Babesia gibsoni* 1.0-2.5 μm) [11,15].

On the basis of geographical distribution of *B. canis* transmitted by different tick species, its antigenic properties and pathogenicity, large canine piroplasms were further subdivided into three subspecies. Namely, Uilenberg et al. [16] proposed that parasites transmitted by *Dermacentor reticulatus* be named *B. canis canis*, those transmitted by *Rhipicephalus sanguineus* be named *B. canis vogeli* and those transmitted by *Haemophysalis leachi* be named *B. canis rossi* [17]. Additional studies showed that these three groups of parasites, although they were morphologically identical, do not just have distinct tick vectors, but also different cross-immunity and pathogenicity [18]. Additionally, with the advent of molecular phylogenetic analysis in the 1980s, in particular genotyping of the small ribosomal subunit 18S gene, it was concluded that these subspecies are actually distinct species, named *B. canis*, *B. rossi* and *B. vogeli* [5,19-21]. Another, fourth large *Babesia* sp (Coco), genetically distinct (and as yet unnamed) has been found

in number of dogs with clinical signs coherent with babesiosis in North Carolina, New Jersey and New York, USA. In addition, this large *Babesia* sp, related to *Babesia bigemina*, has been reported in immunocompromised dogs, many of which had been splenectomized [22].

With regard to small piroplasms, only three genetically and clinically distinct species have been described and currently known to cause disease in dogs: *B. gibsoni*, *Babesia conradae* and *Babesia microti*-like piroplasm (*Theileria annae*) [23-25]. There is a controversy whether *Babesia microti*-like piroplasm, also named *Theileria annae*, is a member of *Babesia* or *Theileria* genus [26]. It is a small piroplasm species closely related to *B. microti*, discovered in dogs in northern Spain. Traditionally, *Babesia* and *Theileria* species are distinguished based on the sites of replication in the vertebrate host and transovarial transmission within the tick vector. While *Theileria* genus is characterized with multiplying in the lymphocytes or macrophages and then the erythrocytes of the vertebrate host and not being transmitted through the ovary in the tick vector, *Babesia* species multiply exclusively in the erythrocytes of the vertebrates and pass through the ovary into the tick eggs [27]. Due to disagreement on placement of *Babesia microti*-like piroplasm in the *Theileria* or *Babesia* genera, several synonyms have been used for the parasite such as *Babesia*-Spanish dog isolate, *Babesia (Theileria) annae*, *Babesia cf. microti*, while Baneth et al. suggested *Babesia vulpes* [28-30]. It was shown on the genetic level that this piroplasm is closely related to the rodent piroplasm *B. microti* and distantly connected to the representative *Theileria* species [26].

Babesiosis is one of the most important globally extended and quickly spreading tick-borne infections of dogs. As far as is known, all species of *Babesia* are transmitted by ticks, and in the case of canine babesiosis there is a close relationship between the *Babesia* species and the tick species. The occurrence of the disease is associated with the seasonal activity of tick vector, with clinical cases mostly in spring and autumn [31], but the dynamics of spreading of canine babesiosis in Europe has markedly changed in the last decade. The cause of these changes is probably due to the global warming, shifting use of the landscape, the increase of wild animal populations, spreading of vectors by wild birds and animals, and the change of habitat structure of wildlife [32]. The occurrence of canine babesiosis has been found to change in an annual seasonal pattern, namely the relatively mild and wet weather in spring and autumn is ideal for ticks although exact time of beginning and ending of the tick activity is strongly correlated with specific local climate conditions [32]. During dry summers babesiosis is almost never seen, but during the rainy summers and mild winter days babesiosis can appear. Geographical distribution of *Babesia* spp. infections in Europe is highly variable and dependent on the presence of the tick vectors in the environment and hosts [31,33]. Reported prevalence of *Babesia* spp. infections depends on various factors, such as various diagnostic techniques used for detection, country and population analyzed, as well as species of *Babesia* under investigation [34]. Seroprevalence depends also on use/lifestyle, and it was significantly higher in hunting/mixed dogs and shelter/outdoor dogs compared to companion/indoor animals [35]. The survey conducted in

Western Romania confirmed that hunting lifestyle is a major risk for acquiring *B. canis* infection [36]. Also, the increase in prevalence of seropositive dogs with age could be related to the cumulative increase of the exposure period to arthropod vectors over the years. Some authors have observed that the prevalence of antibodies to *B. canis* was significantly higher among German Shephards and Komondors, while in another survey *B. gibsoni* was typically associated with American Pit Bull Terriers [36,37]. However, other authors have considered that despite the statistical significance, babesiosis is not connected with the predisposition of particular breeds, but with the living conditions of dogs and the nature of their work [38,39]. In our survey, no significant gender predisposition to the disease was found, in contrast to another investigation which observed babesiosis more frequently in male animals [38].

B. canis is the main cause of canine babesiosis in Europe and sporadically around the world. This large *Babesia* species has been detected in dogs of various northern European countries, as well as in central and southern Europe, due to the abundance of its main vector, *Dermacentor reticulatus* [40]. Thus, the seroprevalence of *B. canis* in Croatia was 20.0%, Serbia 26.17%, France from 14.1% to 20.0% and in Western Romania 19.8% [35,36 41-43]. In other European countries the prevalence was 7.3% and 13% in Albania, 5.7% in Hungary, and from 0.8% to 17% in Italy [44-48]. The highest seroprevalence was obtained by Casini et al. [49] in the central regions of Italy, with the prevalence of 52-57%. The highest recorded seroprevalence is probably overestimated, considering that titers were generally low and cross-reaction is commonly reported for immunofluorescent antibody testing [50]. It is also important to point out that the positive serological results presented in these studies might be because of either current infection or previous contact/exposure to *Babesia*. That is the reason why serological screenings should be complemented with molecular-based detection methods to test if infections are active or not [50].

Molecular studies on canine *Babesia* infection have demonstrated *B. canis* infection in different countries of Europe, with the prevalence ranges from 2.3% in Italy [49], to 3.42% in Croatia [23], 4.6% in Slovenia [51], 25.3% Poland [52] and up to 44.8% in Romania [53]. Global changes of the climate and spreading of vectors caused the first reported case of *B. canis* on the north of Europe in Norway [54].

Babesia vogeli has a global distribution [55] and has been identified in Africa [56, 57], Asia [58], Turkey [59], Australia [60], North America [61] and South America [62]. In Europe, DNA sequences of *Babesia vogeli* were found in Slovenia [51], Albania [48], France [63], Spain [64], Portugal [65] and Croatia [23] with prevalence from 0.01% in Spain, 0.9% in France to 1.3% in Slovenia, 1.9% in Serbia [66], and 16.3% in Central and Southern Italy [67]. Interestingly, in the research conducted in Croatia, the higher prevalence of *B. vogeli* is observed in asymptomatic dogs (7%) versus symptomatic dogs (1.3 %) [23].

Finally, *B. rossi*, one of the most pathogenic species of *Babesia*, is endemic in the southern Africa, but can also be found in the eastern Africa [56,68].

Regarding the small *Babesia* species, *Babesia gibsoni* is the most prevalent, with global distribution. Clinical cases of *B. gibsoni* infection have been reported in Spain [69, 70] Germany [71], Croatia [23], Italy [72] and Serbia [73], but also in other parts of the world such as Asia, United States, Australia and Brasil [60,74,75]. It is known that in some parts of the world *Babesia gibsoni* can be transmitted by dog bites during the fighting. Among small *Babesia* species, *Babesia microti*-like sp. isolates have been identified in Portugal [76], Spain [77], France [78], Croatia [23], Serbia [66] and Sweden [79].

Table 1 presents *Babesia* species currently identified in dogs with relevant vector tick species and geographical distribution. There can sometimes be unexpected findings of piroplasm infections in dogs, such as identification of two piroplasm species which usually infect horses, *T. equi* and *B. caballi*, in two symptomatic dogs from Zagreb, Croatia. These species are usually transmitted by genera of vector ticks living in the Croatian area, such as *Hyalomma*, *Dermacentor* and *Rhipicephalus*, suggesting their role as vectors for canine host [23]. Also, it was previously thought that the specificity of *Babesia* species for the vertebrate host is restricted, but molecular analysis showed that the range of hosts can be wider. For example, there was a finding of *B. canis* using PCR and sequencing methods in free-ranging grey wolf populations in Croatia [13].

Table 1. Large and small *Babesia* species currently identified in dogs with corresponding vectors and geographical distribution

Form	Species	Vector tick	Geographical distribution	References
Large (2.5-5.0 μm)	*Babesia canis*	*Dermacentor reticulatus*	Europe	[23, 35, 36, 41-49, 51-54]
	Babesia vogeli	*Rhipicephalus sanguineus*	global (Africa, Asia, Australia, America, Europe)	[23, 48, 51, 55-67]
	Babesia rossi	*Haemaphysalis* spp.	Southern and Eastern Africa	[56, 68]
	Babesia sp.	Unknown	America (USA)	[22]
	Babesia caballi	Unknown	Croatia	[23]
Small (1.0-2.5 μm)	*Babesia gibsoni*	*Haemaphysalis longicornis*	global (Asia, Africa, Australia, America, Europe)	[23, 60, 69-75]
	Babesia conradae	*Rhipicephalus sanguineus* (assumed)	America (Western USA)	[24, 25]
	Babesia microti-like sp.	*Ixodes* spp. (assumed)	Europe (Spain, Portugal)	[23, 66, 76-79]

LIFE CYCLE AND TRANSMISSION

All *Babesia* species are transmitted via the saliva upon feeding of the tick vector on the vertebrate host. In this way, sporozoites are injected into the bloodstream and directly invade the red blood cells where they differentiate into trophozoites. Trophozoites multiply by binary fission into two or four merozoites (merogony), which exit the

erythrocytes thereby destructing them. The multiplication cycle of merozoites is continuing by the invasion of new erythrocytes until the death of the host or until the drug treatment and/or the immune response abolishes the replication of the parasite [5].

Some of the merozoites transform inside erythrocytes into pre-gametocytes (gamonts), which are ingested by the tick feeding on an infected host. In the tick gut, pre-gametocytes differentiate into male and female gametes, also called ray bodies or Strahlenkörper, and then fuse into an elongated diploid zygote (gamogony) [80]. Zygotes undergo meiosis creating haploid kinetes which multiply and enter the hemolymph, invading different tick organs, including the salivary glands and ovaries. In the salivary glands there is a final multiplication and differentiation of kinetes, which become sporozoites, able to infect the vertebrate host once the tick transforms into the next stage (larvae to nymph; nymph to adult), which is called trans-stadial transmission. As already mentioned, *Babesia* parasites are also transmitted to the next generation of infected ticks, called transovarial transmission, in a way that kinetes pass through the ovary and the eggs, so the sporozoites form in the salivary glands of the next generation larvae [12]. Once the tick attaches to the new vertebrate host, the sporozoites mature in the salivary glands in 2-3 days to become infective and therefore the parasite transmission happens after few days of tick feeding. For example, at least 2 days of hard tick (*Ixodidae*) feeding is necessary before the transmission of *B. canis* or *B. vogeli*, with the exception of male ticks that already fed once and are immediately transmitting parasites upon feeding on the next host [81]. This fact can be used for the prevention of the transmission with fast-acting acaricidal drugs.

In rare cases, *Babesia* can also be transmitted without the tick vector, such as by blood transfusion from an infected dog donor or during dog fighting [21]. Most cases of such vertical transmission in fighting dogs are detected from *B. gibsoni* infected Pit Bull Terriers, but recently also from *B. canis* and *T. annae* infected dogs [34].

PATHOGENESIS OF BABESIOSIS

Two main mechanisms that dominate in the pathogenesis of babesiosis are invasion and lysis of erythrocytes and the immune response of the host to parasitemia. In spite of the disease manifestation, acute canine babesiosis is characterized by low parasitemia observed in the peripheral blood [19,82].

Anaemia

The common manifestation of canine babesiosis is hemolytic anaemia which can occur in two forms, intravascular and extravascular. After entering the erythrocytes, *Babesia* parasites continue their development in trophozoites and merozoites, which after erythrocyte lysis invade nearby erythrocytes to ensure persistence of the infection in the host [83]. The destruction of the erythrocyte is caused by multi-factorial components, including direct parasite damage to the erythrocyte membrane,

splenic removal of damaged and parasitized erythrocytes, as well as activation of the immune system such as complement cascade and/or presence of anti-erythrocyte antibodies [83]. Using a proteomic approach, the role of haemolysis in the course of babesiosis was demonstrated by changes in expression of haptoglobin, hemopexin and serotransferrin [84]. Erythrocyte lysis is associated with a broad spectrum of clinical manifestations, which are not always proportional to the degree of anaemia and are not correlated with the level of parasitemia, which usually remains low [85]. Even in cases with low parasitemia, anaemia can be profound, which suggests that non-parasite factors play an important role. These factors can include peripheral sludging of capillaries, erythrophagocytosis by the spleen and liver, and possibly immunoglobulin and complement-mediated destruction of erythrocytes [86]. In addition to the hemolysis induced by the parasite, dogs with complicated babesiosis can develop autoimmune hemolytic anaemia (AIHA) [8], which is likely to be more clinically important than parasite-induced erythrocyte destruction, since the intensity depends on the host reaction.

Despite hemolysis, some dogs with babesiosis have a high haematocrit (relative haemoconcentration), that represents a rare paradoxical complication, called red biliary sindrome. The cause is thought to be vasculitis and shifting of fluid from the intravascular to the extravascular component, leading to relative haemoconcentration. As a consequence of fluid shift, increased risk of developing acute renal failure (ARF), cerebral complications and organ failures such as acute kidney injury (AKI) may appear, leading to very high mortality rates [87].

Leucopenia and thrombocytopenia

In spite that the leukocyte count can be variable, leucopenia was observed in canine babesiosis caused by all 3 large species of the parasite; *B. canis, B. gibsoni and B. rossi.* The possible causes are formation of platelet-leukocyte aggregates, sequestration, increased utilisation and reduced production [88,89]. One possible mechanism involved in leukopenia includes the ability of platelets to interact with leukocytes and induce their so-called ˝secondary capture˝. The subsequent neutrophil-endothelial interaction could contribute to the initial decrease in leukocyte number and also trigger vascular inflammation [90,91].

The hallmark symptom associated with canine babesiosis is thrombocytopenia [92]. Although almost all infected dogs are presented with severe thrombocytopenia, none develop haemorrhage. Thrombocytopenia may result from immune-mediated platelet destruction, platelet sequestration in the spleen, elevated body temperatures or disseminated intravascular coagulation (DIC) [91]. The severity and rapid recovery of the platelet counts have led to the suggestion that immune-mediated mechanisms are involved. The binding of platelets to histones may lead to platelet activation and propagation of platelet-platelet binding around neutrophil extracellular traps, similar to the platelet aggregation that occurs in thrombosis [93]. Besides their classical role in hemostasis, platelets have an important role in immunologic functions. Cross talk

exists between these two major functions as inflammation influences both coagulation and immune functions of platelets [94].

Coagulation system, fibrinolysis and endothelium

In addition to their role in primary hemostasis, activated platelets provide an efficient catalytic surface for the activation of enzyme complexes of the blood coagulation system. Besides activated platelets, two additional mechanisms could contribute to the coagulation system activation: haemolysis and acute phase response. In *B. bovis* infection, erythrocytes infected by the parasite exhibited procoagulant activity. Also, when uninfected erythrocytes were damaged during the course of the disease, they were capable of activating the extrinsic coagulation pathway [95]. A consequence of the systemic activation of the coagulation system could be disseminated intravascular coagulation . This complex thrombohaemorrhagic disorder was diagnosed on the basis of increased complexes of thrombin-antithrombin, decreased antithrombin activity, thrombocytopenia and shortened activated partial thromboplastin time in dogs with *B. canis* infection. Without any clinical signs of DIC, it was concluded that a compensated form could be present in canine babesiosis [96]. A transient coagulopathy with abnormalities in prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer and thromboelastography was also found in dogs with uncomplicated *B. rossi* infections [97]. Fibrinolysis was also affected in complicated *B. canis* infections, where dogs showed decreased concentrations of fibrinolysis inhibitors, plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor antigen at admission, that lead to increased fibrinolytic activity [98]. In addition to influencing haemostatic activity, the proinflammatory state in babesiosis also has an effect on the function of endothelium. Markers of endothelial activation were increased in babesiosis as a reflection of host inflammatory response and shift the hemostatic activity towards the procoagulant state [91].

Systemic inflammatory response syndrome

Development of the systemic inflammatory response syndrome (SIRS) in babesiosis is caused by an excessive release of inflammatory mediators and considered to be a major feature of the pathophysiology of canine babesiosis [89,99]. A higher mortality rate is present in dogs that develop severe inflammation than those with severe anaemia, indicating that the intensity of the inflammatory response is the dominant mechanism in the outcome of the disease [8].

The immune response in canine babesiosis involves complex signaling networks, which include eicosanoids and cytokines. Eicosanoids are made by oxidation of arachidonic acid or ther polyunsaturated fatty acids. Signaling of eicosanoids is complex and similar to cytokine signaling. Signals transmitted by eicosanoids have been viewed primarily as a pro-inflammatory component of the innate immune response; however, recent data revailed their anti-inflammatory functions [100]. Our research conducted

in dogs naturally infected with *B. canis* confirmed that the eicosanoids, as inflammatory mediators, are involved in the regulation of the immune response and inflammatory reaction [101]. *B. canis* infection induced significant changes in lipid mediators with significant increases in leukotriene B4 and prostaglandin E2, while thromboxane B2 was significantly lower at the beginning of the disease. The study also confirmed an increase in triglycerides and total cholesterol, while HDL cholesterol decreased [101].

Cytokines play a crutial role in the initiation and development of systemic inflammation. Although cytokines are benefitial for the host defence, in the case of excessive production they can act harmfull to the host, initiating widespread tissue injury and organ damage [102,103]. The pathogenesis of babesiosis is dependent on the host response and disease development results from excessive production of proinflammatory cytokines in different animal models [104]. An increased concentration of interleukin-8 with a negative correlation with erythrocytes and haematocrit was found in our study of 20 dogs with an uncomplicated form of *B. canis* infection. Also, the monocyte chemoattractant protein (MCP-1) and the keratinocyte chemotactic-like protein were the cytokines found in the study which could discriminate complicated from uncomplicated cases [105]. Those results are consistent with previously reported data for canine babesiosis caused by *B. rossi* in which non-survivors showed an increased concentration of MCP-1, indicating that this protein could be a marker of poor outcome [88]. A study of Zygner et al. [106] showed an increase of tumor necrosis factor alpha (TNF-α) serum concentration during canine babesiosis caused by *B. canis*. This proinflammatory cytokine has an influence on the development of hypotension and renal failure in canine babesiosis.

Concentrations of acute-phase proteins (APPs) change in a response to inflammatory cytokine secretion. As part of the acute phase response (APR), increased production of positive APPs and decreased production of negative ones occurs. Our recent results indicate that various physiological pathways, including APR, complement and coagulation activation, lipid transport and metabolism, oxidative stress and vitamin D pathway are modulated in canine babesiosis [107]. Results of the study of Matijatko et al. [89] indicated that *B. canis* induces a marked APR, with C-reactive protein (CRP) and serum amyloid A (SAA) being the markers which showed the highest response and may be useful in monitoring the response to treatment. Excessive proinflammatory activity with increased concentrations of CRP was also detected in natural infection with *B. canis* [91] and experimental one with *B. gibsoni* [108]. In our proteomic study of serum changes in canine babesiosis, a number of differentially expressed proteins involved in inflammation mediating the acute phase response, including CRP, were identified in dogs with babesiosis [84].

Multiple organ dysfunction

Complicated babesiosis involves clinical manifestations that are unrelated to haemolytic disease. The Consensus Conference of the American College of Chest Physicians

and the Society of Critical Care Medicine [9] gave definitions for the multiple organ dysfunction syndrome (MODS), which can also be applied in dogs with complicated canine babesiosis. Primary MODS is described as a direct result of an insult and occurs early, while secondary MODS develops as a result of the host inflammatory response [9]. Goris et al. [109] considered that MODS develops as a consequence of dysregulation of proinflammatory and anti-inflammatory mechanisms resulting in overwhelming auto-destructive inflammation. The major mediators of the host inflammatory response are cytokines, nitric oxide, free oxygen radicals, eicosanoids and platelet-activating factor [110]. The most common complications in MODS are AKI, cerebral babesiosis, coagulopathy, icterus and hepatopathy, AIHA, peracute babesiosis, acute respiratory distress syndrome (ARDS), haemoconcentration, hypotension, myocardial pathology, pancreatitis and shock. The number of affected organs in multiple-organ failure correlated with mortality [87].

Hypoxia, which is a common feature in babesiosis, triggers a cascade of pathophysiological events as an adaptive mechanism. Reduced oxygen availability activates the expression of nuclear factor-kappa B, responsible for the release of pro-inflammatory cytokines [111].

Hepatopathy is a common complication in *B. rossi* infection [112]. A transient form of hepatopathy could be the result of hypoxic insults, that cause diffuse hepatocellular swelling [8]. Anaemia is considered as one of the factors causing hypoxia and hypoxic liver injury. On the other hand, the results of a study by Zygner et al. [113] did not show correlations between anaemia and increased aminotransferase and alkaline phosphatase activities in 230 dogs infected with *B. canis*, indicating that subspecies of the parasite may have a role in liver damage and disfunction.

Although acute kidney injury can occur as a complication of canine babesiosis, more often there is a finding of minimal renal damage demonstrated by proteinuria and abnormal urine sediment [114]. The morphologic lesions which can be found in kidneys have been attributed to anaemic hypoxia due to erythrocyte destruction. However, in cases of complicated babesiosis hypovolemia represents a more likely cause than anaemia [89]. It was shown that hypoxia is a more probable cause of renal tubular injury than the toxic effect of haemoglobinuria in dogs [115], while Máthé et al. [116] observed renal lesions typical for hypoxia in dogs infected with *B. canis*. A strong positive correlation between serum TNF-α and serum concentrations of urea and creatinine suggested that TNF-α has an influence on the development of renal failure in canine babesiosis [106]. Renal impairment caused by inflammatory mediators results in reduced renal tissue perfusion and glomerular filtration rate [113].

Cerebral babesiosis (CB) refers to the occurrence of nervous system symptoms associated with parasitized erythrocytes, but relatively small number of dogs with babesiosis develop it [117]. CB carries a poor prognosis and is caused by endothelial damage with subsequent microvascular necrosis, perivascular edema and hemorrhage. Histological lesions appeared in a spectrum of severity and included localized

endothelial injury [112]. The pathogenesis of CB is related to parasitized erythrocytes that become sequestrated in the central nervous system microvasculature and the release of inflammatory mediators, as well as tissue hypoxia, which can lead to neurological signs [118]. CB is usually associated with high mortality [15].

The pathophysiology for ARDS is probably connected with increased alveolar capillary permeability due to SIRS reaction, where reactive oxygen species and inflammatory cytokines seem to play an important role [10].

Rhabdomyolysis, cardiac dysfunction and pancreatitis in canine babesiosis are less frequent complications. The pathogenesis of *Babesia*-induced rhabdomyolysis remains unclear, but inflammatory cytokines and nitric oxide could play an important role. Rhabdomyolysis can be accompanied by other complications including AKI, CB and ARDS [119].

Regarding cardiac dysfunction, increases in N-terminal pro-brain natriuretic peptide (NT-proBNP) in dogs with babesiosis imply there is a reduced cardiac function which becomes more severe as the disease severity increases [120]. NT-proBNP serum levels increase with cardiac volume overload, resulting from myocardial failure or secondary to pulmonary complications. Cardiac troponin I, a sensitive marker for myocardial injury, has also been shown to be increased and proportional to the severity of the disease [121].

In *B. rossi* infection, haemolytic anaemia with ischaemia-reperfusion is proposed as a possible primary pathophysiological mechanism in pancreatitis. Hypotensive shock, immune-mediated haemolytic anaemia, haemoconcentration and possibly altered lipid metabolism in babesiosis may also be involved [122].

Endocrine predictors contribute to the mortality in canine babesiosis. High concentrations of cortisol and adrenocorticotropic hormone, as well as low thyroxine and plasma free thyroxine concentrations have been shown to be predictors of mortality in *B. rossi* infection [123].

CLINICAL SIGNS AND CLINICOPATHOLOGICAL ABNORMALITIES OF BABESIOSIS

Clinical signs of babesiosis are exceedingly variable, although similar for all *Babesia* infections, whether they involve large or small *Babesia*. The wide range of clinical signs of *Babesia* spp. infection depends on several factors such as infecting species, signalment and host immunity, age, splenectomy and concomitant infection or disease [20]. In general, the incubation period of canine babesiosis is around 4-21 days [15].

Mainly depending on the *Babesia* species or subspecies, clinical presentation of canine babesiosis may be peracute, acute or chronic, ranging from subclinical infections to multi-organ failure, with a risk of death [124]. Peracute infection is rare and characterized by significant tissue damage and high mortality rate. Acute babesiosis is

characterized by fever, tachycardia with hyperdynamic pulse pressures, lethargy, varying degrees of hemolytic anaemia, pallor, anorexia, vomiting, dehydration, splenomegaly, lymphadenomegaly, thrombocytopenia, jaundice, pigmenturia, hypotension and water hammer pulse [21,125-129]. Chronic infections are often asymptomatic, since many "carrier" dogs do not have any clinical signs as a result of premunition or concomitant immunity unless their health deteriorates, as a consequence of immunosuppressive therapy, splenectomy, or any other immune-compromised situation [130]. In the same time, some of dogs remain asymptomatic carriers of parasites, with high antibody titres for a period as long as one year [131]. There is a research which showed that dogs which live in endemic areas can synthesize antibodies against *B. canis*, sometimes at high levels, without any signs of the disease [132]. The occurrence of *B. canis* in asymptomatic dogs is very important, because these animals may serve as reservoirs if moved to nonendemic regions [23].

Canine babesiosis can be clinically classified into uncomplicated and complicated forms. An uncomplicated form of babesiosis is considered to be a consequence of anaemia caused by haemolysis [10]. Complicated babesiosis may be a consequence of inflammatory mechanisms that lead to the development of the systemic inflammatory response syndrome and multiple organ dysfunction syndrome, which are cytokine-mediated conditions [87]. Although various mechanisms have been suggested to cause both forms of babesiosis, recent studies have indicated that much of the disease process could be explained by host inflammatory responses to the parasite, rather than the parasite itself [89].

The clinical presentation of uncomplicated babesiosis includes pale mucous membranes, fever, anorexia, depression, splenomegaly and water hammer pulse [133]. Clinical manifestations of the complicated form are variable and related to the complications developed. Abnormalities seen in complicated canine babesiosis cases include acute renal failure, cerebral babesiosis, coagulopathy, icterus and acute liver dysfunction, immune-mediated haemolytic anaemia (IMHA), peracute babesiosis, acute respiratory distress syndrome (ARDS), relative haemoconcentration ("red biliary"), acute pancreatitis, rhabdomyolysis, myocardial dysfunction and shock [87,112,122,134,135].

The wide range of clinical symptoms during the course of the babesiosis depends very much on the species of *Babesia* that causes the infection [136]. There are clinical symptoms and clinicopathological abnormalities that are similar for all *Babesia* species which infect dogs. The most common manifestations are apathy, weakness, anorexia, fever, anaemia, pale mucous membranes, thrombocytopenia, jaundice, pigmenturia, enlarged spleen, hypoalbuminemia, and hyperbilirubinemia [34]. Anaemia, which may be regenerative and nonregenerative, and thrombocytopenia, when present, vary from mild to severe [34], although in our experience dogs with babesiosis showed very often marked thrombocytopenia which is often followed by increasing of mean platelet volume, indicating the stimulation of megacaryocytopoiesis and conservation of the functional platelet mass [137].

Thrombocytopenia is very often one of the first changes during the course of the disease. However anaemia, corresponding to the quantity of the destroyed erythrocytes, is usually much higher than the degree of parasitaemia, suggesting that non-parasited erythrocytes may also be damaged [138]. Namely, several studies have shown that non-parasitized erythrocytes may be damaged, most likely due to a multifactorial pathogenesis including immune mediated processes [139], oxidative damage of erythrocytes [140,141] and a systemic acute inflammatory response [89]. It is also known there is a possible role of the highly reactive oxygen free radicals in the pathogenesis of parasitic infections [142]. Our research confirmed the presence of oxidative stress in dogs infected with *B. canis* by examining serum malondialdehyde (MDA), an end product of lipid peroxidation, and relationship between paraoxonase 1 activity and high-density lipoprotein concentration [143,144]. In addition to the above, our latest research confirmed changes in biomarkers related to the antioxidant status of dogs naturally infected with *B. canis* [145].

Considering all the clinical symptoms associated with all *Babesia* species, there are some clinical signs and clinicopathological abnormalities which differ among *Babesia* species which infect dogs.

Infection with *B. vogeli*

Among the large *Babesia*, infection with *B. vogeli* causes a subclinical to mild or moderate disease, with lack of virulence, probably due to its long association with the domestic dog [18, 146]. Severe anaemia in puppies is possible, while mature dogs often show clinically unapparent infection or subclinical infection [134]. Parasitemia in *B. vogeli* infection is often very low, which may be a problem during routine examinations of blood smears [21]. The main clinicopathological abnormalities are haemolytic regenerative immune mediated anaemia, respectively nonregenerative anaemia, leukocytosis, leucopenia and thrombocytopenia [67,147].

Infection with *B. canis*

B. canis mostly causes a mild to severe disease depending on the particular complications that develop. Parasitaemia is often low and anaemia does not necessarily correlate to the degree of parasitaemia. Experimental infection with *B. canis* resulted in transient low parasitaemia (1-2%). The main acute clinical symptoms are fever, anorexia or decreased appetite, lethargy, weakness, dehydration, jaundice, pale mucous membranes, presence of ticks and pigmenturia [82,148]. Clinicopathological abnormalities in *B. canis* infection are: mild to moderate normocytic normochromic regenerative/non regenerative hemolytic anaemia, thrombocytopenia, leucopenia and neutropenia, lymphopenia, pigmenturia, bilirubinemia, bilirubinuria, hyperfibrinogenemia, splenomegaly [39,82,137,148-150].

B. canis infection usually results in mild disease in the American strains while the African, Australian and European forms are more pathogenic [117,151]. In Europe, higher

mortality has been recorded in countries where complications are similar to those in the South African form of babesiosis, caused by *B. rossi*. The highest mortality rate is noted in Hungary where MODS was reported in 16% of cases [148], while in Croatia MODS ocurred in 10% of cases [152]. Our results confirm that *B. canis* infection is characterized not only by hemolytic anaemia (intravascular and extravascular), which is hallmark manifestation, but also by a number of complications, different clinical syndromes and related clinicopathological abnormalities associated with *B. canis* infections. During intense hemolysis there is developing haemoglobinemia, haemoglobinuria, bilirubinemia and bilirubinuria, which result in tissue hypoxia, followed by hypotensive shock. In a study of *B. canis* infection in Croatia, a considerable number of dogs with hypotensive shock were observed [153,154]. In the same time, an acute phase response occurs, which results with a significant increase in the concentration of major acute phase proteins, C-reactive protein and serum amyloid A [89]. Our data also confirm that during *B. canis* infection there is activation of primary and secondary hemostasis. Namely, TAT complexes were significantly elevated, while antithrombin III, protein C and Hageman's factor activity were significantly decreased and APTT significantly shortened [96,155-157]. Also, research confirmed that proinflammatory condition in babesiosis appears to influence endothelial dysfunction and hemostatic activity. Namely CRP, soluble intercellular adhesion molecule 1 and fibrinogen concentrations were significantly increased before therapy and remained high for 3 days after therapy in dogs with babesiosis while von Willebrand factor activity was significantly decreased in dogs with babesiosis before treatment [91]. Also, research conducted with the amino-terminal portion of C-type natriuretic peptid (NT-pCNP), which is expressed primarily by the vascular endothelium and macrophages in response to several stimuli, confirmed that NT-pCNP can be considered a good prognostic pro-inflammatory marker of the outcome in canine babesiosis [158]. Our latest results confirmed that haemostatic alterations in uncomplicated babesiosis represent a procoagulant state that is mostly reversed during treatment, although biomarkers of endothelial activation and fibrinolysis were also altered in dogs with babesiosis [98,159]. Namely, the concentration of soluble thrombomodulin, high mobility group box-1 protein, vascular adhesive molecule-1, and soluble urokinase receptor of plasminogen activator were increased in dogs with babesiosis at admission while plasminogen and plasminogen activator inhibitor-1 were decreased at presentation compared to day 6 after treatment [159].

Infection with *B. rossi*

Infection with *B. rossi* is considered to cause the most severe disease manifestations between the large babesial species that infect dogs [112]. A large number of dogs develop different complications, so it is extremely challenging to treat dogs with *B. rossi* infection. Published data reported a mortality rate higher than 45% [87]. Dogs with *B. rossi* infection may present clinical manifestations connected with abnormalities seen in complicated cases such as hepathopathy, acute kidney injury, cerebral babesiosis, acute

respiratory distress syndrome, relative hemoconcentration (˝red biliary˝), pancreatitis, rhabdomyolysis and myocardial dysfunction [10,87,112,134]. Many factors have been shown to be associated with high mortality of *B. rossi* infection such as high parasitemia and state of collapse, hypoglycaemia, metabolic acidosis and respiratory alkalosis, high serum lactate, high cortisol and ACTH concentrations, low thyroxine and free thyroxine concentrations, coagulopathy and disseminated intravascular coagulation and immune-mediated haemolytic anaemia [134,160-162]. The pathology of cerebral babesiosis is characterized by ˝sludging˝ of parasitized erythrocytes in the small vessels of the brain, while renal changes have been attributed to hemoglobinuria and also methemoglobin [10]. In experimental studies methemoglobin has been shown as possibly toxic [21]. In one research, authors found a protein named *B. rossi* erythrocyte membrane antigen 1, which is suspected to be a virulence factor in *B. rossi* canine babesiosis [163].

Infection with *B. gibsoni*

Infection with *B. gibsoni*, a small *Babesia*, mostly causes mild signs which are manifested as a subclinical infection or associated with weight loss and weakness [136]. Subclinical infection is common in the USA connected to Pit Bull Terriers [164]. Latest research described azotemia and proteinuria in dogs infected with *B. gibsoni* [165]. In some situations, *B. gibsoni* infection can cause severe anaemia, as a consequence misdiagnosed as IMHA. Namely, dogs can have low parasitemia, and consequently the parasite is not visible on blood smears, while at the same time, there is a low level of suspicion in non-endemic areas [151]. Most dogs with *B. gibsoni* infection have a history of anorexia, lethargy, and diagnosed regenerative anaemia, although there are cases with ˝carrier˝ dogs with chronic infections and without any clinical signs as the result of premunition or immunosuppresive treatment [164, 166]. Chronic infection is common and includes low-grade fever, pallor, splenomegaly and lymphadenomegaly [21].

DIAGNOSIS OF BABESIOSIS

For an accurate diagnosis of canine babesiosis, an integrative approach is recommended based on the clinical picture and examination of blood smears by microscopy and/or serology testing, as well as confirmation of the infecting species by molecular methods.

Microscopy examination of a blood smear

Microscopy is still one of the well-established, low-cost direct methods for the identification of vector-borne pathogens and the method of choice for blood parasites such as *Babesia* or *Plasmodium* [167]. Microscopy examination is the most simple and most accessible diagnostic test for clinical babesiosis in dogs, requiring a well prepared and suitably stained blood smear together with a trained observer. A fresh smear is recommended for the accurate diagnosis of infection and parasite detection could

be improved by examining buffy coat smears or smears made from capillary blood [15,160]. Figure 1 is showing *B. canis* in red blood cells on a blood smear detected using light microscopy.

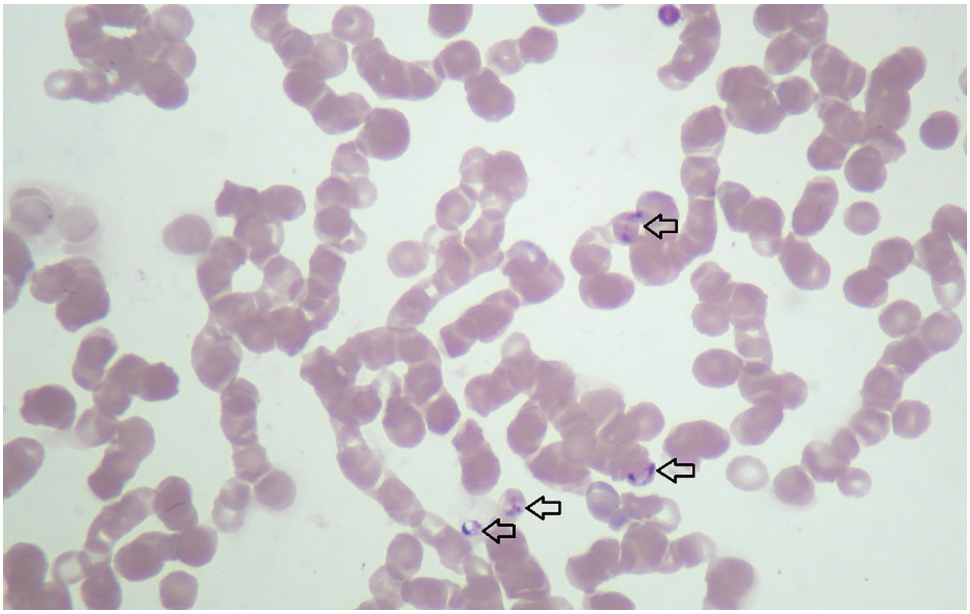

Figure 1. *Babesia* sp. detected on a blood smear using light microscopy. Arrows indicate the parasite in the red blood cells.

Size and morphology of the intraerythrocytic parasite have been the main parameters in diagnosing *Babesia* spp. The large and small form of *Babesia* can be distinguished using a microscopy examination of a blood smear, although the small piroplasms (*B. gibsoni, B. microti*-like sp.) are hard to observe by light microscopy, which has relatively poor to moderate sensitivity [26]. Microscopy is reliable when a moderate to high parasitaemia is present, but it is less sensitive to detect chronic and sub-clinical babesiosis in carrier dogs due to low and often intermittent parasitaemia [26].

Due to these limitations, mainly low sensitivity and inability to identify species of *Babesia* parasite, microscopy examination of the blood smear should be accompanied with more sensitive molecular methods.

Serological testing for the diagnosis of babesiosis

Serological tests are employed to diagnose babesiosis, at the screening level, for both surveillance and research. They have a wide diagnostic time window, as antibodies for a parasite may persist for months or even years. That makes these assays valuable to investigate past exposure to parasites. Immunofluorescent antibody testing (IFAT) has been the most widely supported serological diagnostic test for canine babesiosis, while enzyme-linked immunosorbent assays (ELISA) have been used mostly for research

and epidemiologic surveys [168]. IFAT is considered highly sensitive and moderately specific to detect chronic infection and subclinical infection in carriers.

Serology is unable to distinguish between *B. canis, T. annae* and *B. gibsoni* infection, and blood smears examination cannot distinguish between *T. annae* and *B. gibsoni* [26]. To improve the diagnostic specificity, various recombinant or purified antigens are being widely used. The use of recombinant proteins such as the thrombospondin-related adhesive protein (TRAP) of *B. gibsoni* has been employed as an alternative for the complete parasite antigen with good sensitivities and specificities [169].

However, poor specificity due to cross-reactions between *Babesia* spp. and with other apicomplexan parasites, the inability to differentiate acute from chronic infections and the interpretation of a positive titre are for clinicians working in regions that are endemic for babesiosis the limitations of serological tests. False-negative results are possible in peracute or acute infections, as antibodies usually take 8 to 10 days to develop. In these cases, the use of convalescent antibody titers is strongly recommended to confirm acute infection [136].

Molecular diagnosis of babesiosis

Molecular techniques are extremely useful in determining the identity of blood protozoan parasites infecting dogs and have a higher sensitivity and specificity, over the evaluation of blood smears for detecting canine babesiosis. Through the use of these techniques, our knowledge of the prevalence and incidence of the different *Babesia* species and subspecies infecting dogs has increased considerably [69].

The polymerase chain reaction (PCR) is a sensitive and specific diagnostic technique which is frequently employed for the diagnosis of babesiosis. It is particularly useful for the detection of the infection in dogs with low parasitaemia levels and for differentiation with parasites. Ribosomal RNA genes 18S, 5.8S, 28S and the internal transcribed spacer (ITS) sequences have been used for conventional PCR [20]. A large number of PCR assays and protocols using a variety of gene targets have been described. These include semi-nested PCR [61], reverse line blotting [56,170,171] and PCR-restriction fragment length polymorphism analysis (RFLP) [172]. Furthermore, a number of these PCR methods have been applied to filter-paper technologies such as FTA cards (Whatman Bioscience) and IsoCode Stix (Schleicher and Scheull) for ease of transport of samples to distant laboratories and for epidemiological and other diagnostic studies [172].

PCR assays, based on detection of the small subunit rDNA, and sequence analyses of the amplicons proved powerful in more exact species identification. Because a high degree of 18S rDNA sequence identity exists between many *Babesia* spp., the complete 18S rRNA gene (about 1700 bp) should always be analysed especially in newly recognised organisms. Further refinement in primer design was reported to clearly separate amplicons of 342bp, 546bp, and 746bp target fragments of *B. rossi, B. vogeli, B. canis*, respectively [173]. The PCR-RFLP technique allowed to distinguish

between different large *Babesia* species as previously described [18] and a semi-nested PCR is able to detect and discriminate DNA from *B. canis, B. rossi, B. vogeli* and *B. gibsoni* [61]. A high-resolution melting curve quantitative fluorescence resonance energy transfer-PCR has been developed to discriminate between *B. gibsoni, B. canis, B. vogeli* and *B. rossi* species based on melting curves analysis [174]. Loop-mediated isothermal amplification (LAMP) was found to have advantages of speed and specificity for detecting *B. gibsoni* infections in dogs [175].

False negative PCR results may occur in chronic babesiosis and it is very important to recognise this limitation when screening potential carriers and other asymptomatic dogs such as blood donors. This could determine that in the long term an infection might only be revealed (retrospectively) by serology.

Mass spectrometry based assays

Recently, there are more studies exploring the use of mass spectrometry based methods for diagnosis of babesiosis in dogs. PCR–electrospray ionization mass spectrometry (PCR-ESI/MS) offers a new approach using conventional PCR followed by electrospray ionization MS (ESI-MS) for broad-ranged microbial identification from clinical specimens [176]. A vector-borne panel is available with a small parasite library detecting *Babesia microti, Babesia divergens*, the nonhuman pathogens *Babesia bovis, Babesia gibsoni* and *Babesia canis*, and the filarial nematode *Dirofilaria immitis*, but still for research use only.

The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS technique revealed the presence of a protein fraction of 51–52 kDa in the blood serum of all the animals infected with *B. canis*, which was not found in the serum of healthy dogs [177]. These results were compliant with PCR, showed good sensitivity and specificity, and the costs of the test were lower, while time for analysis shorter than in the case of standard molecular testing.

Other studies employing two dimensional electrophoresis and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) showed potential for using proteomic approach not only for diagnosis, but for monitoring and outcome prediction in canine babesiosis [84, 107].

TREATMENT

Antiprotozoal drugs, antimicrobials and supportive care represent the mainstays of anti-babesial therapy. However, the treatment of canine babesiosis is based on different antibabesial drugs, doses, treatment duration and the reported response to treatment. It is important to point out that some drugs appear to have a greater efficacy against either the large or the small *Babesia*, with the possible exception of diminazene [20]. That is the reason why it is important to determine the species of *Babesia*, or at least determine whether it is the large or small form, at the time of treatment.

Imidocarb dipropionate, a carbanilide member of the diaminidine family, is the treatment of choice for canine babesiosis caused by the large *Babesia* species. It has shown high efficacy against *B. canis.* A single dose of 6.6 mg/kg intramuscularly (IM) or subcutaneously (SC) is the recommended treatment. There are different doses and regime treatments. Some authors suggest a single dose of 7.5 mg/kg IM or a dose of 5-6.6 mg/kg or 7 mg/kg, IM on day 1 and 14 which eliminates clinical signs and decreases the infectivity of tick vectors who fed on treated blood for up to 4 weeks post-therapy [134,178-180]. In France, a specific treatment of canine babesiosis is based on single treatment of imidocarb dipropionate in a dose of 2 mg/kg IM or SC [39]. In our experience, treatment of uncomplicated cases of *B. canis* infection in a dose of 6.6 mg/kg results in significant improvement and the resolution of clinical symptoms within the first 24 - 48 hours, with a prophylactic protective post-therapy effect up to 4 weeks, but in some studies up to 6 weeks [181]. Despite the protective effect, complete disease eradication may not be possible, and there is an often relapse [117]. Administration of imidocarb dipropionate is connected, although uncommon, with adverse effects like pain at the injection side and cholinergic signs, such as salivation, lacrimation, vomiting, diarrhea, muscle tremors, tachycardia, and dyspnea [182]. The side effects generally disappear quite quickly, although some effects can be ameliorated by pre-medication with atropine or glycopyrrolate [180,183]. The toxic effects of imidocarb may occur spontaneously from a dose of 10 mg/kg [39]. The case of overdosing was recorded with massive liver necrosis, and nephrotoxicity [184]. Small forms of *Babesia*, such as *B. gibsoni* and *Babesia microti*-like sp., appear to be more difficult to treat, and this is not the treatment of choice for small *Babesia* [34].

Diminazene aceturate, an aromatic diaminidine derivative, was used traditionally as the most effective treatment for large babesial species, but currently it is not approved for use in many countries. In addition to imidocarb, large *Babesia* (*B. canis*, *B.rosi, B. vogeli*) are most successfully treated with diminazene aceturate in a single dose of 3.5 mg/kg SC, IM, or 3-5 mg/kg IM in a single dose [134,178,185]. Diminazene aceturate is used for the treatment of *B. gibsoni,* although some clinical reports have raised doubts about its efficacy [186]. However, it is important to point out that diminazene is relatively toxic and severe side effects are registered following its use [187]. Namely, it has a very narrow therapeutic range, as the drug is inconsistently cleared and results in possibly high toxicity [178,179]. Side effects can cause central nervous system toxicity in dogs, possibly dose related or as a consequence of repeated administration due to the drug's prolonged elimination half-life [188]. Its use is now limited to clinical cases that are refractory to other treatments [185,188].

Phenamidine isethionate is also effective anti-babesial drug, namely the large babesial species (specifically *B. canis*) show high susceptibility to phenamidine therapy. The drug can be used in a dose of 15-20 mg/kg administered SC once daily for 2 consecutive days [117].

Pentamidine isethionate, another antiprotozoal drug, is approved for use in the United States, and has documented efficacy against *B. canis* and *B. gibsoni*. The recommended

dose is 15-20 mg/kg, SC, every 12 hours for 2 consecutive days. It has adverse effects including pain at the site of injection, hypotension, tachycardia and vomiting [117,179].

Although several treatment protocols are employed for small forms of *Babesia*, treatment is not so efficient and clinical relapses may occur frequently. For example, *B. gibsoni* infection is frequently resistant to treatments with imidocarb dipropionate and diminazene aceturate [136]. Antibiotics are not the treatment of choice for babesiosis, but there are attempts to sterilize infections with *B. gibsoni* using triple antibiotic combinations. There are several combinations of antibiotics such as: doxicycline in a dose of 10 mg/kg/day, administered *per os* (PO) or intravenous (IV); clindamycin (25 mg/kg PO, twice daily); metronidazole (15 mg/kg (PO), twice daily), or doxycycline (7-10 mg PO, twice daily), enrofloxacin (2-2.5 mg/kg PO, twice daily), metronidazole (5-15 mg/kg PO, twice daily in combination with 6 or 12 weeks of oral antibiotics) diminazene aceturate [186,189]. However, antibiotics alone will not eliminate the infection. Some authors suggest to use an alternative therapy with the combination of the anti-malarial drug atovaquone (13.3 mg/kg, three times daily, PO, for 10 days) and the macrolide drug azithromycin (10 mg/kg, once daily, PO, for 10 days) [190]. Other drugs (e.g. quinuronium sulfate, trypan blue solution, parvaquone) are rarely used, or are in the experimental phase such as artesunate and epoxomicin [191,192].

Medical management of infection is sometimes useful and requires supportive treatment, including restoring adequate tissue oxygenation by correction of the anaemia and correction of dehydration and electrolyte disturbances [182]. Supportive treatment is provided only to hospitalized dogs. This is especially important for patients with the complicated form of babesiosis who require aggressive therapy such as: fluid therapy to maintain blood volume and adequate end-organ perfusion, correction of acid-base and electrolyte abnormalities, diuresis, blood transfusion, immunosuppressant drugs in dogs with immune-mediated haemolytic anaemia or thrombocytopenia, and heparin for DIC [182,193]. Many other supportive therapies can be useful depending on the clinical manifestations and/or laboratory abnormalities.

PREVENTION (PROPHYLAXIS)

There are several strategies which can be employed for the prevention of canine babesiosis. These approaches are: use of acaricidal products targeted to transmitting ticks, chemoprophylaxis targeted against the parasites, vaccination of dogs and behavioural prevention by avoiding areas when the ticks are active. Elevated caution is recommended between spring and autumn, when the average air temperature rises above 12 °C, resulting in increased tick activity and easier infestation of dogs [194]. It is important to remove the attached ticks as soon as noticed to prevent the infection before maturation and transmission of infective parasite sporozoites via saliva. Also, since the parasites can be transmitted by blood transfusion, all canine blood donors should be tested for *Babesia* spp. using serological and molecular assays [34].

An important and widely applied approach is tick control based on the use of acaricides. Acaricidal drugs have different mechanisms of action: they repel/irritate the ticks by contact, inhibit the attachment and feeding or have a fast killing effect, which is important to prevent the release of the parasites into the host bloodstream when the tick is already attached. In Europe, there are now licensed drugs containing isoxazolines orally given to dogs, which act on the tick once it attaches and starts to feed, causing fast death or inhibition of feeding [194]. For example, Beugnet et al. [195] showed that use of oral afoxolaner in dogs prevented *B. canis* infection upon experimental infestation with *D. reticulatus* ticks harbouring the parasite, providing protection up to 4 weeks. There are also other acaricides in use, such as collars, spot-on pipettes and sprays. Collars contain flumethrin/imidacloprid or deltamethrin with duration of efficacy for 5-8 months, while the spot-on products applied to the skin contain permethrin or fipronil and have efficacy up to 4 weeks [194]. Acaricides are proven useful for canine babesiosis prevention, with reports showing protection between 88-100% lasting 1-3 months, although their efficacy was mostly tested for *B. canis* transmission by *D. reticulates* [34]. Nevertheless, there is an emerging problem of acaricide resistance, so development of new tick control strategies such as anti-tick vaccines is underway, exploiting new research methodologies such as ˝omics˝ tools [196].

The chemoprophylactic approach using drugs such as imidocarb dipropionate or doxycycline to prevent *B. canis* infection is rarely employed, since adverse effects can occur, such as anaphylaxis and kidney/liver damage. The use of these drugs for prophylaxis is restricted to immunosuppressed dogs, for example those with a removed spleen, which are exposed to ticks in endemic areas [34].

In order to protect dogs from *B. canis* infection, there is also the possibility of vaccination with a commercially available vaccine in Europe called Pirodog® (Merial, France), which contains soluble parasite antigens (SPA) derived from supernatants of *B. canis in vitro* cultures [197]. The immunity against *B. canis* in dogs is largely based on humoral immune reactions, involving specific antibodies production and complement activation. The available SPA-based vaccine induces antibody titres to rise, which doesn´t protect against *B. canis* invasion upon challenge, but results with milder clinical symptoms, such as a shorter period of anaemia, parasitaemia and hyperthermia [197]. It was showed that induced protection develops only against homologous (same strain) infection with *B. canis*, and not against heterologous strains, resulting with a variable degree of vaccine efficacy [198]. Therefore, a broader range bivalent vaccine was developed, containing a combination of SPA of supernatants from *in vitro* cultures of European *B. canis* isolate and South African *B. rossi* isolate, which can provide protection against both heterologous *B. canis* and *B.rossi* infection in terms of reduced clinical signs [199,200]. It was shown that protective immunity induced by the bivalent vaccine differs between challenge with *B. canis* or *B. rossi*, based on different dynamics of parasitaemia, pointing out to biological differences between the isolates [200]. This bivalent vaccine, called Nobivac Piro® was approved for use on the European market

for a few years, but is currently unavailable. Recently a novel protective antigen as new vaccine candidate was discovered and produced as recombinant protein, called canine babesia antigen (CBA). The CBA vaccine provided protection in vaccinated dogs upon challenge infection by limiting parasite multiplication and preventing clinical symptoms and has a potential to replace SPA based vaccines in the future [201]. There are also attempts to produce vaccines against *B. gibsoni* by finding suitable antigens, mostly based on recombinant parasite proteins, such as 50-kDa surface protein, but no commercial vaccine is available yet [202].

CONCLUSION

Canine babesiosis has been recognized for a long time as one of the most important worldwide vector-borne diseases of dogs and ample of data regarding the disease has been collected, as presented herein. Nevertheless, there are still unknowns to be discovered, especially regarding disease pathogenesis, in order to guide the development of improved novel prevention and treatment strategies. This is of particular importance since in the near future higher incidence of babesiosis can be expected due to global warming.

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Authors' contributions

BP, KJ, RBR and MV performed literature searches, wrote and revised the article. All authors read and approved the final manuscript.

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BABEZIOZA PASA: GDE SMO SADA?

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Babezioza pasa je bolest koja se prenosi putem krpelja, koju izazivaju hemoparaziti protozoa koji pripadaju različitim vrstama iz roda *Babesia*. Babezioza je jedna od najvažnijih globalno prisutnih bolesti pasa koja se brzo širi. Ovaj pregledni rad daje sveobuhvatni pregled svih protozoa iz roda *Babesia* koje su identifikovane kod pasa, zajedno sa relevantnim vrstama vektora – krpelja i njihovom geografskom rasprostranjenošću, životnim ciklusom i prenosom parazita. Zajedno sa pregledom nove literature, opisani su glavni mehanizmi patogeneze babezioze. Pošto infekcija babezijama uzrokuje varijabilne kliničke manifestacije bolesti, posebna pažnja posvećena je kliničkim, laboratorijskim i kliničko-patološkim nalazima. Prikazan je pregled dijagnostičkih metoda pomoću mikroskopskih, seroloških i molekularnih metoda, zajedno sa savremenim metodama masene spektrometrije. Pouzdana detekcija i prepoznavanje vrste parazita važni su u izboru odgovarajuće terapije, praćenja stanja i predviđanja ishoda lečenja. Na kraju su prikazane smernice za terapiju i preventivu bolesti.