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ORIGINAL PAPER

Seasonal differences in the intensity of acute phase response in dogs infected with *Babesia canis*

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Abstract

The highest number of acute *Babesia canis* cases in dogs is recorded over the February–May (Feb–May) period, which also represents the optimal climate conditions for tick activity in Belgrade, Serbia. A possibility that the acute phase response is more intense in dogs developing the disease in the Feb–May period compared with the response in other time periods of the year was tested. A total of 63 client-owned dogs with acute *B. canis* infection were enrolled and the routine hematology and biochemistry parameters—serum amyloid A (SAA), IgG against *B. canis*, level of parasitemia, ceruloplasmin (CER), paraoxonase-1 (PON-1), and fibrinogen—were measured. Acute phase indexes (API) were calculated as (SAA×CER)/(Iron×PON-1) and (SAA×CER)/(Albumin×Iron). Statistics included Kruskal-Wallis test and logistic regression analysis. The results showed that in the Feb–May period, the following parameters were lower: creatinine, albumin, iron, and level of parasitemia. Furthermore, increased API values were more probable in the Feb–May than in the other periods. Together, higher acute phase response intensity and presumptive hemodilution in the Feb–May period indicate a more acute course of *B. canis* infection than in other time periods of the year.

Keywords Acute phase indexes · Hemodilution · Iron · Level of parasitemia · Seasonality

Introduction

Dogs in the area of Belgrade (Republic of Serbia, Southeastern Europe) are under a high risk of infection with an intraerythrocytic parasite *Babesia canis* (Potkonjak et al. 2020) and canine babesiosis occurs frequently (Potkonjak and Stošić 2020). Clinical cases are recorded over the whole year, even in the warmest period, i.e., June, July, and August, albeit only sporadically (Janjić et al. 2019). The frequency of clinical cases seen in the Belgrade region between 2013 and 2016 established a basis for discriminating three periods of the occurrence of canine babesiosis (Janjić et al. 2019). An expansion of cases appeared from mid-February to May (Feb–May period). The disease was less frequent in January and the first part of February (Jan–Feb period), as well as between October and December (Oct–Dec period) (Janjić et al. 2019). Furthermore, the Feb–May period coincides with the part of the year when ticks are most active (Milutinović and Radulović 2002), which could imply that dogs suffer from multiple tick bites and are infected with a higher number of sporozoites, than in the other two periods. Therefore, we postulated that there was a difference in the intensity of inflammatory response between the dogs presenting with the disease in the three periods of the babesiosis occurrence.

When a babesia "naïve" dog gets infected with *B. canis*, acute phase response (APR) develops within 1 to 3 weeks (Bilić et al. 2018; Leschnik 2020). In the majority of cases, clinical disease is without complications, although intensity of the fever together with the alterations in total and differential leukocyte count indicate that more than half of the diseased dogs have systemic inflammatory response syndrome

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(SIRS) (Beletić et al. 2021). However, if they develop, common complications are impairment of the renal and liver function, immune-mediated anemia, and in the most severe cases, the septic shock, multiple organ dysfunction, and lethal outcome (Leschnik 2020). In experimental conditions, the relationship between the severity of the disease and parasite load is rather comprehensive. The magnitude of APR is similar in dogs with different parasitemia levels, but the incubation time shortens as the level of parasitemia increases. Increase in body temperature and clinical outcome correlate with the level of parasitemia (Schetters et al. 2009). Regarding host factors, clinical severity in natural infection may depend on the dog's immune response, age, and comorbidities (Milanović et al. 2017; Singer et al. 2016). However, in case of infection with *B. canis*, these factors have not been investigated thoroughly.

Using the fact that in the Belgrade region, a high number of overt canine babesiosis cases is diagnosed each year, we designed this study with an aim to compare the magnitude of the acute phase response in cases diagnosed over three periods of the babesiosis occurrence: Jan–Feb, Feb–May, and Oct–Dec. We analyzed clinical data, hematology and biochemistry results, levels of acute phase proteins (APPs) and their indexes, serology status, and level of parasitemia.

Material and methods

Animals and blood sampling

We included 63 dogs in this observational, cross-sectional study conducted between October 2017 and May 2019: 7, 42, and 13 cases were diagnosed in Jan–Feb, Feb–May, and Oct–Dec, respectively. The median age was 14 months, with the range from 2 to 180 months.

The patients were selected according to the criteria described in our previous papers (Beletić et al. 2021; Milanović et al. 2019). For 2 or 3 days, the dogs suffered from the acute illness presenting with fever, anorexia, lethargy, tachycardia or tachypnea, and mild icterus. The confirmation of the infection with *B. canis* was based on the microscopic detection of the large *Babesia* organisms on the blood film slides (Romanowsky staining), which were afterwards identified as *B. canis* using the polymerase chain reaction (PCR) technique. The exclusion criteria were the history of a chronic disorder and a positive result of the modified Knott test.

Furthermore, we considered the development of SIRS if the patient showed at least two of the following signs: body temperature above 39.2 °C or below 37.2 °C, tachycardia (more than 140 beats/minute), tachypnea (more than 40 breaths/min), total leukocyte count higher than 19.5×10^9/L or lower than 5×10^9/L, and more than 5% of band neutrophils (DeClue 2017).

Imidocarb-dipropionate was administered as a single dose to each dog (6 mg/kg *sc*). All the patients successfully recovered within 15 days.

Laboratory methods

Blood samples were collected from cephalic vein into EDTA and plain vacuum collection tubes (BD Vacutainer; Becton Dickinson, Franklin Lakes, USA). The samples in the EDTA tubes were used for the complete blood count (CBC), the blood film slide preparation, the measurement of fibrinogen concentration, and the PCR analysis. For the CBC, we used Abacus Junior Vet hematology analyzer (Diatron). The differential CBC and presence of the large *Babesia* forms were assessed during microscopic examination of the blood film slide.

The samples in plain tubes were centrifuged for 15 min at 1500 *g* to obtain the serum. For some patients, the quantity of the serum was insufficient for all analyses. Therefore, the "n" in figures stands for the number of analyzed samples. The serum samples were kept at −20 °C until the analysis, not longer than 2 months. We used ready-to-use reagents (Elitech) on Technicon RA-XT automated analyzer (Bayer) to measure the concentration of glucose, urea, creatinine, phosphate, total proteins, albumin, total bilirubin, cholesterol, triglycerides, iron, and the activity of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, amylase, lipase, and creatine kinase. Also, laboratory methodology included ELISA (Tridelta Development Ltd) for measurement of SAA level, the spectrophotometric methods for paraoxonase-1 (PON-1) activity and ceruloplasmin (CER) concentration (Beletić et al. 2021), and the heat precipitation method for fibrinogen level (Athanasiou et al. 2013). In accordance with Gruys et al. (2006), acute phase index (API) was defined as:

$$\mathrm{API} = \frac{(\text{concentration of rapid positive reactant}) \times (\text{concentration of slow positive reactant})}{(\text{concentration of rapid negative reactant}) \times (\text{concentration of slow negative reactant})}$$

We calculated two APIs. Both of them used SAA and CER as the rapid and slow positive reactant, respectively (Cray et al. 2009; Gabay and Kushner 1999), and iron as the

rapid negative reactant (Spottiswoode et al. 2014). The difference between the APIs was in the choice of the slow negative reactants—one used albumin, and the other one PON-1 (Gabay and Kushner 1999; Tvarijonaviciute et al. 2012).

The commercial real-time PCR method (Tick/Vector Comprehensive RealPCR Panel–Canine, IDEXX Laboratories, Inc.) amplifying the hsp70 gene was used to verify the infection with *B. canis* as the cause of the disease. In addition, the method allowed us to estimate the level of parasitemia, which is inversely proportional to the crossing point (Cp) value. The presence of the IgG against *B. canis* was tested with indirect immunofluorescence test (MegaFLUO BABESIA canis, MEGACOR Diagnostik GmbH). The positive fluorescence pattern upon dilution 1:160 was the indicator of the positive test result.

Statistical analysis

The quantitative data were presented as the medians with minimum and maximum. The obtained results were compared among different periods using Kruskal-Wallis tests with the post hoc analysis according to Conover. The categorical data were provided as the ratio and the chi-square test was used to analyze difference in their distribution across the periods. Finally, univariate and multivariate logistic regression analysis allowed us to establish the independent associations between the changes of the investigated parameters and periods. *P* value less than 0.05 was considered statistically significant. All the testing was done with the MedCalc® software version 16.2.1.

Results

Age of the dogs (given in months) did not differ across the investigated time periods ($P = 0.30$): the median (min–max) values were 14.5 (2–52) in Jan–Feb, 18 (4–180) in Feb–May, and 9.5 (4–84) in Oct–Dec. Similarly, body temperature did not differ across the periods ($P = 0.274$): the median (min–max) values were 40.1 °C (39.2–41.0 °C) in Jan–Feb, 40.0 °C (35.5–41.1 °C) in Feb–May, and 40.0 °C (40.0–40.6 °C) in Oct–Dec.

Table 1 presents the results of the hematology, biochemistry, and molecular analyses. The concentrations of creatinine, albumin, and iron were lower in the Feb–May period than in the other two. In contrast, the activities of alanine aminotransferase and alkaline phosphatase were higher when the Jan–Feb period was compared with Feb–May and Oct–Dec. The dogs diagnosed with *B. canis* infection during the Jan–Feb and Feb–May periods had higher amylase activity in comparison with those presenting between October and December, when the results were within the reference range. The concentration of total proteins during the Feb–May period was lower than that measured in the Jan–Feb period. Level of parasitemia, on the other hand, was the lowest in the Feb–May period.

We also observed specific changes in the APP concentrations during the Feb–May period. The concentration of SAA was higher compared with the Jan–Feb, and the fibrinogen levels were lower than those in the Oct–Dec period. Furthermore, both APIs showed an evident distinction between the Feb–May and the other two periods (Fig. 1). However, the frequency of SIRS and the presence of IgG against the *B. canis* did not differ significantly among the periods (Fig. 2).

We further evaluated an association between the time periods and changes in the laboratory parameters using logistic regression (Table 2). We took the medians calculated from all the investigated dogs as the cut-off values. The univariate analyses showed that the characteristic of the Feb–May period was a very high probability that several parameters—level of parasitemia, albumin, creatinine, and iron—are decreased below the cut-off value. The highest probability characterized the occurrence of the ferremia below 37 µmol/L. However, the multivariate analysis identified only a decrease in albumin and iron as independently associated with the Feb–May period. The probabilities associated with the APIs assessed with the univariate analyses were even higher with the highest value, almost 60, associated with the (SAA×CER)/(Iron×PON-1). Nevertheless, since indexes were calculated using the values of APPs having the opposite dynamics during the APR, the multivariate analysis was not applicable. The probability that the dogs will show higher amylase values was several times higher in the periods Jan–Feb and Feb–May than during the Oct–Dec.

Discussion

Our results revealed a pathology feature of canine babesiosis that can be associated with the time period of the infection occurrence. Namely, between February and May, when the number of clinical cases was the highest, the patients developed more intense APR, evaluated through APIs, although the parasite load in the venous blood was lower compared with cases diagnosed in the other two time periods. Still, our results showed no difference among three observed time periods in the frequency of the dogs that developed SIRS and those that were seroreactive against *B. canis*.

The level of individual acute phase reactants did not have uniform differences among the three time periods. The lowest concentrations of two negative acute phase reactants, albumin and iron, indicated the highest intensity of APR in the Feb–May period. Hypoalbuminemia probably resulted from a combination of the decreased synthesis, a well-recognized feature of APR (Moshage et al. 1987), and

Table 1 Laboratory results for the investigated seasons

Parameter (unit)	Reference range	Season median (min–max)			*P*
		Jan–Feb	Feb–May	Oct–Dec	
RBC (10^{12}/L)	5.5–8.5	5.1 (3.1–6.7)[8]	4.8 (1.4–7.4)[32]	4.1 (2.4–5.9)[13]	0.464
HGB (g/L)	120–180	128 (72–159)[8]	117 (33–178)[32]	104 (59–139)[13]	0.447
HCT (%)	37–55	32 (18–40)[8]	31 (10–48)[32]	29 (17–37)[13]	0.494
WBC (10^9/L)	6–17	5.6 (3.0–8.0)[8]	4.8 (1.6–14.5)[39]	5.9 (3.0–21.6)[13]	0.640
NEUT (10^9/L)	3–12	4.3 (2.0–5.7)[8]	3.3 (2.1–12.6)[29]	4.4 (2.3–17.5)[13]	0.523
MON (10^9/L)	0.2–1.2	0.3 (0.1–1.2)[8]	0.3 (0.0–1.9)[29]	0.5 (0.0–2.4)13	0.460
LYM (10^9/L)	1–4.8	0.9 (0.4–2.3)[8]	1.3 (0.3–4.5)[32]	0.8 (0.3–2.7)[13]	0.161
BANDS (10^9/L)	0.0–0.3	0.03 (0.00–0.30)[8]	0.01 (0.00–0.50)[29]	0.12 (0.00–0.60)[13]	0.098
PLT (10^9/L)	200–500	0 (0–237)[8]	0 (0–179)[32]	2 (0–118)[13]	0.305
GLU (mmol/L)	4.2–6.6	5.4 (4.3–6.8)[8]	5.6 (1.7–9.6)[32]	5.9 (3.0–7.3)[12]	0.610
Urea (mmol/L)	2.9–10.0	6.9 (2.4–25.9)[8]	5.6 (1.0–32.5)[31]	6.3 (2.6–13.0)[12]	0.354
CRE (µmol/L)	54–150	123 (58–184)[8]	85 (24–173)[32]	106 (67–205)[12]	0.010†
PHOS (mmol/L)	0.8–1.9	1.6 (1.3–3.4)[8]	1.6 (0.6–2.8)[32]	1.7 (0.9–2.3)[12]	0.460
TP (g/L)	54–75	67 (56–92)[8]	57 (41–73)[32]	60 (49–79)[12]	0.040*
Albumin (g/L)	29–35	36 (31–47)[8]	29 (17–42)[32]	33 (23–40)[12]	0.001†
T-BIL (µmol/L)	1.7–5.1	5.9 (2.4–24.1)[8]	3.2 (1.0–58.8)[23]	3.3 (1.7–15.9)[12]	0.110
CHOL (mmol/L)	3.5–7.5	5.9 (3.1–8.6)[8]	4.6 (2.5–8.0)[32]	4.8 (3.9–7.4)[12]	0.191
TRIG (mmol/L)	0.3–1.5	0.8 (0.4–1.6)[8]	0.8 (0.1–4.3)[32]	0.7 (0.5–1.0)[12]	0.494
ALT (U/L)	10–109	86 (19–198)[8]	26 (3–130)[32]	28 (4–72)[11]	0.026#
AST (U/L)	13–60	53 (24–141)[8]	29 (3–158)[32]	57 (9–98)[12]	0.084
ALP (U/L)	11–114	613 (306–1413)[8]	244 (86–568)[32]	214 (140–367)[12]	< 0.001#
GGT (U/L)	1–12	6 (3–9)[7]	6 (1–15)[31]	4 (1–8)[10]	0.317
Amylase (U/L)	219–1215	1159 (406–6555)[8]	1050 (471–5944)[29]	802 (288–1167)[12]	0.032#
Lipase (U/L)	25–250	69 (20–1091)[8]	33 (11–2432)[30]	43 (16–263)[12]	0.188
CK (U/L)	50–400	95 (5–252)[8]	98 (25–326)[32]	99 (54–210)[12]	0.928
Iron (µmol/L)	15–40	51 (33.0–69.3)[8]	24 (4.5–88.6)[30]	51 (37.4–84.3)[12]	< 0.001†
Parasitemia (C_p)	N/A	23.4 (21.2–25.9)[7]	25.8 (20.9–33.2)[43]	23.4 (21.2–32.0)[13]	0.022†

Abbreviations: *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *AST* aspartate aminotransferase, *BANDS* neutrophil bands, *Cp* crossing point value, *CHOL* cholesterol, *CK* creatine kinase, *CRE* creatinine, *GGT* gamma-glutamyltransferase, *GLU* glucose, *HCT* hematocrit, *HGB* hemoglobin, *LYM* lymphocytes, *MON* monocytes, *N/A* not applicable, *NEUT* neutrophils, *PHOS* inorganic phosphate, *PLT* platelets, *RBC* red blood cells, *T-BIL* total bilirubin, *TP* total protein, *TRIG* triglycerides, *WBC* white blood cells. Symbols: # – $P < 0.05$ for Oct–Dec vs. Jan–Feb/Feb–May, † – $P < 0.05$ for Feb–May vs. Jan–Feb/Oct–Dec, * – $P < 0.05$ for Feb–May vs. Jan–Feb, ♯ – $P < 0.05$ for Jan–Feb vs. Feb–May/Oct–Dec. The number in the italic superscript gives the number of analyzed samples

hemodilution due to water retention attempting to compensate for hypotension (Schetters 2019). Iron concentrations were close to the upper reference limit or increased during the Jan–Feb and the Oct–Dec periods, presumably as a consequence of the iron export from the red blood cells harboring *B. canis* (Ganz 2018; Ross 2017). Higher intensity of inflammatory response may be a promotor for the reduction of the iron values (Spottiswoode et al. 2014) in the Feb–May period, in comparison with the other two. Higher concentration of SAA pointed towards a stronger APR (Schmidt and Eckersall 2015) in the Feb–May in comparison with the Jan–Feb period. Nevertheless, the SAA concentration did not suggest the difference in the APR intensity between the Feb–May and Oct–Dec periods.

The fluctuations in the fibrinogen levels among the time periods seemed rather complex. Although in the majority of cases fibrinogen was above the reference range, the concentrations were lower in the Feb–May than in the Oct–Dec period. As fibrinogen belongs to the "slow" APPs (Cray 2012), it is possible that the clinical onset of the disease was so fast in the Feb–May period that it did not allow enough time for the synthesis of fibrinogen to increase. Also, lower fibrinogen levels might result from the systemic consumption. Namely, the conversion of fibrinogen to fibrin

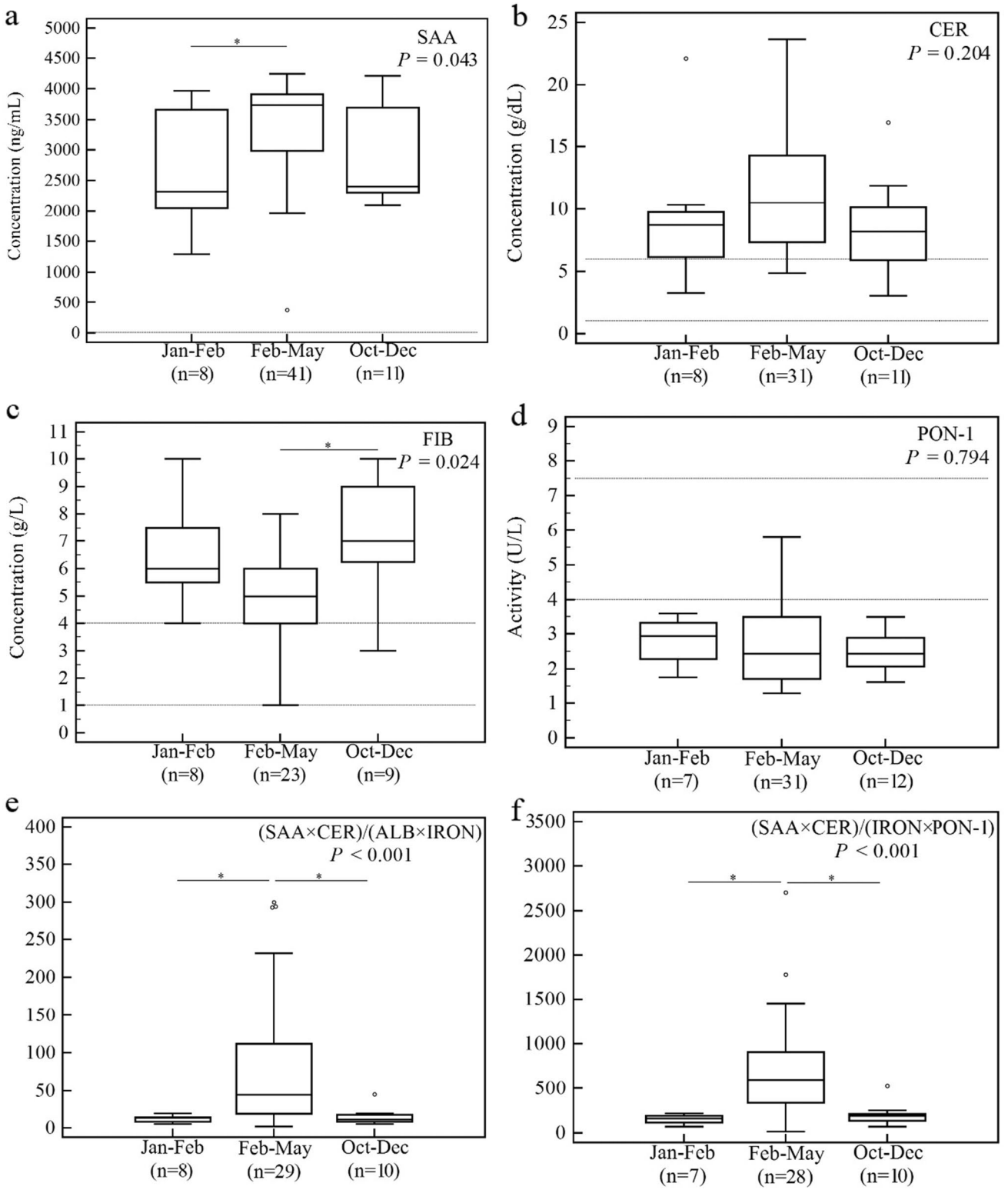

Fig. 1 Concentration of **a** serum amyloid A (SAA), **b** ceruloplasmin (CER), and **c** fibrinogen (FIB); **d** activity of paraoxonase-1 (PON-1); and values of the inflammatory indexes: **e** (SAA×CER)/(Albumin×Iron), and **f** (SAA×CER)/(Iron×PON-1). Kruskal-Wallis test, $P < 0.05$ is considered significant. Box represents the values from the lower to upper quartile. The middle line in the box represents the median. A line extends from the minimum to the maximum value. Circles represent outliers. The asterisk symbol * indicates a statistically significant difference between the groups connected by a line at the top. The dotted lines give the reference values from the clinical laboratory at the Faculty of Veterinary Medicine, University of Belgrade, Serbia

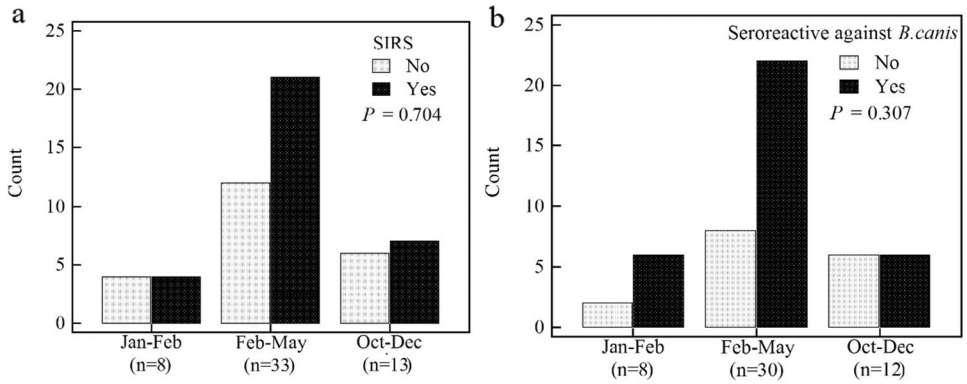

Fig. 2 Frequency of **a** systemic inflammatory response syndrome (SIRS), and **b** seroreactivity against *B. canis*. Chi-square test, $P < 0.05$ is considered significant

Table 2 Logistic regression analysis with babesiosis season as dependent variable

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Feb–May				
Parasitemia (C_p) > 26	3.81 (1.09–13.30)	0.035	4.39 (0.43–44.32)	0.210
Albumin < 31 g/L	17.00 (3.93–73.58)	0.002	17.26 (1.41–211.34)	0.026
CRE < 94 µmol/L	5.45 (1.56–19.06)	0.008	3.08 (0.36–26.25)	0.304
Iron < 37 µmol/L	24.75 (4.66–131.48)	0.002	62.82 (4.69–841.89)	0.002
(SAA x CER)/(Albumin x Iron)	25.14 (4.60–137.40)	< 0.001	N/A	
(SAA x CER)/(Iron x PON-1)	58.67 (6.42–536.30)	< 0.001	N/A	
Oct–Dec				
Amylase < 950 U/L	0.23 (0.05–0.98)	0.047	0.10 (0.02–0.61)	0.012

Abbreviations: C_p crossing point value, *CER* ceruloplasmin, *CRE* creatinine, *CI* confidence interval, *N/A* not applicable, *OR* odds ratio, *PON-1* paraoxonase 1, *SAA* serum amyloid A

contributes to the clearance of the *B. canis* parasites from circulation, because the infected erythrocytes become stored in the fibrin layer on the capillary margins. Moreover, a decrease in fibrinogen level closely accompanies a decrease in level of parasitemia (Schetters 2019). In line with that, the quantification of parasite DNA presence in the venous blood showed the lowest values detected in the Feb–May period. In some cases, level of parasitemia in internal organ capillaries can reach 100% (Schetters 2019), but it is not known whether *B. canis* sequestration and eventual proliferation in capillaries influence their number in the larger blood vessels. Thus, a degree of venous blood level of parasitemia should be interpreted with caution, as it is possible that in the Feb–May period, a significant number of parasites reside in fibrin layers in the capillaries.

Our results further showed that although the CER level and PON-1 activity were not different among the time periods, they underlined an omnipresent reaction to oxidative stress in acute canine babesiosis (Crnogaj et al. 2017). The CER concentrations above and the PON-1 activity below the reference limit were comparable across the time periods. This pattern might reflect the uniform involvement of CER as one of the main protective antioxidants (Bildik et al. 2004). The activity of PON-1 in the serum has a similar role, but it decreases as a consequence of self-inactivation while neutralizing reactive oxygen species (Aviram and Rosenblat 2004). These findings additionally point out a possibility that oxidative stress during *B. canis* infection does not correlate with intensity of inflammation quantified using the SAA and albumin concentrations.

The APIs were superior to individual acute phase proteins/reactants in differentiating the period of babesiosis occurrence on the basis of the APR intensity. When APPs are used to compare the degree of inflammation or tissue injury among naturally infected animals, a single protein/reactant is often not sensitive enough to detect the differences (Gruys et al. 2006). Various clinical courses (or stages in the evolution of the disease), incubation times of disease, and dogs' immune system specifics might affect the uniformity of APP or acute phase reactant changes (Gabay and Kushner 1999). In our study, both APIs undoubtedly denoted a distinct and more intense inflammatory response in dogs diagnosed in the Feb–May period, when the occurrence of their increased values was 20, or even 60 times, more probable than in the other two time periods. Still, our results raise two questions, which

necessitate further studies about the clinical significance of the observed temporal pattern in the intensity of APR in canine babesiosis. The first question is the absence of a significant difference in the frequency of the dogs that develop SIRS across the three observed time periods. Next, although the IgG against *B. canis* can originate from previous clinical infections or exposure, the similar rate of the dogs seroreactive against *B. canis* across different periods needs to be further addressed.

The Feb–May period also differed from the other two by the lower creatinine concentration. This feature is regarded as an additional indicator of hemodilution in the early onset of the clinical disease. In that context, the decreased creatinine concentration could be more specific than hypoalbuminemia (Schetters 2019).

Our study implies that the fast acute phase reactants SAA and iron, and albumin as a marker of hemodilution, are the main drivers of significant API change, so we hypothesized that the course of the disease is "more acute" in the Feb–May period. However, our study offers no answer to the ensuing question: Why is canine babesiosis "more acute" in that period? The fact that ticks are most active during the Feb–May period (Milutinović and Radulović 2002) does not fully answer the question. For example, we can presume that as a result of the high tick activity, our patients in the Feb–May period suffered from multiple tick bites—an assumption that would be highly challenging to ascertain—and it still would not help explain the lowest level of parasitemia detected over this period. A future investigation could address other possible key factors, such as a frequency of ticks hosting *B. canis*, the number of sporozoites per tick, and a likelihood that a tick saliva itself might cause APR (Strong-Klefenz and Gaskill 2008). It is also interesting to mention that a detachment of a male *D. reticulatus* after feeding on an infected dog, and a subsequent reattachment to a *Babesia*-naïve dog increases a possibility for horizontal transmission (Varloud et al. 2018).

The markedly elevated amylase activity was an additional parameter that distinguished the babesiosis diagnosed early in the year (Jan–Feb and Feb–May) from the one in autumn (Oct–Dec). As hyperamylasemia in dogs is not highly specific (Xenoulis 2015), the origin and clinical significance of this change need to be further investigated.

The potential limitation of this study represents the lack of data about the previous exposure to *B. canis* and immune status of the dogs. Apart from Knott test, these dogs were not tested to rule out other vector-borne diseases. Although clinical condition of the dogs in this study did not indicate the existence of associated diseases, this does not exclude the possibility of comorbidities that could affect APR.

Conclusion

Our results show that the strongest inflammatory response, quantified through APIs, is associated with acute *B. canis* infection developed in the Feb–May period. Taken together with the results indicating hemodilution in the same time period, our study implies that the most acute course of canine babesiosis develops between the months of February and May, i.e., in late winter and spring.

Author contribution MKF designed the study; FJ, KS, and VR collected the samples; FJ, MR, KS, JFA, MD, and AB performed the analyses; FJ and AB performed and interpreted the statistical analyses; FJ, MKF, AB, and JA interpreted the data; MKF, AB, and FJ contributed in the writing of the manuscript. All authors have read and approved the manuscript.

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Availability of data and material

The datasets analyzed in this study are available upon request from the corresponding author.

Declarations

Ethics approval The Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade, approved the study and permission was also obtained from the Ministry of Agriculture and Environmental Protection, Republic of Serbia (number 323-07-03455/2015-05/3).

Consent to participate The dogs' owners signed an informed consent that the surplus of samples collected during the diagnostic procedure could be used in the study.

Conflict of interest The authors declare no competing interests.

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