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Original Article

Molecular and Serological Prevalence of *Anaplasma phagocytophilum, A. platys, Ehrlichia canis, E. chaffeenses, E. ewingii, Borrelia burgdorferi, Babesia canis, B. gibsoni and B. vogeli* among Clinically Healthy Outdoor Dogs in Serbia

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ABSTRACT

Data concerning combined molecular and serological prevalence of emerging canine tick-borne pathogens in Serbia are lacking. A large population of outdoor living dogs in Belgrade, Serbia's' capital, present an excellent population for epidemiology study. Blood samples were collected from 111 dogs, including 46 shelter, 31 free roaming, and 34 hunting dogs. Species-specific real-time polymerase chain reaction (PCR) (IDEXX Laboratories, Inc., Westbrook Maine, USA) was applied for the molecular detection of *Anaplasma phagocytophilum*, *A. platys*, *Ehrlichia canis, Babesia canis*, *B. gibsoni* and *B. vogeli*. A research based SNAP assay (SNAP® M-A, IDEXX Laboratories, Inc., Westbrook Maine, USA) that uses genus and species-specific peptides was used to asses *Anaplasma* spp., *A. phagocytophilum*, *A. platys, Ehrlichia spp., E. canis, E. chaffeensis, E. ewingii and Borrelia burgdorferi* antibody status. *B. canis*, *B. gibsoni and B. vogeli* antibody status was assessed with an indirect immunofluorescence test (MegaCor Diagnostic, Horbranz, Austria). *Anaplasma* spp. and *Ehrlichia* spp. DNA was not amplified. One quarter of the dogs were *A. phagocytophilum,* one dog was *A. platys,* one was *E. ewingii* and two dogs were *B. burgdorferi* seroreactive with the SNAP® M-A. *Babesia canis* or *B. gibsoni* DNA was amplified by PCR from 16.2% of dogs, whereas 67.6% were seroreactive to one or more *Babesia* spp. *Babesia vogeli* was not PCR amplified. We conclude that outdoor dogs in this territory are reservoirs for *B. canis* and *B. gibsoni* and are frequently co-exposed to combinations of *Anaplasma* and *Babesia* spp.

1. Introduction

The Republic of Serbia, located in central and south-eastern Europe, has an increasing population of outdoor dogs, many of which are strays that roam freely throughout urban, suburban and rural environments. Their largest population is found in Belgrade, the capital of Serbia. Despite the growing canine population, there is a lack of comprehensive awareness about the prevalence of canine tick-borne pathogens (TBP), such as *Anaplasma, Ehrlichia, Borrelia* and *Babesia* spp. Also, it is not clear whether free roaming dogs serve as major reservoirs of named TBP relative to the prevalence of these organisms in shelter and hunting dogs, which are occasionally treated for ectoparasites.

In the context of canine health, two *Anaplasma* spp. are of clinical interest: *A. phagocytophilum* and *A. platys*. Molecular studies indicate that *Ixodes ricinus* is the dominant tick species that harbours *A. phagocytophilum* in Serbia (Milutinović et al., 2008; Tomanović et al., 2013; Potkonjak et al., 2016.). *A. phagocytophilum* causes granulocytic anaplasmosis, with disease most often characterized by few and mild clinical signs, including lethargy and fever (Carrade et al., 2009; Sainz et al., 2015). The short period that *A. phagocytophilum* persists in the blood following tick transmission limits its detection by blood smear examination as well as PCR confirmation (Egenvall et al., 2000; Carrade et al., 2009). Antibodies against *A. phagocytophilum* appear 8 to 28 days after tick exposure and can persist for months or even years after

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clearance of microorganism from the blood (Egenvall et al., 2000; Carrade et al., 2009). Most often, seroconversion has not occurred at the time of illness onset (Chandrashekar et al., 2017). Regarding dogs in Serbia, only one *Anaplasma* spp. seroprevalence study has been published. Using an indirect immunofluorescence antibody test (IFAT), approximately 15% of outdoor and indoor dogs tested had been exposed to *A.phagocytophilum* (Potkonjak et al., 2015). Molecular studies involving *A. phagocytophilum* in dogs in Serbia have not been published previously.

Anaplasma platys is most likely transmitted by *Rhipicephalus sanguineus*. This rickettsial species resides in platelets, causing canine cyclic thrombocytopenia, often a subclinical infection, or associated with haemostatic abnormalities (Sainz et al., 2015). *A. platys* has been occasionally identified in dogs in Europe, particularly from countries in the Mediterranean basin and those neighbouring Serbia (Sainz et al., 2015; Huber et al., 2017).

Canine ehrlichiosis is caused with three *Ehrlichia* species: *E. canis*, *E. chaffeensis,* and *E. ewingii*. *E. canis* is transmitted by *R. sanguineus* and the latter two species by *Amblyoma americanum*. *E. canis,* a major canine tick-borne pathogen in Southern Europe, causes canine monocytic ehrlichiosis (Mylonakis and Konstantina, 2017) and is frequently characterized by sever clinical signs and a fatal outcome, but lacks zoonotic potential. Also, *E. canis* was not isolated from ticks collected in Serbia (Potkonjak et al., 2016) or from apparently healthy or deceased dogs with a history of anaemia and thrombocytopenia in Croatia (Huber et al., 2017). *E. chaffeensis* causes canine and human monocytic ehrlichiosis, while *E. ewingii* causes canine and human granulocytic ehrlichiosis, most often reported in immunocompromised individuals (Sainz et al., 2015; Mylonakis and Konstantina, 2017). Seroreactivity to *E. chaffeensis* and *E. ewingii* have been occasionally reported in European human population (Topolovec et al., 2003; Sainz et al., 2015), but only one patient in Serbia was recognized to have clinical illness (Arsić et al., 2014). Both pathogens cause less severe diseases in dogs than *E. canis* and neither has been reported in European canine populations (Sainz et al., 2015).

Among numerous *Borrelia* species (> 52) only *B. burgdorferi sensu stricto* (s.s.) is an established canine pathogen transmitted by ticks from genus *Ixodes* (Littman et al., 2018). After exposure, majority (> 95%) of dogs remain asymptomatic (Little et al., 2010). Well defined clinical manifestations are only arthritis and nephritis (Littman et al., 2018). Dogs are considered as reservoirs and indicators of *Borrelia* spp. spreading (Little et al., 2010). It is shown that stray dogs in Serbia are exposed to this pathogen (Obrenović et al., 2015; Potkonjak et al., 2016a), but further studies are needed to establish the impact of *Borrelia* species on the health status of dogs in this region.

Babesiosis was recognized as a frequent canine disease in Serbia 25 years ago (Krstic et al., 1994), but the molecular and serological prevalence of *Babesia* at the species level are just being defined. *Babesia canis* is primarily vectored by *Dermacentor reticulatus* ticks, *B. vogeli* by *R. sanguineus,* and the transmission of *B. gibsoni* is still under investigation (Solano-Gallego et al., 2016). *Babesia canis* is considered the most prevalent species that infects dogs in Serbia (Davitkov et al., 2015) and Croatia (Bilić et al., 2018). *B. canis* and *B. gibsoni* are documented (by PCR) in dogs with clinical manifestation of babesiosis in Serbia (Davitkov et al., 2015) and neighbouring Croatia (Bilić et al., 2018). Another study documented *B. vogeli, B. gibsoni,* and *B. microti*-like DNA in the blood of clinically healthy outdoor dogs; however, the authors did not provide *B. canis* prevalence data (Gabrielli et al., 2015). Furthermore, published data on *B. canis*, *B. vogeli,* and *B. gibsoni* seroprevalence in clinically healthy dogs in Serbia are not available, with the exception of *B. canis* in hunting dogs (Spasojevic-Kosic et al., 2015); therefore, additional epidemiologic data is needed.

Concerning limited combined data of TBP in canine population in Serbia, the aim of the present study was to determine the molecular and serological prevalence and the frequency in which co-infection and co-exposure with *A. phagocytophilum, A. platys, E. canis*, *E. chaffeensis, E. ewingii* and *B. canis*, *B. gibsoni* and *B. vogeli*, occurs in clinically healthy dogs in Serbia, using the commercially available assays. Exposure of dogs with *Borrelia burgdorferi* was determined with serology. Molecular and serology results for shelter, free roaming, and hunting dogs were compared to determine whether free roaming dogs are at greater risk of infection with or exposure to TBPs.

2. Material and Methods

2.1. Animals and Samples

Adult male (N = 61) and female (N = 50) dogs from suburban and rural Belgrade municipalities were enrolled during March and April of 2015. Among the enrolled animals, 46 dogs were from two shelters (with indoor/outdoor facilities) located at geographically distant sites, 30 km from each other, each surrounded by grass area and woods. Shelter dogs had been maintained in either facility for at least 6 months; some of the dogs had been in the facility for several years. Most shelter dogs previously were free roaming. Ivermectin s.c. (200 μg/kg) was applied to shelter dogs at least three times a year. Diazinon was used monthly for ectoparasite control. Free roaming dogs (N = 31) enrolled in this study were removed from the streets of urban and suburban city municipalities by Belgrade city communal service. Blood samples were collected at the time of surgical neutering at the Hospital of the Faculty of Veterinary Medicine, University of Belgrade and a surplus of blood was used in this study. Compared to shelter or hunting dogs, free roaming dogs were unlikely to have received any ectoparasite or endoparasite treatments; however, their historical care was unknown. Hunting dogs (N = 34) from two rural city municipalities, 20 km apart, participated in the study. Hunting dogs had received occasional ivermectin treatments and/or had antiparasitic collars. Before initiation of the study, in accordance with European Union declaration 63/2010 and based on Serbian law for protection of animal welfare, permission for the study was obtained from the Ministry of Agriculture and Environmental Protection (number 323-07-03455/2015-05/3). The blood samples from shelter and hunting dogs were collected with the informed consent of responsible shelter management personnel or the dog owners.

The study enrolled only dogs that appeared clinically healthy. Clinical examinations were performed by a clinician, and when available, the dog's medical history was recorded. Previous disease states were often unknown and were not exclusive or inclusive criteria for enrolment in this study. Median age of hunting dogs was 3 years (range: 1–14 years). Age of shelter and stray dogs could not be estimated with accuracy, and thus, age was omitted from statistical analysis.

Blood samples were obtained from the saphenous vein. Samples collected in tubes with ethylenediaminetetraacetic acid were used for blood smear preparation and stored at −20 °C. Blood collected in the tubes with no anticoagulant was allowed to clot, then was centrifuged for 15 min at 1500*g*. Serum was separated and stored at −20 °C. All molecular and serological analyses were performed within 120 days following collection.

2.2. Laboratory Methods

After staining with Romanowsky stain (Bio-Diff kit®, Biognost Ltd.; Croatia), blood smears from each dog were examined by the two experienced veterinarians. Smears were inspected for presence of *Babesia* spp. in erythrocytes and *A. phagocytophilum*, *A. platys* and *E. canis* in neutrophils, platelets and monocytes, respectively.

For PCR analyses commercial species-specific assays were used (IDEXX Canine RealPCR™ Tests, IDEXX Laboratories, Westbrook, Maine, USA).

B. canis, *B. vogeli* and *B. gibsoni* antibodies were assayed with indirect immunofluorescence antibody test (IFAT) (MegaScreen®FLUO *Babesia canis*, MegaScreen®FLUO *Babesia gibsoni* and MegaScreen®