ABSTRACT: Prevalence of blood parasites in dogs in the Belgrade area has been investigated continuously during the last 20 years, especially in clinically suspicious dogs. In the period from 2014 to 2015, 249 blood samples of pets (182) and shelter dogs (67) were examined. Using Giemsa-stained blood smears, the presence of *Babesia spp.* was examined in erythrocytes and the presence of morulae of *Ehrlichia spp.* and *Anaplasma spp.* in circulating monocytes and granulocytes. To confirm positive findings of ehrlichiosis and anaplasmosis in blood smears, CaniV-4 Test Kit or IDEXX SNAP 4DX test was used. Infection with two pathogens was found in 78/249 (31.32%) cases; in all cases, the infection with one of the protozoa or bacteria was in combination with heartworms. In blood-smears, babesiosis was found in 39.75% of pet dogs and in 71.64% of shelter dogs, ehrlichiosis in 15.93% and 28.35%, and anaplasmosis in 6.04% and 19.40%, respectively. From collected ticks, relative abundance analysis revealed that the species *Ixodes ricinus* was absolutely dominant and found in 50.53% (47/93), followed by *Rhipicephalus sanguineus* – 38.70% (36/93), *Dermacentor marginatus* – 9.67% (9/93), *D. reticulatus*, and *Ixodes persulcatus* found in 3.22% (3/93), which for the first time occurred in dogs in the Belgrade area and in Serbia.

KEYWORDS: dogs, babesiosis, ehrlichiosis, anaplasmosis, ticks

INTRODUCTION

Blood parasites represent an actual health problem in dogs in the Belgrade area. Of the tick-borne diseases of protozoan and bacterial etiology – babesiosis, ehrlichiosis and anaplasmosis are the most important (Ristic and Holland, 1993)
and have a permanent increase (Pavlović et al., 2014). The principal vector of babesiosis and ehrlichiosis are ticks: i.e. *Rhipicephalus sanguineus*, which occurs throughout the world; species of the genus *Dermacentor* – *D. marginatus* and *D. reticulatus*, which have been incriminated in Europe, including Russia (Bowman, 2008). *Ixodes ricinus* is a principal vector of anaplasmosis and the most abundant tick species in Europe and occurs throughout the world (Milutinović et al., 2012).

These diseases have a seasonal character primarily because the population density of ticks varies throughout the year. Ticks tend to be more active during warmer months, though this varies by geographic region and climate. Areas with woods, bushes, high grass, or leaf litter are likely to have more ticks (Bowman, 2008; Pavlović et al., 2012a).

During the examination performed in the Belgrade green areas, in the central city parks, picnic areas and walkways along the rivers *I. ricinus, R. sanguineus, D. marginatus* and *D. reticulatus* tick species have been reported (Milutinović et al., 1997a, b; Dimitrić, 1999; Pavlović et al., 1999, 2011; Pavlović, 2016). The presence of *I. persulcatus* in Serbia for the first time was reported in 2014 and 2015, first in foxes and later in dogs in the Belgrade area (Stojanov et al., 2014).

At the same time, an increased number of cases of tick-borne diseases in dogs has been registered. Babesiosis in dogs was for the first time recorded in the Belgrade area during early 1990s (Pavlović et al., 1999); ehrlichiosis was for the first time recorded in 2002 (Pavlović et al., 2002b) and anaplasmosis in 2012 (Pavlović et al., 2012c).

**MATERIALS AND METHODS**

In the period 2014–2015, 249 blood samples of dogs have been examined. Examinations were performed in 182 pet dogs and in 67 dogs with no owner in shelters. Positive results were confirmed by identification of species of blood parasites in the laboratory of parasitology at the Scientific Veterinary Institute of Serbia.

Of the examined animals 37 originated from old housing district (central Belgrade municipalities where there is the largest number of parks). In the western part of Belgrade, near the Sava and the Danube Rivers (New Belgrade and Zemun), there are large green areas and walkways along the rivers where the owners are walking dogs. From these areas originated 61 of the examined dogs. In the northern part of Belgrade near the Danube River (Ovča and Borča) there is a space with rural character, with a large arable area, forests, partially swampy areas and shrub. There is also a hunting ground for small wild game, roe deer and wild boar, with lots of ticks and mosquitoes. Blood samples of 84 dogs from these places were examined. At same time, blood samples of 67 non-owner dogs from dog shelters were examined.

Tested dogs were exposed to tick bite and ticks were collected from some of them during the observation. The ticks were removed and then species
identification was performed. The tick species were determined using the keys given by Pomerancev (1950) and Kapustin (1955).

Using Giemsa-stained blood smears, the presence of babesia in erythrocytes and morulae of erlichia and anaplasma in circulating monocytes and granulocytes was examined. The affirmative tests (CaniV-4 TEST KIT and SNAP (IDEXX 4Dx)) were used for \textit{in vitro} diagnostics for the detection of \textit{Dirofilaria immitis} antigen, and antibody to \textit{Anaplasma phagocytophilum}, antibody to \textit{Anaplasma platys}, antibody to \textit{Borrelia burgdorferi}, antibody to \textit{Ehrlichia canis} and antibody to \textit{Ehrlichia ewingii} in canine serum, plasma or whole blood. In laboratory of parasitology at the Scientific Veterinary Institute, blood samples were tested to \textit{Ehrlichia spp.} by ELISA test using Ingezim Ehrlichia 1.5.EHR.K1 plate kit. In the same laboratory was performed the identification of \textit{Babesia}, \textit{Anaplasma} and \textit{Ehrlichia} species in all positive samples using morphometric methods.

\textbf{RESULTS AND DISCUSSIONS}

\textit{Ticks findings}

During the study, ticks were found in 93/249 (37.34\%) of the examined dogs. All ticks were collected from owner dogs. Relative abundance analysis revealed that the species \textit{I. ricinus} was dominant, found in 50.53\% (47/93), and followed by \textit{R. sanguineus} 38.70\% (36/93), \textit{D. marginatus} 9.67\% (9/93), \textit{D. reticulatus} and \textit{I. persulcatus} found in 3.22\% (3/93). Usually 2–3 ticks occurred per animal and in several cases were found two tick species at the same animal. In total, 271 ticks were collected. Overall male-female ratio in the course of the study was 61.02\% : 38.98\% in favor of females for the two most commonly found species \textit{Ixodes ricinus} and \textit{Rhipicephalus sanguineus}. This ratio was 69.50\% : 30.50\% and 63.42\% : 36.58\% in favor of females.

The population dynamics of \textit{I. ricinus} and \textit{I. persulcatus} shows two phases of season fluctuation: spring and autumn, because two generations mature every year. \textit{R. sanguineus} reached their maximum in June and \textit{D. marginatus} and \textit{D. reticulatus} had a spring peak (Pavlović \textit{et al.}, 2015b). Occurrence of infection was directly correlated with the seasonal dynamics of ticks. Seasonal variations and changes in the microclimate caused the permanent presence of ticks throughout the year but the degree of infection was higher in the periods when the tick population was the densest (Pavlović \textit{et al.}, 2011).

\textit{Results of blood examination}

In blood-smears of pet dogs, babesiosis was found in 39.75\% (99/182), ehrlichiosis in 15.93\% (29/182), and anaplasmosis in 6.04\% (11/182) of the samples (Table 1). In non-owner dogs from shelters, dog babesia was found in 71.64\% (48/67), ehrlichiosis in 28.35\% (21/67), and anaplasmosis in 19.40\% (13/67) of the samples (Table 2). During blood examination with CaniV-4 TEST KIT or
SNAP (IDEXX 4Dx) tests infection with the two pathogens was found in the 78/249 (31.32%) cases. In all cases, infection with one of protozoa/bacteria was in combination with heartworms. By ELISA test using Ingezim Ehrlichia 1.5.EHR.K1 plate kit only the presence of ehrlichiosis was confirmed.

Table 1. Prevalence of blood parasites infections in pet dogs in the Belgrade area

<table>
<thead>
<tr>
<th>Location in Belgrade</th>
<th>No. exam.</th>
<th>No. infected</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total</td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
</tr>
<tr>
<td>Old housing districts of Belgrade</td>
<td>37</td>
<td>17</td>
<td>6.82</td>
<td>6</td>
<td>3.29</td>
</tr>
<tr>
<td>New Belgrade and Zemun</td>
<td>61</td>
<td>39</td>
<td>15.66</td>
<td>4</td>
<td>2.19</td>
</tr>
<tr>
<td>Ovča and Borča</td>
<td>84</td>
<td>43</td>
<td>17.26</td>
<td>19</td>
<td>10.43</td>
</tr>
<tr>
<td>Total</td>
<td>182</td>
<td>99</td>
<td>39.75</td>
<td>29</td>
<td>15.993</td>
</tr>
</tbody>
</table>

Table 2. Prevalence of blood parasites infections in non-owner dogs from shelters

<table>
<thead>
<tr>
<th>Shelters</th>
<th>No. examined</th>
<th>No. infected</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total</td>
<td>Positive</td>
<td>%</td>
<td>Positive</td>
</tr>
<tr>
<td>Shelter dogs</td>
<td>67</td>
<td>48</td>
<td>71.64</td>
<td>21</td>
<td>28.35</td>
</tr>
</tbody>
</table>

Babesiosis

In shelter dogs, prevalence was 71.64% (48/67) and in pet animals prevalence was 39.75% (99/182). *B. canis* was found in 117 and *B. gibsoni* in 30 animals (distinguished in the smear). In the Belgrade area, *B. canis* for the first time occurred in dogs during 1993 and *B. gibsoni* in 2009 (Pavlović et al., 2002a, 2012). Pathogenicity increased in young dogs, heavily parasitized and immunosuppressed dogs and when there was exposure to a virulent strain or concurrent infection with other tick-borne pathogens.

Commonly encountered was the acute form accompanied with weakness, fever, lethargy, haemolytic anaemia, pale of mucous membranes, a yellow coloring of the eyes (and skin), and red or orange urine color (haemoglobinuria). These symptoms, in various combinations, were found in 79 pet dogs. In only five dogs were found ascites, peripheral edema, and gastroenteritis (Pavlović et al., 2012a). If parasites infest the central nervous system, a dog with babesiosis can display neurological problems, as well as local inflammation, which rarely occurred in seven cases. Acute infections of virulent strains of *Babesia canis* have been associated with induction of the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) secondary to massive immunostimulation and cytokine release. Signs of MODS can include
coagulopathies (DIC), adult respiratory distress syndrome (ARDS), cerebral dysfunction, and acute renal failure (Riek, 1968). These symptoms were observed in one dog, which was infected also with heartworm. In shelter dogs, an acute form of babesiosis occurred with moderate level of mortality (29.85%).

Figure 1. Babesia canis

Ehrlichiosis

Average determined prevalence for ehrlichiosis in pet dogs was 15.93% (29/182) but in shelter dogs it was 28.35% (21/67). In the Belgrade area, E. canis was for the first time identified in dogs in 2002 and E. ewingii in 2006 (Pavlović et al., 2002b, 2006, 2012a). Dog ehrlichiosis caused by E. canis was more common than E. ewingii. Determination was performed by morphometric characteristic and location in adequate blood element. However, E. canis parasitizes monocytes and E. ewingii is smaller than E. canis and produces morulae in granulocytes rather than in monocytes. It is easy to determine but requires qualification and adequate experience.

During the examination, E. ewingii was identified in four cases in pet dogs, which travelled previously to Croatia and Montenegro. Clinically, the infection is characterized by acute, subclinical, and chronic stage of infection. In this study, there were nine cases of acute stage of the disease. Ehrlichiosis was determined during blood smear examination (at first, symptoms look like babesiosis). Dogs may resolve the disease, but develop subclinical persistent infections, and thus, become asymptomatic carriers of the infection.

The acute stage of the disease is due largely to vasculitis. This was found in 12 pet dogs and all shelter dogs. The organism replicates in circulating monocytes, and subsequently in mononuclear phagocytic cells throughout the body.
The infected monocytes bind to vascular endothelial cells and initiate vasculitis and subendothelial cell infection. Acute phase of the disease is characterized with fever (found in 37 shelter dogs), anorexia (4 cases), and lethargy (12 cases). In all infected pet dogs we confirmed lymphadenopathy and thrombocytopenia by blood analyses performed at “Vetlab” in Belgrade.

These symptoms were similar to those given by Shipov et al. (2008). The thrombocytopenia in ehrlichiosis may be due to consumption of platelets, sequestration of platelets in the spleen, immune-mediated destruction of platelets, decreased bone marrow production of platelets, or some combination of these mechanisms. Overall, however, the basis for ehrlichia thrombocytopenia remains unclear (Waner et al., 1997).

Anaplasmosis

Average determined prevalence for anaplasmosis in pet dogs was 6.04% (11/182) and in shelter dogs 19.40% (13/67). Anaplasmosis is a tick-borne disease caused by bacteria Anaplasma phagocytophilum. In the Belgrade area, anaplasmosis was for the first time reported in dogs in 2012 (Pavlović et al., 2012c).

Infection often causes lameness, joint pain, fever, lethargy, and loss of appetite (Carrade et al. 2009). During the examination, ten infected dogs had these symptoms for 1 to 7 days; however, four dogs had only minor symptoms or no symptoms (Pavlović et al., 2015b). Other less commonly observed clinical signs include gastrointestinal problems such as vomiting (found in one dog), diarrhea, or both (found in seven dogs). Respiratory signs described by Kohn et al. (2008) were not determined. There were no data for non-owner dogs about clinical signs of anaplasmosis.
Because animals had clinical signs of polyarthritis and possibly a history of tick exposure, clinical signs of canine anaplasmosis may be indistinguishable from those seen in Lyme disease. In addition, the same tick transmits both diseases. During this research, there were 19 suspect cases, of which 15 were positive to anaplasma and in the other four cases Lyme boreliosis was determined by SNAP test.

**Prevention and control**

The best way to prevent these diseases is by preventing the exposure to the ticks that carry all tick-borne diseases. This is especially important during the peak tick season or if a dog spends time in the woods or tall grass (these areas should be avoided in tick season).

Owners should inspect their dogs daily for ticks. Prompt removal of ticks within 24 hours should prevent disease transmission, because it has been reported that a tick must be attached for two to three days to transmit the organism. In kennels where puppies are being lost to disease, aggressive tick-control measures should be instituted including spraying the environment as well as treating animals.

One of the best measures for protection against ticks is the use of spot on drugs, which is applied to the skin, like fipronile or other drugs. Drop on method is used when a small amount of the solution is squeezed from a tiny tube and gently rubbed into the pet’s coat at the base of the neck, right where it connects to the shoulders at the back of the head. Through a process called translocation, the ointment works its way through the pet’s coat. The oil in the coat slowly dispenses through the hair over 30 days. The initial translocation normally completes in about 24 hours.

**CONCLUSIONS**

In the period 2014–2015 in the Belgrade area, 249 blood samples of dogs were examined for the presence of babesiosis, ehrlichiosis and anaplasmosis. Rate of established infection with babesia, ehrlichia and anaplasma was significantly higher in shelter (non-owner) dogs, compared to pet dogs.

During the study, ticks were found in 93/249 (37.34%) of examined dogs. *Ixodes persulcatus* occurred for the first time in dogs from the Belgrade area and in Serbia.

**ACKNOWLEDGEMENTS**

We thank all our colleagues from veterinary clinics and ambulances in Belgrade who helped us in collecting samples and data so that we can conduct this research.
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КРВНИ ПАРАЗИТИ ПАСА НА ПОДРУЧЈУ ГРАДА БЕОГРАДА У ПЕРИОДУ 2014–2015. ГОДИНЕ

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РЕЗИМЕ: Испитивање преваленце крвних паразита код паса с подручја Београда континуирано се врши у последњих 20 година, поготову код клинички сумњивих животиња. У периоду 2014–2015. испитано је 249 узорака крви од којих су 182 потицала од животиња љубимца а 67 од невласничких паса. Вршен је пре- глед нативних крвних размаза бојених по Гимси при чему смо присуство Babesia spp. утврђивали у еритроцитима а морула Ehrlichia spp. и Anaplasma spp. у моноцитима и неутрофилима. Позитивне налазе на ерлихиозу и анаплазмозу потврђивали смо применом CaniV-4 TEST KIT или SNAP (IDEXX 4Dx) теста. Током прегледа у 31,32% (78/249) узорка установљено је присуство два патогена – уобичајени протозоа или рикеција у комбинацији с дирофиларијама. Током прегледа присуство бабезиозе је установљено код 39,75% власничких и 71,64% невласничких паса, ерлихиоза код 15,93% и 28,35% и анаплазмозе код 6,04% и 19,40% паса. Истовремено, установљено је и присуство крпеља који су вектори ових патогена при чему је Ixodes ricinus био доминанта врста, нађена у 50,53% (47/93), следе Rhipicephalus sanguineus 38,70% (36/93), Dermacentor marginatus 9,67% (9/93), D. reticulatus док је Ixodes persulcatus нађен у 3,22% (3/93) по први пут установљен код паса с подручја Београда и у Србији.

КЉУЧНЕ РЕЧИ: пси, бабезиоза, ерлихиоза, анаплазмоза, крпељи