Characterization of serum protein fractions of dogs naturally infected with *Ehrlichia canis* or *Anaplasma platys* associated with uveitis

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Abstract. Uveitis associated with *Ehrlichia canis* or *Anaplasma platys* infections were reported in dogs. However, only two *E. canis*-infected dogs with hypergammaglobulinemia showed acute blindness were reported. There were limited data of the species of *Ehrlichia* or *Anaplasma* and the alteration of serum protein fractions in infected dogs. Thus, the species of causative pathogen were investigated and compared the serum protein fractions between infected dogs associated with anterior uveitis and panuveitis in clinical situations. All 103 studied dogs were brought into the ophthalmology clinic which each dog showed signs of unilateral or bilateral uveitis related to ehrlichial infection. Dogs were divided into anterior uveitis and panuveitis groups. The species of *Ehrlichia* or *Anaplasma* were identified using nested-PCR based on the 16S rRNA gene and DNA sequencing from blood samples. The serum protein fractions were analyzed using electrophoresis. Fifty-eight dogs (56.31%) were positive of which *E. canis* and *A. platys* were detected in 51 and 7 dogs, respectively. The total serum protein and globulin levels were higher in the infected dogs associated with panuveitis than anterior uveitis while the albumin levels were significantly lower in the panuveitis group. The A/G ratios significantly decreased in both groups. Gamma globulin was detected at high levels in both groups while beta globulin significantly increased in the panuveitis group. Hypergammaglobulinemia was detected in 76.92 and 90.90% of infected dogs associated with anterior uveitis and panuveitis, respectively. Most of the infected dogs associated with panuveitis showed significantly levels of hyperproteinemia, hyperbetaglobulinemia and hypergammaglobulinemia compared with anterior uveitis group. *E. canis* was found as the major pathogen in infected dogs associated with uveitis in this study.

INTRODUCTION

Ehrlichia and *Anaplasma* are obligatory intracellular alpha-proteobacteria. In Thailand, based on molecular techniques, only three species have been reported in infected dogs: *Ehrlichia canis*, *Anaplasma platys* and *Anaplasma phagocytophilum* (formerly *Ehrlichia equi* or *Ehrlichia phagocytophila*) (Suksawat *et al.*, 2001; Pinyoowong *et al.*, 2008; Jirapattharasate *et al.*, 2012; Saeng-Chuto *et al.*, 2016;

Kaewmongkol *et al.*, 2017; Piratae *et al.*, 2019). *E. canis*, *A. platys* and *A. phagocytophilum* reside within the cytoplasm of the monocyte, platelet and neutrophil, respectively, as tick-borne pathogens which are transmitted by the ixodid tick with worldwide distribution. *A. phagocytophilum* also causes human granulocytic anaplasmosis (HGA) and this pathogen spreads the disease in several animals, including horse, sheep and dog (Severo *et al.*, 2012). However, only *E. canis*

and *A. platys* infections have been reported as pathogens associated with uveitis in dogs (Massa *et al.*, 2002; Saeng-Chuto *et al.*, 2016). *E. canis* infection causes canine monocytic ehrlichiosis and appears in three stages: acute, subclinical and chronic which may be obvious by fever, depression, lymphadenopathy, splenomegaly, anemia, leukopenia and thrombocytopenia (Harrus *et al.*, 1999; Skotarczak, 2003). The ocular signs are a common feature of the disease and can be presented in all stages (Leiva *et al.*, 2005). The most common ocular disorders from *E. canis* infection include hyphema, anterior uveitis, panuveitis, optic neuritis, retinal hemorrhage and retinal detachment. Substantial levels of anterior uveitis (29.40–39.47%) and posterior uveitis (44.12–64.7%) with retinal detachment leading to acute blindness in *E. canis*-infected dogs have been reported (Leiva *et al.*, 2005; Saeng-Chuto *et al.*, 2016). *A. platys* can cause the clinical signs compatible with canine erhliciosis (hemorrhagic manifestation and thrombocytopenia) in infected dogs but generally it is mild-to-moderate clinical signs (Abarca *et al.*, 2007). *A. platys* was also reported as the etiology of both anterior and posterior uveitis in infected dogs (Glaze & Gaunt, 1986; Saeng-Chuto *et al.*, 2016).

Biochemical abnormalities in dogs infected with *E. canis* have been mentioned. From the previous studies, serum protein fractions analyses using electrophoresis showed hypoalbuminemia, hyperglobu-linemia and hypergammaglobulinemia in infected dogs but the ocular signs were not mentioned in these studies (Breitschwerdt *et al.*, 1987; Harrus *et al.*, 1996). Moreover, the association between hyperproteinemia with hypergammaglobulinemia and panuveitis leading to acute blindness in *E. canis*-infected dogs were reported. Serum protein electrophoresis of two Labrador retrievers revealed monoclonal gammopathy and hypoalbuminemia (Harrus *et al.*, 1998; Gould *et al.*, 2000). To the best of our knowledge, a wider investigation for an association between serum protein fractions and the type of uveitis (anterior uveitis or panuveitis) has never been reported. Thus, the aims of the present study were to investigate the species of *Ehrlichia* and *Anaplasma* causing uveitis in clinical situations and to compare the serum protein fractions between infected dogs associated with anterior uveitis and panuveitis.

MATERIALS AND METHODS

Animal selection and criteria

One hundred and three dogs were brought into the ophthalmology clinic of a veterinary teaching hospital. Each dog showed signs of unilateral or bilateral uveitis related to ehrlichial infection which had been screened by the SNAP 4Dx test (IDEXX Laboratories, USA) or the dogs were suspected of having *Ehrlichia* or *Anaplasma* infections. Dogs were divided into two groups according to ocular signs, defined as anterior uveitis (n = 41) and panuveitis (n = 62). All dogs fulfilled the 3 criteria of (1) having no history of ocular trauma (2) having no systemic diseases and neoplasia and (3) having no dehydration status. Control dogs were selected from 20 healthy dogs all of which were blood donors. Blood samples were collected from cephalic or saphenous veins and kept in EDTA tubes and clotted blood tubes (Fushino®, Japan) for PCR assay and serum protein analysis, respectively. This study was approved by the Animal Experimental Committee of Kasetsart University (ACKU 59-VET-007).

DNA extraction

Blood samples (100 µl each) were lyzed with 600 µl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, 0.5% N-lauroylsarcosine), added with proteinase K 20 µl and shaken for 5 min. DNA was extracted using phenol-chloroform extraction and then precipitated in absolute ethanol, as previously described (Sambrook & Russell, 2001). DNA products were re-suspended in TE buffer (50 mM Tris, pH 8.0, 1 mM EDTA) and stored at -20°C until use.

Nested-PCR and DNA sequencing

DNA products were used as a template to amplify the 16S rRNA gene of the species of

Ehrlichia or *Anaplasma* using nested-PCR as previously described (Saeng-Chuto *et al.*, 2016). The first PCR, primers EF: 5'-TTGTA GCTAACGCGTTAAGCACT-3' and ER: 5'-AACTCGAAGCTGGTGYGCYAACC-3' amplified a 551-bp region. The second PCR, primers IEF: 5'-GTTCGGCTGGAYCTYRCA CAGGT-3' and IER: 5'-CTGMAACTCGAGAG CATGAAGTC-3' amplified a 310-bp region of the 16s rRNA gene. In brief, the thermal cycling conditions for both PCR reactions were 94°C for 5 min, 45 cycles of [94°C for 20 sec, 57°C for 20 sec, and 72°C for 40 sec], and the final extension 72°C for 10 min. The positive control consisted of DNA from blood samples of dogs known to be naturally infected by *E. canis* and *A. platys*. Both PCR protocols were processed in a MyCycler™ Thermal Cycler (BioRad Laboratories, USA).

Babesia canis infection was also checked using PCR assay. Mixed infection between *E. canis* or *A. platys* and *B. canis* can occur naturally in dogs. DNA amplifi- cation of *B. canis* was performed using the specific primers Can172F: 5'-GTTTATTAG TTTGAAACCCGC-3' and Can626R: 5'-GAA CTCGAAAAAGCCAAACGA-3' to amplify PCR product approximately 550-bp. Two microliters of DNA extract from each sample was used as the template DNA for PCR in a final volume of 25 µl. The PCR conditions followed the protocol as previously described with an annealing temperature at 55°C and 35 cycles (Inokuma *et al.*, 2004). Twenty microliters of amplified nested-PCR product of *Ehrlichia* and PCR product of *B. canis* were added to the 5 µl of 6X loading buffer and loaded in 1.5% agarose (SeaKem ME:FMC, USA) gel electrophoresis for 40 min. Amplicons were processed using UltraClean™ GelSpin DNA purification Kit (MO BIO LABORATORIES Inc., USA) and submitted for sequencing (1st Base Labora- tory, Malaysia). DNA sequences were compared for the similarity of the nucleotide base with the genomic database in Genbank using the BLAST algorithm.

Total protein level and serum protein electrophoresis
PCR-positive samples for *Ehrlichia* or *Anaplasma* infection were randomly selected for the study of serum protein electro- phoresis. Serum samples in clotted blood tubes were centrifuged at 978 xg for 5 min and then sera were harvested and stored at -40°C until use. The serum protein concentration was determined using spectrophotometry following the protocol of the Bradford method. The separation of protein fractions was performed using 13 and 22 serum samples of infected dogs associated with anterior uveitis and panuveitis, respectively and 20 serum samples of the control group using the cellulose acetate electrophoresis Helena system (Helena Laboratories 2001, USA) with TRIS-barbital-sodium barbital buffer pH 8.8 (Electra® HR buffer, Helena, USA). Human serum protein standard was used as control sera in a separated membrane (Helena, USA). The electrophoresis was performed for 15 min at 180 V and then moved for dyeing in Ponceau S staining solution: serum protein electrophoresis fractions were analyzed using the Image Lab software (BioRad Laboratories, USA).

Statistical Analysis
The Tukey-Kramer Test and Dunn's Test Statistical System (NCSS) ver. 2000 (Kaysville, USA) programs were used to compare the relationship between the concentration of serum protein fractions and ocular signs (anterior uveitis/panuveitis). A *p-value* $\leq$ 0.05 was considered as statistically significant. The results were presented as the mean ± standard deviation.

RESULTS

In total, 58/103 dogs (36 males and 22 females) or 56.31% were positive for *Ehrlichia* or *Anaplasma* infection (Table 1). The age range of infected dogs was 0.5 to 14 years, with a mean of 6.47 years. DNA sequencings showed 47/58 (81.03%) and 7/58 (12.07%) positive dogs were infected with *E. canis* and *A. platys*, respectively. *B. canis* infection was found in four *Ehrlichia*-positive dogs (4/58, 6.9%). The mixed infections between *E. canis* and *B. canis* were only found in the present study.

Table 1. Results of PCR and DNA sequencing of 58 positive dogs grouped by the type of uveitis

Group	Number of infected dogs		
	E. canis (%)	*A. platys* (%)	*B. canis* and *E. canis* (%)
Anterior uveitis (n=41)	17 (41.46)	4 (9.75)	2 (4.87)
Panuveitis (n=62)	30 (48.38)	3 (4.83)	2 (3.22)
Total (n=103)	47 (45.63)	7 (6.79)	4 (3.88)

Dogs with this mixed infection were excluded from the study of serum protein fraction analysis. Both of *A. platys* and *E. canis* caused anterior uveitis and panuveitis in the present study. The ocular disorders in infected dogs associated with panuveitis were hyphema, vitreous hemorrhage, retinal hemorrhage and retinal detachment.

The mean values of total serum proteins significantly increased in infected dogs associated with anterior uveitis or panuveitis compared with the control dogs. Comparison between the two groups of infected dogs indicated that the total serum protein was also significantly higher in infected dogs associated with panuveitis than anterior uveitis. Mean values of albumin concentration were significantly low in infected dogs associated with panuveitis compared with the control dogs. There was no significant difference in the albumin level between infected dogs associated with anterior uveitis and the control groups. The mean values of globulin concentration were significantly higher in infected dogs associated with anterior uveitis or panuveitis compared with the control group. Moreover, the level of globulin in infected dogs associated with panuveitis was significant higher than for anterior uveitis ($p \leq 0.05$). The albumin/globulin (A/G) ratio significantly decreased in both groups of infected dogs associated with uveitis compared with the control group (Table 2).

Comparison between groups of infected dogs associated with anterior uveitis or panuveitis and the control dogs indicated that the mean concentration values of gamma globulins were statistically higher ($p \leq 0.05$). Hypergammaglobulinemia was detected in 10/13 (76.92%) and 20/22 (90.90%) of the infected dogs associated with anterior uveitis

and panuveitis, respectively (Figure 1). The mean concentration values of beta globulin significantly increased ($p \leq 0.05$) in infected dogs associated with panuveitis compared with anterior uveitis or the control groups. Elevated levels of beta globulin were detected in 14/22 (63.63%) of the infected dogs associated with panuveitis. However, there was no statistical difference of the concentration levels of alpha1 or alpha2 globulins between the two groups of infected dogs associated with uveitis and the control dogs (Table 2 and Figure 1) ($p > 0.05$). All infected dogs with hyperglobulinemia showed polyclonal gammopathy (Figure 2).

DISCUSSION

From the present study, 23 and 35 infected dogs were associated with anterior uveitis and panuveitis, respectively. All infected dogs were in the acute or subclinical stages. Only *E. canis* and *A. platys* were detected in infected dogs: the other species of *Ehrlichia* (*E. ewingii*, *E. chaffeensis*) and *A. phagocytophilum* were not detected. The prevalence of infections with *E. canis* or *A. platys* were found in male dogs more than female dogs which are similar to the previous results in Thailand (Saeng-Chuto *et al.*, 2016; Piratae *et al.*, 2019). *E. canis* was a major pathogen for anterior uveitis and panuveitis of dogs in the present study. This is similar to previous studies reporting that only *E. canis* and *A. platys* infections can cause uveitis in a clinical situation (Glaze & Gaunt, 1986; Leiva *et al.*, 2005; Saeng-Chuto *et al.*, 2016). The ocular disorders in *E. canis* or *A. platys*-infected dogs associated with panuveitis were similar to the previous report (Saeng-Chuto *et al.*, 2016). However, the infection

Table 2. Concentrations of total serum protein, albumin, globulin, A/G ratio, α_1-, α_2, β- and γ-globulin presented as mean ± SD of 13 and 22 infected dogs associated with anterior uveitis and panuveitis, respectively, and 20 normal control dogs. The different lowercase superscript letters indicate statistically significant differences at $p \leq 0.05$

| Parameter	Control	Anterior uveitis	Panuveitis
Total protein (g/dl)	6.23 ± 0.37 [a]	7.16 ± 0.87 [b]	8.02 ± 1.15 [c]
Albumin (g/dl)	3.85 ± 0.40 [a]	3.20 ± 1.21 [a]	2.67 ± 0.93 [b]
Globulin (g/dl)	2.38 ± 0.45 [a]	3.96 ± 1.38 [b]	5.35 ± 1.69 [c]
A/G ratio	1.72 ± 0.60 [a]	1.03 ± 0.76 [b]	0.62 ± 0.46 [b]
α_1-globulin (g/dl)	0.12 ± 0.07 [a]	0.15 ± 0.07 [a]	0.11 ± 0.05 [a]
α_2-globulin (g/dl)	0.66 ± 0.21 [a]	0.96 ± 0.54 [a]	0.71 ± 0.32 [a]
β-globulin (g/dl)	1.06 ± 0.28 [a]	0.85 ± 0.34 [a]	1.53 ± 0.81 [b]
γ-globulin (g/dl)	0.53 ± 0.23 [a]	2.00 ± 1.52 [b]	3.00 ± 1.56 [b]
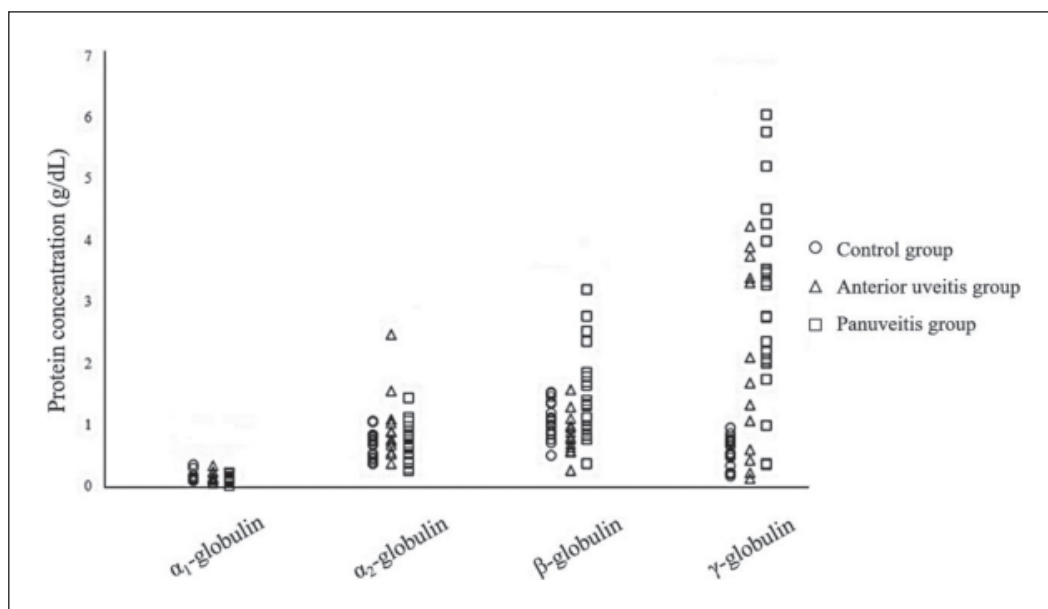
Figure 1. Concentrations of four serum protein fractions of 13 and 22 *Ehrlichia*-infected dogs associated with anterior uveitis and panuveitis, respectively, and 20 normal control dogs.

rate in the present study using nested-PCR detection was 56.31% which was lower than the suspected case numbers with canine ehrlichiosis or anaplasmosis. It is possible that the detection of ehrlichial DNA in blood may result in a lower succession than expected, as spleen aspiration is a good source of DNA for PCR because the spleen is probably the last place to harbor *E. canis* in the subclinical stage (Harrus *et al.*, 1999, 2004). *E. canis* seems to persist longer in splenic macrophages more than blood monocytes. Mixed infections between *E. canis* and *B. canis* or *A. platys* with *B. canis* can naturally occur in dogs because these pathogens are tick-borne, sharing the same vector in Thailand. In the present study, the mixed infections between *E. canis* and *B. canis* was detected at a low percentage which was similar to the previous report that identified this mixed infections in only 2.5% of studied dogs using nested-PCR (Piratae
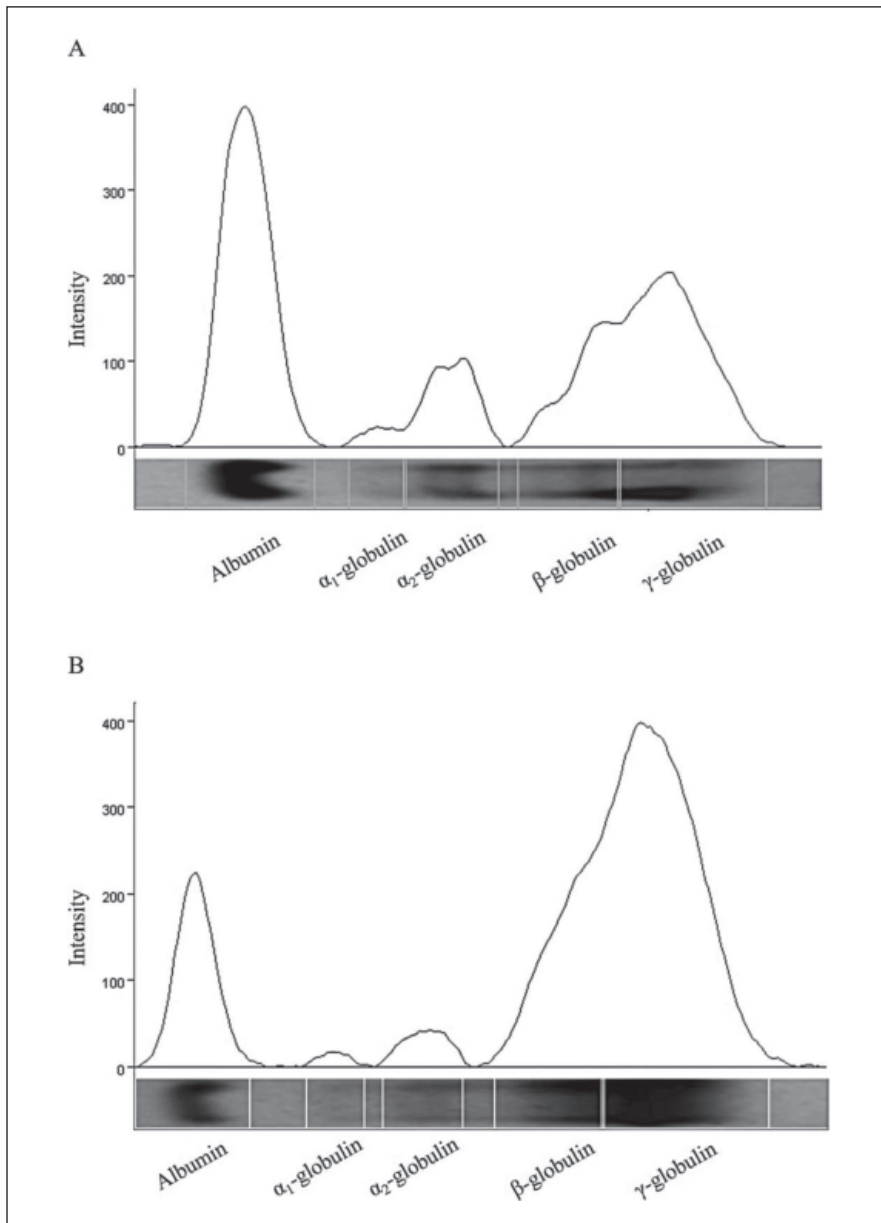
Figure 2. Serum protein electrophoresis from two dogs presenting with anterior uveitis (A) and panuveitis (B) associated with *E. canis* infection. Serum protein pattern shows the elevation of beta- and gamma globulins, particularly in the panuveitis group. Polyclonal gammopathy was detected in these dogs.

et al., 2015). Dogs with *E. canis* and *B. canis* infections were excluded from the serum protein fraction analysis because *B. canis* infection could cause serum protein alterations including, hypergamma-globulinemia, hyperbetaglobulinemia and hypoalbuminemia (Solona-Gallego *et al.*, 2008).

Albumin and globulin (α_1, α_2, β, γ) are the major serum proteins in dogs. Hypergamma-globulinemia in *E. canis* or *A. platys*-infected dogs indicates an increase in immunoglobulin production. Hypoalbuminemia associated with acute stage of *E. canis* infection in dogs was reported which may be attributable primarily to increase renal loss of protein, or

blood loss, rather than decreased hepatic synthesis (Codner & Maslin, 1992; Harrus *et al.*, 1999). The decrease in albumin concentration may act as a compensatory mechanism for hyperglobulinemia to maintain the oncotic pressure. In general, a normal range for the A/G ratio is 0.8-2.0 in adult dogs (Klaassen, 1999). The A/G ratio was significantly low in infected dogs associated with uveitis, particularly in the panuveitis group for which the A/G ratio was lower than 1, indicating that the infection or disease is present in these dogs. Moreover, hyperbetaglobulinemia was detected in infected dogs associated with panuveitis. Beta globulin (beta1 and beta2) is composed of C-reactive protein (CRP), transferrin, and complement factors among other things. Unfortunately, the type of beta globulin was not identified in the present study. However, it is possible that elevated beta globulins in panuveitis groups come from the increased production of CRP. Increased concentrations of CRP in blood were reported in dogs infected with *E. canis* at the acute stage of infection (Rikihisa *et al.*, 1994; Shimada *et al.*, 2002). CRP is synthesized in the liver following tissue damage caused by infection or inflammation. It was reported to causes the activation of macrophages, it may help to kill *E. canis* in the macrophages of infected dogs, controlling the infection at a subclinical stage (Rikihisa *et al.*, 1994).

Hyperproteinemia, hypergamma-globulinemia and hyperbetaglobulinemia may result in increased blood viscosity in *E. canis* or *A. platys*-infected dogs. Serum hyperviscosity increases the oncotic pressure in blood vessels and probably results in ischemic changes and hemorrhage in the posterior uvea. The most important gamma globulins are immunoglobulins. Hypergammaglobulinemia in infected dogs may come from the production of antibodies including anti-*E. canis* antibodies, anti-platelet antibodies (APA) and antinuclear antibodies (Harrus *et al.*, 1999; Smith *et al.*, 2004). A high level of immunoglobulin may result in the deposition of immune complexes in ocular blood vessel walls and subsequently cause vasculitis (Panciera *et al.*, 2001). The

appearance of free *E. canis* antigen in the plasma has been previously reported which presented initially at 15-20 days after infection (Waner *et al.*, 1996). High titers of antinuclear antibodies were detected in dogs naturally infected with *E. canis* (Smith *et al.*, 2004). Moreover, thrombocytopenia was detected in 29/33 (87.87%) of the infected dogs associated with panuveitis in the present study (data not shown). There are many mechanisms of thrombocytopenia, including immune-mediated platelet destruction, hypersplenism, vascular endothelial damage and direct infection of platelets by *A. platys* (Harrus *et al.*, 1998). APA production in dogs after experimental infection with *E. canis* supported the hypothesis that immune-mediated platelet destruction may contribute to the patho-genesis of thrombocytopenia (Waner *et al.*, 1995; Harrus *et al.*, 1999). Serum hyper-viscosity, vasculitis and thrombocytopenia could result in intraocular hemorrhage in *E. canis* or *A. platys*-infected dogs in the present study. Thus, in the future, the detection and levels of anti-*Ehrlichia* antibodies, antinuclear antibodies, APA and the type of beta globulins should be investigated in infected dogs associated with uveitis, particularly in dogs with intraocular hemorrhage.

In the present study, most of the *E. canis* or *A. platys*-infected dogs associated with anterior uveitis or panuveitis showed hypergammaglobulinemia in which poly-clonal gammopathy was detected. This accords with previous reports that most of the studied dogs infected with *E. canis* had hypergammaglobulinemia with polyclonal gammopathy, while monoclonal gammopathy rarely occurred (Harrus *et al.*, 1996, 1999). However, two Labrador retrievers were reported to have acute blindness associated with monoclonal gammopathy induced by *E. canis* infection (Harrus *et al.*, 1998; Gould *et al.*, 2000).

In conclusion, higher levels of total serum protein, gamma- and beta globulins were detected in *E. canis* or *A. platys*-infected dogs associated with uveitis, particularly with panuveitis. These higher

levels are probably associated with panuveitis, intraretinal or subretinal hemorrhages as well as retinal detachment leading to acute blindness in dogs.

Author's contributions

B.N., S.P. and P.K. designed the experiment. B.N. and A.T. carried out blood sample collection. S.P. performed laboratory work (PCR, serum protein electrophoresis). C.K. carried out DNA sequencing and analysis. B.N. and S.P. prepared and revised the manuscript. All authors read and approved the final manuscript.

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