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Original article

Acute-phase response in *Babesia canis* and *Dirofilaria immitis* co-infections in dogs

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ABSTRACT

Babesia canis and *Dirofilaria immitis* are emerging and geographically overlapping vector-borne pathogens in dogs. Infection with *B. canis* leads to acute-phase response (APR) that can be mild to severe and results in either non-complicated or complicated forms of the disease. The aim of this study was to determine whether acute *B. canis* infection is more severe in dogs with underlying asymptomatic *D. immitis* infection. Dogs of both sexes, different ages and breeds, with naturally occurring mono-infections with *B. canis* (n = 13) and *D. immitis* (n = 18) and co-infected dogs (n = 7) were enrolled as well as healthy controls (n = 15). Routine haematology and biochemistry, agarose gel electrophoresis (agEF) protein fraction separation and enzyme-linked immunosorbent assay (ELISA) for serum amyloid A (SAA) were performed. Based on clinical and laboratory findings, sepsis was diagnosed in the majority of dogs with acute *B. canis* infection with or without underlying asymptomatic *D. immitis* infection. Overall, haematology, biochemistry and agEF pattern changes were induced and dictated by acute *B. canis* infection whether or not the dogs had an asymptomatic *D. immitis* infection. *D. immitis* infection slightly influenced the level of anaemia, slightly aggravated the level of dehydration and increased the concentration of γ-globulins in acute-phase *B. canis* infection. *D. immitis* infection prevented *B. canis*-induced leukopenia. SAA equally increased in dogs with acute *B. canis* infection with or without underlying *D. immitis* infection. The level of SAA was not changed in dogs with asymptomatic *D. immitis* when compared to the controls. In conclusion, asymptomatic *D. immitis* infection does not influence overall APR after acute *B. canis* infection.

1. Introduction

Babesia canis and *Dirofilaria immitis* are widely distributed vector-borne pathogens in dogs (Alho et al., 2014; Gabrielli et al., 2015; Solano-Gallego et al., 2016; Tasić et al., 2008). While *B. canis* often induces acute clinical infection, *D. immitis* usually provokes chronic asymptomatic infection that can persist for several years before clinical signs become apparent (Shaw and Day, 2005). Several recent reports have described simultaneous *Babesia* spp. and *Dirofilaria* spp. infections detected with molecular and serological methods in dogs in Central and Southern Europe (Kovacevic-Filipovic et al., 2016; Pantchev et al., 2015; Víchová et al., 2014). It is supposed that global warming will lead to a further spread of mosquitoes and ticks and the pathogens they transmit (Beugnet and Chalvet-Monfray, 2013; Genchi et al., 2005). This implies that there will be an increasing number of acute *B. canis* clinical cases with underlying asymptomatic *D. immitis* infection in the future.

Non-complicated and complicated forms of *B. canis* infections have been described (Shaw and Day, 2005). The common feature of both forms is acute-phase response (APR) characterised by clinical signs, such as anorexia, fever, mucosal pallor and/or icterus. Pathophysiological events underlying complicated forms of *B. canis* infection are immune-mediated anaemia and sepsis with acute renal failure (Matijatko et al., 2007; Schetters et al., 2009) with a 10 (Davitkov et al., 2015) to 20% mortality rate (Máthé et al., 2006). Signs of *D. immitis* infection are coughing, exercise intolerance and chronic weight loss, but as stated, the majority of dogs are asymptomatic in stage I disease (Shaw and Day, 2005) and infection is discovered during annual health exams. Until recently, it was not known whether asymptomatic *D. immitis* infection changed the severity of *B. canis*-induced APR and the outcome of *B. canis* infection.

Acute-phase proteins (APPs) are useful markers for stratification of

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APR (Schmidt and Eckersall, 2015) and α1-acid glycoprotein (Lobetti et al., 2000), ceruloplasmin, C-reactive protein (CRP) (Ulutas et al., 2005) and serum amyloid A (SAA) have been shown to be increased in acute *B. canis* infection in dogs (Matijatko et al., 2007). Until recently, SAA has not been evaluated in asymptomatic *D. immitis* infection, nor was it determined during acute *B. canis* infection in dogs with underlying *D. immitis* infection.

A more comprehensive view of the sum of APPs, can be obtained on agarose gel electrophoresis (agEF). In acute-phase *B. canis* infection from South Africa, α1-globulin and α2-globulin fractions were decreased and γ-globulins were increased (Lobetti et al., 2000). In *B. canis* cases from Poland, α-globulins were decreased and β-globulins were increased, while γ-globulins were variable (Zygner et al., 2011). The cause of this decrease in α-globulins is not clear. The only agEF performed on sera of *D. immitis* microfilaria-positive dogs, revealed increases in β2 and γ-globulin fractions (Barsanti et al., 1977). That finding is consistent with the chronic inflammation agEF pattern.

Concurrent infections often pose a problem in retrospective or prospective natural infection outcome analysis. Therefore, it would be of interest to stratify APR using SAA and to investigate major haematology, biochemistry and agEF changes in cases of acute *B. canis* infection with and without underlying asymptomatic *D. immitis* infection.

2. Materials and methods

2.1. Animals

The study was conducted on privately owned dogs naturally infected with *B. canis* and/or *D. immitis* in Belgrade, Serbia. Dogs were housed outdoors at a location known to be a district endemic for both *B. canis* and *D. immitis*. The study was designed prospectively, and dogs that were chosen for study were surveyed up to one month after presentation. Inclusion criteria for the four groups were:

1) "Bab" group – the presence of clinical signs of babesiosis (fever, anorexia and golden-orange colour of faeces), blood smear positive for large babesia forms, negative on modified Knott test and positive *Tick/Vector Comprehensive RealPCR Panel Canine*, (IDEXX *Laboratories* for *B. canis* and SNAP®4Dx®) test system, negative for *D. immitis*, *Ehrlichia* sp., *Borrelia* sp. and *Anaplasma* sp. (IDEXX Laboratories Westbrook, ME, USA). After collection of blood samples, dogs were treated with imidocarb-dipropionate (protocol recommended by Solano-Gallego et al., 2016). All dogs survived and recovered. The number of dogs in this group was 13 (11 males/2 females) with a median (minimum–maximum) age of 4 (1–13) years.
2) "BaD" group – fulfilled inclusive criteria of "Bab" group with exception that they were positive on SNAP®4Dx® test system for *D. immitis* and positive on modified Knott test for microfilaria. Further inclusion criteria were obtained from anamnestic data and clinical examination of dogs. This group comprised of dogs that did not have signs of *D. immitis* infection (coughing, exercise intolerance and weight loss). After blood collection, dogs were treated and all dogs survived with fast clinical recovery after anti-babesial treatment. After 15 days, dogs were also treated with microfilaricidal ivermectin according to "slow-kill" protocol (https://heartwormsociety.org/images/pdf/2014-AHS-Canine-Guidelines.pdf). The number of dogs in this group was 7 (5 males/2 females) with a median (minimum –maximum) age of 4.5 (2–10) years.
3) "Dir" group – asymptomatic dogs, blood smear negative and polymerase chain reaction (PCR)-negative for *Babesia* sp., but positive for *D. immitis* antigen on SNAP®4Dx® test and for microfilaria on modified Knott test. After blood collection, *D. immitis*-positive dogs were treated according to the "slow-kill" protocol (https://heartwormsociety.org/images/pdf/2014-AHS-Canine-Guidelines.pdf). The number of dogs in this group was 18 (9 males/9 females), with a median (minimum–maximum) age of 2 (1–7) years.
4) "HeC" group – healthy control dogs negative on all test systems stated above. Blood samples from "HeC" dogs served to obtain values to make comparisons for agEF and SAA, as these parameters do not have internationally recognised reference values. The number of dogs in this group was 15 (6 males/9 females), with a median (minimum–maximum) age of 2 (1–6) years.

Table 1
Survival, sepsis incidence and icterus among examined dogs.

Groups (n)	Survival	Sepsis	Anorexia	Icterus
Bab	13/13	10/13	3/13	1/13
BaD	7/7	6/7	6/7	1/7
Dir	18/18	0/18	0/18	0/18
HeC	15/15	0/15	0/15	0/15

Abbreviations: (Bab) – *Babesia canis*-positive group, (BaD) – *Babesia canis* and *Dirofilaria immitis*-positive group, (Dir) – *Dirofilaria immitis*-positive group, (HeC) – Healthy control group of dogs.

All dogs that presented on March and April 2016 and fulfilled the stated criteria were enrolled in this study. According to a previously published paper describing criteria for systemic inflammatory response syndrome – SIRS (Okano et al., 2002) and sepsis in dogs with acute *B. canis* infection (Matijatko et al., 2009), dogs within the "Bab", "BaD" and "Dir" groups were classified to have sepsis if they had two or more of the following criteria fulfilled: 1) body temperature of 39.7 °C or higher or 37.8 °C or lower, 2) heart rate of 160/min or higher, 3) respiration rate of at least 40 breaths/min, a white blood cell (WBC) count of 4×10^9/L or less, or 12×10^9/L or more, or in which at least 10% band neutrophils could be counted.

Owners signed an informed consent stating that the surplus of material and data obtained during the study will be used for scientific purposes. The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine University of Belgrade and according to the Law for Animal Welfare; permission number 323-07-03455/2015-05/3 from the Ministry of Agriculture and Environmental Protection of the Republic of Serbia was obtained.

2.2. Blood sample collection

Blood was collected for complete blood count (CBC), biochemistry, agEF and PCR analysis in accordance with standard laboratory sampling procedures. Blood smear, SNAP®4Dx® test and modified Knott test were performed immediately after blood collection. Semi-quantitative evaluation of platelet number was obtained from blood smear. CBC analysis was done on an impedance haematology analyser (Abacus Junior Vet, Diatron, Austria) within 2 h of sampling. The remaining whole blood samples and sera were stored for further analysis at −20 °C. Blood smear stained with BioDiff® (BioGnost, Croatia) served for determination of band neutrophils percentage and detection of *Babesia* sp. After separation of sera samples, haemolysis was inspected, and the visual score was calculated according to http://www.eclinpath.com/chemistry/interference-indices/. Tingle pink colour was estimated to be present in 7/13 "Bab" and 5/7 "BaD" samples. This level of haemolysis does not affect biochemical assays (http://www.eclinpath.com/chemistry/interference-indices/) but affects agEF. This feature is further analysed in the discussion section.

2.3. Agarose gels electrophoresis

Agarose gels were prepared by pouring 1% of agarose on 9 × 11 cm foils. Electrophoresis was performed in a SAS-MX horizontal electrophoresis chamber (Helena Laboratories, Beaumont, Texas) in barbital buffer for 45 min at 80 V. After electrophoresis, gels were dried at 60 °C for 20 min, stained in 0.1% Comassie brilliant blue stain and distained