The electrophoretic pattern of serum proteins in dogs with babesiosis

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Abstract

This study was aimed at the evaluation of the electrophoretic pattern of serum proteins in dogs naturally infected with Babesia canis. Blood samples were collected from 37 dogs infected with B. canis and showing clinical signs consistent with the disease. The sick animals were classified as dogs with physiologic and decreased red blood cell (RBC) values. Twenty-five healthy dogs formed the control group. The concentrations of total proteins and protein fractions were measured in blood serum. The values of total proteins, albumin and albumin/globulin (A/G) ratio in dogs with babesiosis were significantly lower than in healthy ones (P < 0.001). In the globulin fractions, significantly higher relative concentrations of α1-, β1- and β2-globulins (P < 0.01), and non-significantly higher values of α2- and γ-globulins were found in dogs with babesiosis with a double α2-zone in six out of 37 animals. Marked differences were observed also between the two groups of sick animals, with significantly lower values of albumin and A/G ratio (P < 0.05), and significantly higher values of α1- and β1-globulins in dogs with decreased RBC (P < 0.05 and P < 0.01, respectively). Presented results indicate marked alterations in the electrophoretic pattern of serum proteins in dogs with babesiosis suggesting its usefulness for the evaluation of pathophysiological changes caused by the disease and for diagnostic of disease severity.

Blood parasites, canine, zone electrophoresis, protein fractions, red blood cells

Canine babesiosis belongs to frequently occurring tick-transmitted diseases in Europe with a clinical importance and is predominantly caused by the haemoproteozoan apicomplexan parasite Babesia canis (Uilenberg 2006). It is characterized by erythrocyte destruction resulting in varying degrees of haemolytic anaemia with a wide variety of associated clinical signs, including fever, lethargy, pigmenturia, coagulopathies, icterus, pale mucous membranes, as well as enlarged lymph nodes and spleen, tremors and organ failure (Mathe et al. 2006; Schoeman 2009). The severity of anaemia due to erythrocyte destruction varies from mild to severe, but according to some authors, the clinical manifestations of the disease are not always proportional to the degree of anaemia and are not always a consequence of haemolysis alone (Furlanello et al. 2005; Irwin 2010). Systemic inflammatory reactions may be also observed in dogs infected with B. canis characterized by increased production of some acute phase proteins (Ulutas et al. 2005; Matijatko et al. 2007; Schetters et al. 2009). On the other side, the infection with B. canis may be accompanied by other immune and inflammatory reactions of the body, manifested also by an increase of some lipid mediators in cases with severe complications (Mrljak et al. 2014). However, less information is available about the effect of pathophysiological alterations associated with babesiosis on the distribution of serum protein fractions in dogs naturally infected with B. canis. Similarly, little is known about the magnitude of changes in the serum protein pattern in dogs with various alterations in haematologic indices associated with the disease.

The objective of this study was to evaluate the electrophoretic pattern of serum proteins in dogs naturally infected with B. canis, and to describe the differences in the concentrations...
of major protein fractions between dogs with physiologic and decreased red blood cell (RBC) count caused by the infection.

Materials and Methods

Into the evaluation we included blood samples from 37 client-owned dogs naturally infected with *B. canis*, that were admitted to the Small Animal Clinic of the University of Veterinary Medicine and Pharmacy in Košice, Slovak Republic, during the year 2017. The dogs were of various breeds and both sexes (26 males and 11 females) in the age range of 6 months to 14 years. The evaluated animals showed clinical signs consistent with canine babesiosis, characterised by apathy, fever, loss of appetite, pale mucous membranes, and pigmenturia. At the time of admission, blood samples were collected for haematologic, biochemical and microscopic evaluation. All animals were positive for babesia confirmed by the detection of parasites within the infected erythrocytes in blood smears. According to the RBC count, the dogs suffering from babesiosis were categorized into 2 groups, defined as dogs with RBC values within the physiological range (5.5–8.5 T/l, mean haemoglobin concentration of 15.29 g/dl, mean haematocrit of 43.4%, n = 14) (Kraft and Dürr 2005) and dogs with decreased RBC values (less than 5.5 T/l, mean haemoglobin concentration of 9.27 g/dl, mean haematocrit of 25.2%, n = 23). In these groups of dogs, we evaluated also the outcome of the disease. Out of the sick animals, twenty-five clinically healthy dogs without any signs of diseases, negative for babesiosis and in good general condition were selected as control animals. These dogs were admitted to the University Veterinary Hospital for regular preventive examination and vaccination. They were considered healthy based on the physical examination and routine laboratory testing (haematology and serum biochemical analysis). Informed written consent was obtained from all dog owners.

Blood samples for protein analyses were taken before any treatment from the cephalic vein into serum gel separator tubes without any additives or anticoagulants (Sarstedt, Nümbrecht, Germany). The blood for haematologic examination was collected into tubes with ethylenediamine tetraacetic acid (EDTA) as anticoagulant (Sarstedt, Nümbrecht, Germany). The blood for microscopic evaluation was taken from capillaries of the earlobe. Permission to complete blood samples was obtained from each dog owner. The blood samples for biochemical analyses were centrifuged at 3,500 g for 10 min. After the separation of sera, haemolysis was inspected. Haemolysis was present in 9 from 37 samples from dogs with babesiosis. One aliquot of the serum was dispensed into plastic tubes for protein analyses, and stored at -20 °C until it was analysed.

Diff-Quick stain (Medion Diagnostics AG, Düdingen, Switzerland) was used for the demonstration of *Babesia* organisms within RBC in the blood smears. Haematologic analyses were done on an automatic haematologic analyser ProCyte Dx (IDEXXX Laboratories, Westbrook, Maine, USA). To evaluate the changes in the protein profile, serum samples were analysed for the concentrations of total proteins and main protein fractions. The total proteins (TP, g/l) were determined using an automated biochemical analyser Alizé (Randox, Crumlin, United Kingdom). Zone electrophoresis on an agarose gel using an automated electrophoresis system Hydrasys and commercial diagnostic kits Hydragel 7 Proteine (Sebia Corporate, Lisses, Evry Cedex, France) was used for the separation of serum protein fractions (Nagy et al. 2015). The following protein fractions were identified: albumin, α1-, α2-, β2- and γ-globulins. They were expressed as relative values (%) according to the optical density and their absolute concentrations (g/l) were quantified from the TP concentrations. The ratios of albumin to globulins (A/G) were also calculated.

The statistical analyses of the data were processed in the programme GraphPad Prism V5.02 (GraphPad Software Inc., California, USA). Descriptive statistical procedures were used to calculate arithmetic means (x) and standard deviations (SD) for each evaluated variable and group of animals. The distribution of data was evaluated by Kolmogorov-Smirnov test for normality. All parameters showed normal distribution. Unpaired *t*-test was used to assess the significance of differences in values between dogs with babesiosis and healthy animals, as well as between sick dogs with physiological and lower RBC values.

Results

The relative concentrations of albumin were significantly lower in the dogs with babesiosis compared to healthy animals (*P* < 0.001), with significantly lower values in dogs with lower RBC than in those with normal RBC (*P* < 0.05, Tables 1 and 2). An opposite trend was found in the relative concentrations of α2-globulins. The α2-globulins concentration was significantly higher (*P* < 0.01), while the α1-globulins concentration was non-significantly higher in sick dogs than in healthy ones. Further analysis of the relative concentrations of α-globulins showed significantly higher α1-globulins (*P* < 0.05) and non-significantly lower relative values of α2-globulins in dogs with lower RBC. Similarly, the dogs with babesiosis were found to have significantly higher relative concentrations of β1- and β2-globulins (*P* < 0.01) compared to healthy animals. Comparison of the relative concentrations of
β-globulins between the two groups of sick animals showed significantly higher β₁-globulins ($P < 0.01$) and non-significantly higher β₂-globulins in dogs with lower RBC. In the relative concentrations of γ-globulins a trend of non-significantly higher values in dogs with babesiosis was observed, with no further significant differences between the two groups of sick animals. The mean value of A/G ratios was significantly lower in dogs with babesiosis compared with clinically healthy animals ($P < 0.001$), being significantly lower in dogs with lower RBC than in dogs with normal RBC values ($P < 0.05$). Representative examples of the electrophoretic pattern of serum proteins with differences in clinically healthy dogs and dogs with babesiosis are presented in Fig 1.

The dogs with babesiosis had significantly lower concentrations of TP than the clinically healthy animals ($P < 0.001$, Tables 3 and 4). The evaluation of TP concentrations in the two groups of sick animals showed non-significantly lower values in dogs with lower RBC than in dogs with normal RBC values ($P < 0.05$). Representative examples of the electrophoretic pattern of serum proteins with differences in clinically healthy dogs and dogs with babesiosis are presented in Fig 1.

The dogs with babesiosis had significantly lower concentrations of TP than the clinically healthy animals ($P < 0.001$, Tables 3 and 4). The evaluation of TP concentrations in the two groups of sick animals showed non-significantly lower values in dogs with lower RBC than in dogs with normal RBC values ($P < 0.05$). The absolute concentrations of α₁-globulins were non-significantly higher in dogs affected by babesiosis. The mean value obtained in dogs with lower RBC was significantly higher ($P < 0.05$). On the other hand, no significant differences were observed in the absolute concentrations of α₂-globulins between healthy and sick animals, as well as between

Table 1. Differences in the relative concentrations of serum protein fractions (%) and albumin/globulin ratio (A/G) between dogs with babesiosis and clinically healthy dogs (mean ± standard deviation).

| Variable   | Groups of dogs | P value |
|------------|----------------|---------|
|            | With babesiosis | Healthy |
|            | (n = 37)       | (n = 25) |
| Albumin    | 45.0 ± 6.1     | 52.7 ± 4.4 | < 0.001 |
| α₁-globulins | 5.1 ± 1.1     | 4.2 ± 0.8  | < 0.01  |
| α₂-globulins | 16.5 ± 3.8    | 15.0 ± 1.8 | n.s.    |
| β₁-globulins | 12.9 ± 4.0    | 10.0 ± 1.9 | < 0.01  |
| β₂-globulins | 11.1 ± 2.6    | 9.4 ± 1.2  | < 0.01  |
| γ-globulins   | 9.4 ± 2.4     | 8.6 ± 2.4  | n.s.    |
| A/G         | 0.84 ± 0.21   | 1.14 ± 0.22 | < 0.001 |

P value – significance of the differences, n.s. – not significant.

Table 2. Comparison of the relative concentrations of serum protein fractions (%) and albumin/globulin ratio (A/G) between sick dogs with physiological (N) and lower (L) RBC values (mean ± standard deviation).

| Variable   | Groups of dogs | P value |
|------------|----------------|---------|
|            | N (n = 14)     | L (n = 23) |
| Albumin    | 48.0 ± 4.8     | 43.2 ± 6.2 | < 0.05  |
| α₁-globulins | 4.6 ± 0.4     | 5.4 ± 1.3  | < 0.05  |
| α₂-globulins | 17.3 ± 4.3    | 16.1 ± 3.5 | n.s.    |
| β₁-globulins | 10.3 ± 2.4    | 14.4 ± 4.1 | < 0.01  |
| β₂-globulins | 10.3 ± 2.0    | 11.5 ± 2.9 | n.s.    |
| γ-globulins   | 9.6 ± 3.2     | 9.3 ± 1.8  | n.s.    |
| A/G         | 0.94 ± 0.18   | 0.78 ± 0.20 | < 0.05  |

P value – significance of the differences, n.s. – not significant, RBC – red blood cell

Table 3. Differences in the concentrations of total serum proteins (TP, g/l) and absolute values of protein fractions (g/l) between dogs with babesiosis and clinically healthy dogs (mean ± standard deviation).

| Variable   | Groups of dogs | P value |
|------------|----------------|---------|
|            | With babesiosis | Healthy |
|            | (n = 37)       | (n = 25) |
| TP         | 56.4 ± 7.9     | 63.3 ± 5.8 | < 0.001 |
| Albumin    | 25.5 ± 5.2     | 33.3 ± 3.8 | < 0.001 |
| α₁-globulins | 2.8 ± 0.4     | 2.6 ± 0.4  | n.s.    |
| α₂-globulins | 9.4 ± 2.6     | 9.5 ± 1.3  | n.s.    |
| β₁-globulins | 7.2 ± 2.3     | 6.3 ± 1.3  | n.s.    |
| β₂-globulins | 6.3 ± 1.8     | 6.0 ± 1.2  | n.s.    |
| γ-globulins   | 5.3 ± 1.6     | 5.5 ± 1.7  | n.s.    |

P value – significance of the differences, n.s. – not significant
Table 4. Comparison of the concentrations of total serum proteins (TP, g/l) and absolute values of protein fractions (g/l) between sick dogs with physiological (N) and lower (L) RBC values (mean ± standard deviation).

| Variable  | Groups of dogs with babesiosis |  | P value |
|----------|-------------------------------|---|---------|
|          | N (n = 14)                    | L (n = 23) |       |
| TP       | 58.5 ± 7.7                    | 56.1 ± 7.9 | n.s.   |
| Albumin  | 28.1 ± 4.7                    | 23.8 ± 4.9 | < 0.05 |
| α₁-globulins | 2.6 ± 0.3                   | 2.9 ± 0.5 | < 0.05 |
| α₂-globulins | 10.1 ± 2.6                   | 8.9 ± 2.6 | n.s.   |
| β₁-globulins | 6.0 ± 1.5                    | 7.9 ± 2.4 | < 0.05 |
| β₂-globulins | 6.1 ± 1.6                    | 6.4 ± 1.9 | n.s.   |
| γ-globulins | 5.6 ± 2.1                     | 5.2 ± 1.3 | n.s.   |

P value – significance of the differences, n.s. – not significant, RBC – red blood cell

Seven of the total number of dogs with babesiosis died spontaneously, of which six were from the group with lower RBC values (23/6) and only one from those with the normal RBC count (14/1).

Fig. 1 a-d. Representative electrophoretograms in dogs: a – healthy, b – dog with babesiosis, red blood cell (RBC) values within the physiological range, c – dog with babesiosis, lower RBC values, d – dog with babesiosis, haemolytic sample with double α₂-zone
Discussion

Several biochemical parameters and clinical biomarkers have been investigated in dogs affected by babesiosis in order to evaluate their diagnostic significance, that potentially may be helpful in the determination of the severity of the disease. However, the impact of Babesia infections in dogs on the changes of serum protein fractions is not completely understood and the data are not uniform. The results presented in our study showed lower concentrations of TP in dogs with babesiosis, with non-significantly lower values in animals with decreased RBC values. Lower TP level has been reported also by Lobetti et al. (2000) in dogs with mild and severe babesiosis and by Camacho et al. (2005) in dogs with renal failure infected with B. annae when compared to healthy animals. Furthermore, lower TP concentrations were found by Eichenberger et al. (2016) in nonsurvivor dogs affected by babesiosis compared with those that survived. This may be explained by potential protein-losing nephropathy caused by a hypoxic renal damage (Zygner and Gójska-Zygner 2014). Markedly decreased TP concentrations were observed also in sheep naturally infected with B. ovis (Apaydin and Dede 2010). In contrast to these results, increased TP concentrations have been reported in goats and horses with babesiosis, as well as in dogs infected with large Babesia probably resulting from the dehydration due to lethargy and anorexia (Barrera et al. 2010; Esmaeilnejad et al. 2013; Zygner et al. 2007, 2011). Renal changes, including haemoglobinuric nephropathy, acute kidney injury, glomerulonephritis, renal failure, as well as renal insufficiency belong to possible complications in canine babesiosis (Defauw et al. 2012). Thus, lower concentrations of TP observed in our study in dogs with babesiosis might result from the loss of proteins through the kidneys as an effect of the aforementioned renal changes.

The mean concentration of albumin in the study was significantly lower in dogs with babesiosis, which was more marked (only 23.8 g/l) in animals with lower RBC values. Similarly, Sudhakara Reddy et al. (2016) found reduced serum albumin concentrations in dogs with Babesia infections compared to healthy animals. Protein-losing nephropathy associated with glomerular leakage of proteins and membranoproliferative glomerulonephritis, as well as renal impairment due to the damage of renal cells by inflammatory mediators may result in decreased albumin concentrations (Littman 2011). Hepatopathy with marked icterus, or centrilobular hepatitis with hypoxic liver damage as possible complications in canine babesiosis is another cause of reduced albumin synthesis by the liver (Taboada and Lobetti 2006). On the other hand, albumin is a major negative acute phase protein, its lower concentrations in dogs with babesiosis, therefore, may be attributed to the systemic inflammatory response syndrome caused by a marked cytokine release in the disease (Schetters et al. 2009). Marked hypoaalbuminaemia was observed also in ovine and caprine babesiosis and was related to hepatopathy caused by the disease, development of anorexia, or urinary loss of albumin due to renal failure (Apaydin and Dede 2010; Esmaeilnejad et al. 2013). Furthermore, Eichenberger et al. (2016) found lower concentrations of albumin in nonsurvivor dogs suffering from babesiosis compared with survived animals. Thus, lower albumin values in dogs with lower RBC observed in our study may suggest that albumin could be a useful marker to evaluate the magnitude of changes caused by the disease.

In addition, some other proteins associated with the activation of a host immune response were observed in canine babesiosis, including increased concentrations of serum amyloid A, haptoglobin, as well as ceruloplasmin (Ulutas et al. 2005; Matijatko et al. 2007). Lobetti et al. (2000) reported that despite systemic inflammatory response in dogs with babesiosis evidenced by increased concentrations of the aforementioned acute phase proteins, this pattern is not detectable on serum protein electrophoresis. In our study, the electrophoretic separation of serum proteins resulted in higher concentrations of \(\alpha_1\)-, as well as \(\alpha_2\)-globulins in dogs with babesiosis when compared with healthy ones. Increased
α₁- and α₂-globulins were obtained also by Furlanello et al. (2005) in dogs naturally infected with *B. canis*. The majority of acute phase proteins (α₁-antitrypsin, α₁-acid glycoprotein, serum amyloid A, haptoglobin, α₂-macroglobulin, ceruloplasmin) belong to the α-globulins (Bossuyt 2006). Thus, the increases of the alpha fractions in canine babesiosis may reflect the increases in the concentrations of some acute phase proteins, resulting from the activation of the host inflammatory responses due to the infection and tissue destruction caused by the disease. The α₂-globulin fraction may typically increase in cases with the nephrotic syndrome (associated with babesiosis), as a result of the increased synthesis of α₂-macroglobulin from this fraction, which due to its size is unable to pass through glomeruli and is retained in the bloodstream (de Sain-van der Velden et al. 1998). On the other hand, Zygner et al. (2011) observed decreased concentrations of α₁- and α₂-globulins in dogs with babesiosis, probably caused by free haemoglobin due to intravascular haemolysis or liver damage caused by the disease (Martinez-Subiela et al. 2002). Similarly, babesiosis in sheep was accompanied by decreased concentrations of α-globulins (Apaydin and Dede 2010). Furthermore, a double α₂-zone was observable in our study in six out of 37 dogs with babesiosis, while four of them were from the group of dogs with a lower RBC count. This pattern was probably caused by more severe intravascular haemolysis due to babesiosis.

In the present study, a trend of higher relative values was observed in dogs with babesiosis also in the β₁- and β₂-globulin fractions. Similarly, an increase of β-globulins was described by Solano-Gallego et al. (2008) in 9 out of 24 dogs (37.5%) infected with *B.canis* and by Furlanello et al. (2005) in 13 out of 23 dogs (56.5%) infected with a large form of *Babesia*. Increases in the concentrations of β-globulins may be caused by elevated production of transferrin associated with anaemia in the infected dogs (Zygner et al. 2011). Increased synthesis of the C3a complement belongs among other possible causes of hyper-β-globulinaemia, which may be related to the development of intravascular haemolysis and thrombocytopenia in canine babesiosis (Zygner et al. 2007). Furthermore, complement is involved in the regulation of inflammatory processes and, thus, may be attributed to the marked elevation of β-globulins due to infection and tissue damage in the affected dogs (Kuleš et al. 2014). C-reactive protein (CRP) is another protein that belongs to the β-globulin fraction. According to the magnitude of its response during inflammatory processes, CRP was classified as a major positive acute phase protein in dogs (Yamamoto et al. 1992). It has been shown that CRP concentrations increase in *B. rossi* and *B. gibsoni* infections (Ulutas et al. 2005). Thus, marked elevation of β-globulin fractions in dogs with babesiosis may be attributed to the increase of the aforementioned serum proteins from this fraction. According to Lobetti (1998), the severity of canine babesiosis is related to the degree of replication of parasites in the host’s erythrocytes and their subsequent lysis. In the study presented by Ulutas et al. (2005), the concentrations of CRP and ceruloplasmin were markedly higher in dogs with complicated babesiosis (although in a small sample size) compared to dogs with an uncomplicated disease process, suggesting the relation of their concentrations to the disease severity. On the other hand, Köster et al. (2009) identified no association between CRP concentrations and the outcome of the disease. Our results showed higher concentrations of α₁-, β₁- and β₂-globulins in dogs with lower RBC reflecting the response of the body to the infection and severity of the disease. It should be taken into consideration that the concentrations of the evaluated parameters may be related to the stage of the disease at the time of sample collection. Furthermore, additional studies on larger animal groups are required to yield satisfactory results.

Lobetti et al. (2000) observed marked differences in the concentrations of γ-globulins between dogs with mild, severe and complicated babesiosis with the highest values in severe and complicated cases. Increased γ-globulins were obtained also by Zygner et al. (2011) in 25.8% of the infected dogs, as well by Esmaeilnejad et al. (2013) in goats and
by Barrera et al. (2010) in horses with babesiosis, which was related to the activation of humoral immunity by the antibody responses to the *Babesia* antigens. Our results also showed higher relative concentrations of γ-globulins in dogs with babesiosis. On the other hand, the values recorded in dogs with lower RBC were slightly lower compared with dogs with normal RBC, probably due to haemolysis caused by the disease (Giot 2010). The above mentioned changes in the concentrations of albumin and globulin fractions resulted also in alterations in the A/G ratio. The values recorded in dogs with babesiosis were significantly lower compared with clinically healthy animals, being lower in dogs with lower RBC. These low A/G values are consistent with the loss of albumin due to protein-losing nephropathy as a possible complication of babesiosis in dogs, or with the overproduction of globulins caused by the infection (Kaneko 1997).

Because of insufficient and ununiform data regarding the impact of *Babesia* infections in dogs on the changes of serum protein fractions, the results of the present study represent important broadening of knowledge in this area of research. They suggest a significant effect of babesiosis on the protein profile characterised by alterations in the electrophoretic pattern of serum proteins and changes in the concentrations of separated protein fractions. The values of TP, albumin and A/G ratio were lower in the infected dogs compared to those in healthy animals, whereas the concentrations of α₁-, α₂-, β₁-, β₂- and γ-globulins were higher. Marked differences in the results were observed between dogs with normal and lower RBC. The electrophoretic pattern of serum proteins showed a double α₂-zone in six out of 37 dogs with babesiosis, while four of them were from the group of dogs with lower RBC. These results suggest a possible diagnostic importance of serum protein electrophoresis in the evaluation of the severity of the disease, reflecting the inflammatory responses and magnitude of changes caused by the disease.

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