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Thrombocytopenia and anemia related to retinal detachment in dogs infected with *Ehrlichia canis* and *Anaplasma platys*

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Abstract. In Thailand, five species of *Ehrlichia* (*E. canis*, *E. chaffeensis*, *E. equi*, *E. risticii* and *Anaplasma platys*) have been reported to infect dogs. Although ehrlichial infections can cause ocular disorders, the severity and type of ocular disorder varies between individual infected dogs. The aims of this study were to determine the factors associated with retinal detachment and to investigate the species of *Ehrlichia* that cause ocular disorders in natural infected dogs. In the present study, ocular examination, complete blood count and total protein measurement were performed in 134 dogs brought into an ophthalmology clinic. A 310 bp fragment of the *Ehrlichia* 16s rRNA gene was amplified by nested-PCR and direct DNA sequenced. Thirty-eight of these dogs were found to be positive for *Ehrlichia* 16s rRNA, of which the sequence analysis suggested 34 and 4 dogs were infected with *E. canis* and *A. platys*, respectively, with no multiple infections or other *Ehrlichia* species detected. The most common ocular disorders in dogs infected with *E. canis* were blindness, keratoconjunctivitis sicca and retinal detachment, while blindness and retinal detachment were found in *A. platys*-infected dogs. Hematological disorders were found anemia, thrombocytopenia and hyperproteinemia. Odd ratio analysis showed that thrombocytopenia and anemia were likely important factors for increasing retinal detachment risk. In this study, only *E. canis* and *A. platys* closely relate to be causative agents of ocular disorders in infected dogs. To the best of our knowledge, this is the first report of *A. platys* as a causative pathogen of both anterior and posterior uveitis in clinical situations.

INTRODUCTION

Ehrlichia species and *Anaplasma platys* are obligatory intracellular bacteria that reside within the cytoplasmic vacuoles of monocytes or granulocytes and the platelets of dogs, respectively. Dogs can be infected with several species of *Ehrlichia*, including *E. canis* (Donatien & Lestoguard, 1935), *E. chaffeensis* (Dawson *et al.*, 1996), *E. equi* (Madewell & Gribble, 1982), *E. ewingii* (Ewing *et al.*, 1971), *E. risticii* (Kakoma *et al.*, 1994), and *A. platys* (Harvey *et al.*, 1978). In Thailand, five of these six species, namely *E. canis*, *E. chaffeensis*, *E. equi*, *E. risticii* and *A. platys* have been reported in dogs (Suksawat *et al.*, 2001; Pinyoowong *et al.*, 2008).

E. canis is a causative agent of canine monocytic ehrlichiosis and is transmitted by the brown dog tick, *Rhipicephalus sanguineus*. The disease appears in the three clinical stages of acute, subclinical and chronic infections (Woody & Hoskins, 1991). Infected dogs can exhibit several clinical signs depending on the stage of the disease. The most frequent symptoms consist of a high fever, anorexia, emaciation, epistaxis,

hepatomegaly, splenomegaly, lymphadenopathy and ocular changes (Harrus *et al*., 1997). The ocular disorders reported to be caused by *E. canis* infection are hyphema, panuveitis, anterior uveitis, optic neuritis, retinal hemorrhages and retinal detachment (Leiva *et al*., 2005). *A. platys* has also been reported to cause anterior uveitis in an infected dog (Glaze & Gaunt, 1986). Retinal detachment is the separation of the inner layers of the retina from the underlying retinal pigment epithelium. This ocular sign is very important, especially complete retinal detachment leads to acute blindness which it reduces the quality of life of dogs. The most important hematological abnormalities caused by *E. canis* and *A. platys* are anemia (Hoskins, 1991; Moreira *et al*., 2003) and thrombocytopenia (Troy & Forrester, 1990; Chang *et al*., 1996). Moreover, hyperproteinemia has also been reported in dogs infected with *E. canis* (Harrus *et al*., 1997). To the best of our knowledge, no study on the correlation between thrombocytopenia, anemia and hyperproteinemia with retinal detachment in dogs infected with *E. canis* has been reported. Thus, the aims of this study were to determine whether hyperproteinemia, thrombocytopenia and anemia are associated with retinal detachment in infected dogs and to investigate the species of *Ehrlichia* that cause ocular disorders in clinical situations.

MATERIALS AND METHODS

Studied samples

One-hundred and thirty-four dogs that were brought into the ophthalmology clinic, Veterinary Teaching Hospital, Kasetsart University with ocular disorders related to ehrlichial infection and confirmed by a serological test (SNAP 4Dx, *IDEXX* Laboratories, Westbrook, ME, USA), or were suspected for canine ehrlichiosis, were examined. All dogs fulfilled three criteria of (1) having no history of ocular trauma or other systemic diseases, (2) having no dehydration status, (3) having a tick infestation or a history of tick infestation. EDTA blood samples collected from cephalic or saphenous vein were submitted for hematology, for the determination of the total protein level and DNA extraction for the nested-PCR amplification. The medical histories, sex and age of these dogs were documented. This study was approved by the animal experimental committee of Kasetsart University (ACKU 03556).

Detection of *Ehrlichia* by nested-PCR and direct DNA sequencing

Blood samples were lysed in denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol, 0.5% (w/v) N-lauroylsarcosine). DNA was then extracted with phenol-chloroform extraction and precipitated in absolute ethanol as previously described (Sambrook & Russell, 2001). DNA products were resuspended in TE buffer (50 mM Tris, pH 8.0, 1 mM EDTA) and stored at -20°C until use. Primers for amplification of *Ehrlichia* 16S rRNA gene were designed by ClustalW Version 2. Outer EF- (5'-TTGTAGCTAACGCGTTA AGCACT-3') and ER- (5'-AACTCGAAGCTGGTGY GCYAACC-3') primers, and the inner IEF- (5'-GTTCGGCTGGAYCTYRCACAGGT-3') and IER- (5'-CTGMAACTCGAGAGCAT GAAGTC-3') primers were designed for *nested-PCR*. The first PCR, the EF- and ER-primer pair amplified a 551-bp region while the second PCR, the IEF- and IER-primer pair amplified a 310-bp region. For the first PCR, 2 μL of DNA template was added to the PCR mixture to give a final volume of 20 μL containing 1x buffer (10 mM Tris– HCl pH 8.8, 50 mM KCl and 0.1% (v/v) Triton X-100), 2.0 mM $MgCl_2$, 1 *p*mol of each primer, 0.2 mM of each dNTP, and 2.5 U of *Taq* DNA polymerase. For the second PCR, 2 μL of the first PCR product was used as the template, comprised as the first PCR except for using the inner primer pair and 1.5 mM $MgCl_2$. 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 then a final extension at 72°C for 10 min. The positive and negative controls consisted of DNA from a blood sample of a dog known to be infected by *A. platys* and water, respectively. Both PCR reactions were

processed in a MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). The second PCR products were loaded in 1.5% agarose (SeaKem ME:FMC, USA) gel electrophoresis. Amplicons were extracted with the UltraCleanTM GelSpin DNA purification Kit (MO BIO LABORATORIES Inc, CA, USA) and submitted for commercial direct sequencing (1st Base Laboratory, Malaysia). The obtained DNA sequences were compared with those in the GenBank database using the BLAST algorithm.

Ophthalmic examination

Standard ocular examination, including the schirmer tear test, slit lamp biomicroscopy (model SL-15, Kowa Pharmaceutical Co. Ltd.), tonometry (TonoVet®, Icare Finland Oy, Helsinki, Finland), fluorescein staining, and indirect ophthalmoscopy were performed on all dogs (Welch Allyn, Skaneatales, NY, USA). Retinal detachment was observed via slit lamp biomicroscopy and indirect ophthalmoscopy. Ocular ultrasound was also performed in those dogs with an opaque ocular media to diagnose the retinal detachment.

Hematology and total protein level

Total protein measurement and complete blood counts were performed by refractometer (SUR-NE 300, Atago, Tokyo, Japan) and an automated hematology analyzer (Abbott Cell Dyn 3700R, Abbott Park, IL, USA), respectively. The results of the complete blood count from the automated hematology analyzer were confirmed by systematic analysis of a blood smear stained with modified Wright-Giemsa. To evaluate the factors associated with the retinal detachment, dogs with retinal detachment were divided into groups depending on their (i) total protein level; normal (5.3- 7.8 g/dL) and hyperproteinemia (more than 7.8 g/dL), (ii) platelet number; normal (200-500 x $10^3/\mu$L) and thrombocytopenia (less than 200 x $10^3/\mu$L), or (iii) PCV; normal (35-55%) and anemia (less than 35%).

Statistical analysis

The chi-squared and odds ratios (OR) tests with 95% confidence intervals (CI) were calculated for the association of age or sex with retinal detachment and association of the hyperproteinemia, thrombocytopenia and anemia with retinal detachment. Statistical significance was accepted at the $p \leq 0.05$ level.

RESULTS

A total of 38/134 dogs (23 males and 15 females) were found to be positive for *Ehrlichia* infection, as determined by specific 16s rRNA gene amplification from blood DNA extracts. The average age of the infected dogs was 10.08 years and ranged from 3 months to 13 years. Twenty-three dogs were purebreds, representing 12 different breeds and 15 dogs were crossbred. The chi-squared test did not show any significant differences ($p > 0.05$) between the age or sex with retinal detachment.

The positive PCR amplicons in the second round PCR were of the expected size (310 bp; Fig. 1) and comparison of their sequences following direct sequencing revealed that 34 of the 38 positive dogs (89.47%) were infected with *E. canis* and four dogs (10.53%) with *A. platys*, with no other *Ehrlichia* species detected and no multiple infections. The PCR amplified DNA sequences (310 bp) shared 99% similarity to *E. canis* (accession number EF139458) or *A. platys* (accession number AF286699), respectively.

The medical records showed that eight of these infected dogs were prescribed with doxycycline at 5 mg/kg twice a day prior to sample collection. In these cases the blood samples analyzed by here had been collected at 3 - 10 days after this treatment, except for one blood sample that was collected on 89 days after treatment but was still found to be positive by nested-PCR.

Ocular disorders in the dogs infected with *E. canis* included keratoconjunctivitis sicca (KCS), keratitis, anterior uveitis, posterior uveitis, panuveitis, hyphema, vitreous hemorrhage, retinal hemorrhage, retinal detachment and blindness. The most common ocular disorders in *E. canis* infected dogs were blindness (19/34, 55.88%), KCS (16/34,

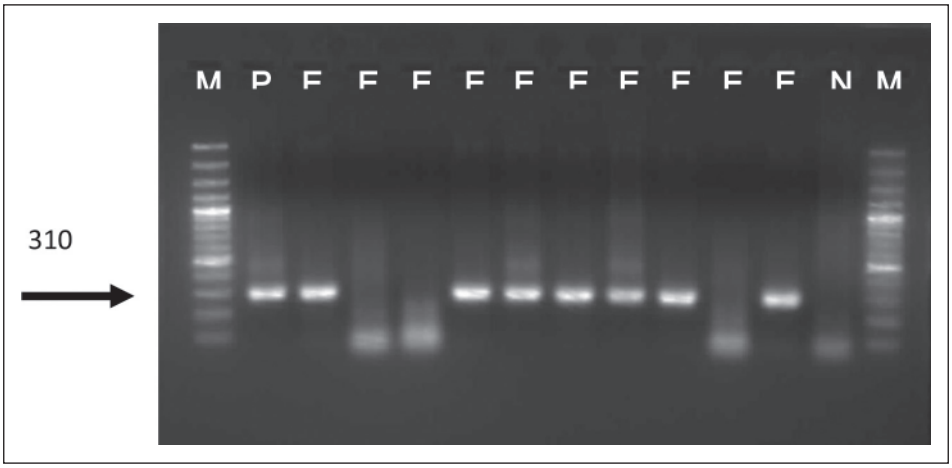

Figure 1. Representative nested PCR reactions after agarose gel electrophoresis showing the positive DNA amplicon bands (310 bp) of *Ehrlichia* 16s rRNA gene. MK: DNA marker 100 bp plus ladder, P: positive control, N: negative control, E1–E10: PCR products of samples No. 1–10, where samples E1, E4–8 and E10 are deemed to be positive and samples E2, E3 and E9 to be negative.

47.06%), and retinal detachment (15/34, 44.12%). Ocular disorders in the four dogs infected with *A. platys* were KCS, anterior uveitis, panuveitis, hyphema, retinal hemorrhage, retinal detachment and blindness. Bilateral ocular disorders were more frequent than unilateral (Table 1), especially for KCS (17/17, 100%), anterior uveitis (13/15, 86.67%) and retinal detachment (13/17, 76.47%).

Hematological disorders in the 38 infected dogs included anemia in 29 dogs (mean PCV = 23.4%, range = 12.0 – 33.0%), thrombocytopenia in 20 dogs (mean platelet count = 89.53 x $10^3/\mu$L, range = 1.12 – 160 x $10^3/\mu$L) and hyperproteinemia in 23 dogs (mean total protein = 9.4 g/dL, range = 8.0 – 12.0 g/dL). Twenty-three out of the 34 dogs (67.65%) infected with *E. canis* presented with hyperproteinemia, 27/34 (79.41%)

Table 1. Ocular disorders in the 34 dogs infected with *E. canis* and the four dogs infected with *A. platys*

Ocular disorder	Number of infected dogs (%)			
	E. canis (n = 34)		*A. platys* (n = 4)	
	Unilateral	Bilateral	Unilateral	Bilateral
Keratoconjunctivitis Sicca		16 (47.1%)		1 (25.0%)
Keratitis	2 (5.88%)	5 (14.7%)		
Anterior uveitis	2 (5.88%)	12 (35.3%)		1 (25.0%)
Posterior uveitis	1 (2.94%)			
Panuveitis			1 (25.0%)	
Hyphema	7 (20.6%)	2 (5.88%)	1 (25.0%)	
Vitreous hemorrhage	2 (5.88%)	5 (14.7%)		
Retinal hemorrhage	6 (17.7%)	6 (17.7%)	1 (25.0%)	
Retinal detachment	3 (8.82%)[a]	12 (35.3%)	1 (25.0%)	1 (25.0%)
Blindness	4 (11.8%)	15 (44.1%)	1 (25.0%)	1 (25.0%)

[a]Phthisis bulbi on the left eye in 1 dog

Table 2. Total protein level, platelet number and PCV with retinal detachment in the 38 infected dogs

Ehrlichia infection species	Number of dogs					
	Total protein level[a] (g/dL)		Platelet number[b] (x 10^3/µl)		PCV[c] (%)	
	≤ 7.8	> 7.8	< 200	≥ 200	< 35	≥ 35
E. canis	3/7	11/23	13/18[d]	2/13[d]	16/27[d]	1/7[d]
A. platys			1/2	1/2	1/2	1/2

[a] Normal range = 5.3–7.8 g/dL
[b] Normal range = 200–500 x 10^3/µl
[c] Normal range = 35–55%
[d] Chi-squared test showed significant differences ($p \leq 0.05$)

with anemia and 18/34 (52.94%) with thrombocytopenia. Two out of the four dogs infected with *A. platys* showed anemia (PCV = of 25.6% and 31.9%) and 2/4 dogs showed thrombocytopenia (platelet number = 1.12 x $10^3/\mu$L and 120 x $10^3/\mu$L) but all four dogs infected with *A. platys* had total protein levels in the normal range. The retinal detachment in dogs infected with *E. canis* or *A. platys* were grouped by the total protein level, platelet number, and PCV (Table 2). The chi-squared test showed significant differences in the relationship between the PCV levels or platelet numbers and the retinal detachment ($p \leq 0.05$). In addition, the OR analysis revealed that thrombocytopenia and anemia were important factors for an increased retinal detachment risk (OR = 14.3 and 8.0, respectively). Nevertheless, the chi-squared test did not show any significant differences in the relationship between hyperproteinemia and retinal detachment ($p > 0.05$).

DISCUSSION

In the 38 *Ehrlichia*-infected dogs in this study in Thailand (out of 134 dogs examined with ocular disorders), only *E. canis* and *A. platys* infections were found and so were the potential pathogenic agents. This supports previous studies which concluded that *E. canis*, not *E. ewingii*, *E. chaffeensis* and *E. equi*, was able to cause ocular disorders in experimental dogs (Panciera *et al*., 2001) and that *A. platys* was the causative pathogen of anterior uveitis in a dog (Glaze & Gaunt, 1986). However, the dogs infected with *A. platys* in this study presented ocular disorders other than in the anterior part, such as retinal hemorrhage and retinal detachment. To the authors' knowledge, this is the first report of *A. platys* that relate to cause both anterior and posterior uveitis in infected dogs.

In the present study, dogs infected with *E. canis* presented with KCS in a high percentage (47.06%) of cases. This data supports previous reports that canine monocytic ehrlichiosis can cause low tear production (Komnenou *et al*., 2007), which in turn leads infected dogs to suffer from KCS. However, it is unclear why dogs infected with *E. canis* would develop KCS. Additional studies are needed to investigate the pathology of the lacrimal gland in *E. canis*-infected dogs suffering with KCS.

Hematological findings in this study showed thrombocytopenia and anemia, not hyperproteinemia, were important factors for the increase the risk of retinal detachment. This is congruent with a previous study that thrombocytopenia is related to the prevalence of ocular lesion, but is in contrast to the data that anemia is unrelated to ocular disorders in dogs (Shelah-Goraly *et al*., 2009). This apparent discrepancy may arise from the difference in the cut-off point between the present and previous study, since it was higher. In the present study (PCV < 35%) was higher than that used in the previous study (PCV ≤ 20%). However, our information was

similar to the previous study in humans that described anemia related with ocular disorders, which determined anemia as a hemoglobin concentration of < 8 g/dL (Carraro *et al.*, 2001). Anti-platelet antibodies (APA) bind to platelet glycoprotein receptors leading to platelet dysfunction was proposed for the cause of thrombocytopenia in acute stage of canine ehrlichiosis. Then, platelet dysfunction may be a contributing factor to the tendency to ocular bleeding (Harrus *et al.*, 1996). Other mechanisms rather than thrombocytopenia alone were also suggested in the pathogenesis of subretinal haemorrhage leading to retinal detachment. The relationship between hyperproteinemia and retinal detachment in the present study is in contrast to a previous study that the monoclonal hypergammaglobulinemia in dogs was related with ocular lesions, including hyphema, retinal hemorrhage and retinal detachment (Harrus *et al.*, 1998a). However, 11 out of 23 dogs (47.82%) with hyperproteinemia showed retinal detachment whilst eight out of nine (88.88%) dogs with severe hyperproteinemia (total protein ≥ 10 g/dL) had retinal detachment. This tends to support that blood hyperviscosity (secondary to hyperproteinemia) could lead to subretinal haemorrhage and retinal detachment in *Ehrlichia*-infected dogs.

Generally, doxycycline treatment does not clear *E. canis* infections, as noted previously where a persistent *E. canis* infection in one of six dogs after treatment for 6 weeks with doxycycline at 10 mg/kg/day (Harrus *et al.*, 1998b). In the present study, *E. canis* DNA was detected in one dog at 89 days after treatment, in accord with the previous study. However, it is not clear if this dog had a persistent infection, rather than it had monitor received a new *E. canis* infection.

From the present study, thrombocytopenia and anemia would appear to be the risk factors for retinal detachment. Only *E. canis* and *A. platys* were found in infected dogs with ocular disorders. Moreover, *A. platys* relates to cause both anterior and posterior uveitis in infected dogs in the clinical situation. Dogs infected with *E. canis* or *A. platys* which show thrombocytopenia and anemia should be examined intensively by ophthalmologist to monitor a chance of retinal detachment.

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