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THESIS

DETECTION AND CHARACTERIZATION OF *EHRLICHIA* SPP. IN INFECTED DOGS WITH OCULAR LESIONS

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Kepalee Saeng-chuto 2015: Detection and Characterization of *Ehrlichia* spp. in Infected Dogs with Ocular Lesions. Master of Science (Veterinary Parasitology), Major Field: Veterinary Parasitology, Department of Parasitology. Thesis Advisor: Assistant Professor Burin Nimsuphan, Ph.D. 63 pages.

In Thailand, five species of *Ehrlichia* (*E. canis, E. chaffeensis*, *E. equi*, *E. risticii* and *Anaplasma platys*) have been reported in infect dogs. Although ehrlichial infections can cause ocular disorders, the severity and type of ocular disorder varies between individual infected dogs. The present study performed ocular examination, complete blood count and total protein measurement in 134 dogs brought into the Ophthalmology clinic. A 310 bp and 220 bp fragment of the *Ehrlichia* 16s rRNA and *p30* gene, respectively, were 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. Thirty-four dogs were found to be positive for *E. canis p30* gene, of which the sequence analysis suggested 18, 3 and 13 dogs were infected with *E. canis* strain Jaboticabal, Jake, and unidentified strain, respectively. 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.

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Student's signature Thesis Advisor's signature

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Kepalee Saeng-chuto

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	=	Degree of Celsius
µg	=	Microgram(s)
µl	=	Microliter(s)
bp	=	Base pair(s)
cumm	=	Cubic millimetre
dl	=	Deciliter
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediamine tetraacetic acid
et al.	=	*et. alii* (and others)
fL	=	Fluidounce
g	=	Gram(s)
gm% or g/dl	=	Gram(s) per
L	=	Liter(s)
M	=	Mole
mg	=	Miligram(s)
min	=	Minute(s)
ml	=	Mililiter(s)
mM	=	Millimolar(s)
PCR	=	polymerase chain reaction
pH	=	negative logarithm of hydrogen ion activity
pmol	=	Picomole
RNA	=	Ribonucleic acid
rpm	=	Round(s) per minute
sec	=	Second(s)
v/v	=	Volume/volume
w/v	=	Weight/volume
U	=	Unit/microliter

DETECTION AND CHARACTERIZATION OF *EHRLICHIA* SPP. IN INFECTED DOGS WITH OCULAR LESIONS

INTRODUCTION

Ehrlichia species and *Anaplasma platys* are obligatory intracellular bacteria residing within the cytoplasmic vacuoles of monocytes or granulocytes, and platelets of dogs, respectively. Dogs can be infected with several species of *Ehrlichia*, including *E. canis* (Donatein and Lestoguard, 1935), *E. chaffeensis* (Dawson *et al*., 1996), *E. equi* (Madewell and Gribble, 1982), *E. ewingii* (Ewing *et al*., 1971), *E. risticii* (Kakoma *et al.*, 1994), and *A. platys* (Harvey *et al.*, 1978). In Thailand, the previous studies showed that dogs were infected with 5 species including *E. canis, E. chaffeensis*, *E. equi*, *E. risticii*, and *A. platys* (Suksawat *et al*., 2001; Pinyoowong *et al*., 2008). *E. canis* is a causative agent of canine monocytic ehrlichiosis (CME) transmitted by brown dog tick, *Rhipicephalus sanguineus*. The disease from *E. canis* infection appears in three stages, including acute, subclinical, and chronic infections (Woody and Hoskins, 1991). Infected dogs show several clinical signs depending on the stage of the disease. The most frequent symptoms consist of high fever, anorexia, epistaxis, hepatomegaly, splenomegaly, lymphadenopathy, and ocular changes (Harrus *et al*., 1997). The ocular disorders that were caused by *E. canis* were hyphema, panuveitis, anterior uveitis, retinal hemorrhages, and retinal detachment (Leiva *et al*., 2005). Retinal detachment is the separation of the inner layers of the retina from the underlying retinal pigment epithelium that leads to blindness. *A. platys* was also reported to be a cause of anterior uveitis in an infected dog (Glaze and Gaunt, 1986). The most important hematological abnormalities caused by *E. canis* and *A. platys* were thrombocytopenia (Troy and Forrester, 1990; Chang *et al.*, 1996) and anemia (Hoskins, 1991; Moreira *et al.*, 2003). Moreover, hyperproteinemia was also reported in dogs infected with *E. canis* (Harrus *et al*., 1997). To the best of our knowledge, no study on the correlation between anemia, thrombocytopenia, and hyperproteinemia and retinal detachment in dogs infected with *E. canis* were reported.

OBJECTIVES

1. To investigate the species of *Ehrlichia* and strain of *E. canis* that cause ocular disorders in the clinical situations.

2. To determine whether hyperproteinemia, thrombocytopenia and anemia are associated with retinal detachment in infected dogs.

LITERATURE REVIEW

1. *Ehrlichia* species

1.1 *Ehrlichia canis*

E. canis (Figure 1) is the primary etiologic agent of canine monocytic ehrlichiosis (CME), a serious and sometimes fatal, globally distributed disease of dogs (Keefe *et al.*, 1982). *E. canis* is transmitted by the brown dog tick, *Rhipicephalus sanguineus* (Figure2) (Groves *et al.*, 1975), and infects monocytes/macrophages in dogs.

The disease is divided into 3 phases includes acute, subclinical, and chronic. *E. canis* can infect all breeds of dogs but the German Shepherd dog appears to be more susceptible, showing the more severe form of the disease with a higher morbidity and mortality compared to other breeds (Nyindo *et al.*, 1980). No age or gender predilection has been established. Disease manifestations may be affected by the pathogenicity of different *E. canis* strains and co-infections with other parasites such as *Babesia canis vogeli* and *Hepatozoon canis* transmitted by the same vector (Gal *et al.*, 2007)

The common clinical signs of the acute phase are high fever, depression, lethargy, anorexia, lymphadenomegaly, splenomegaly and hemorrhagic tendencies, dermal petechiae and ecchymoses, and epistaxis. Ocular disorders are frequent, including anterior uveitis, chorioretinitis, papilloedema, retinal hemorrhage, presence of retinal perivascular infiltrates, and bullous retinal detachment (Komnenou *et al.*, 2007).

The subclinical phase, no clinical signs are evident (Waner *et al.*, 1997). For reasons still unclear, certain dogs will progress to the chronic phase of CME.

The common clinical signs of the chronic disease are weakness, depression, anorexia, chronic weight loss, emaciation, pale mucous membranes, fever and peripheral edema, especially of the hind limbs and the scrotum. Platelet-related bleeding, such as petechiae and echymoses of the skin and mucous membranes and epistaxis are common findings (Huxsoll *et al.*, 1970; Smith *et al.*, 1975).

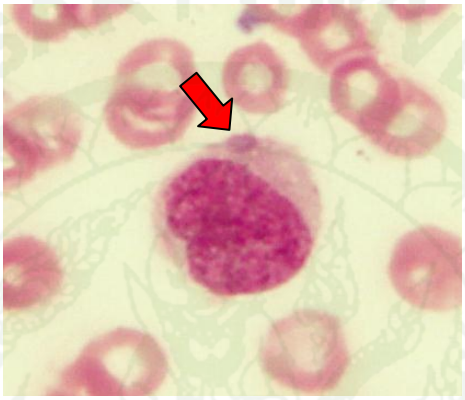

Figure 1 Morula stage of *E. canis* cytoplasm of monocyte (arrow)

Figure 2 Adult stage of *R. sanguineus*

1.2 *Anaplasma platys*

A. platys (Figure 3) is a causative agent of canine cyclic thrombocytopenia (CCT). *A. platys* is an obligate intracellular bacteria that infects platelets. It is thought to be transmitted by *R. sanguineus* (Woody and Hoskins, 1991). It was first described in the USA in 1978 as the agent, and infection has been reported worldwide (Harvey *et al.*, 1978; Kontos *et al.*, 1991; Sainz *et al.*, 1999; Inokuma *et al.*, 2002; Beaufils *et al.*, 2002). The pathogenesis of *A. platys* generally is not severe, although clinical abnormalities such as fever, anorexia, petechial haemorrhage, and uveitis have been reported (Hoskins, 1991; Kontos *et al.*, 1991; Glaze and Gaunt, 1986; Bradfield, 1996).

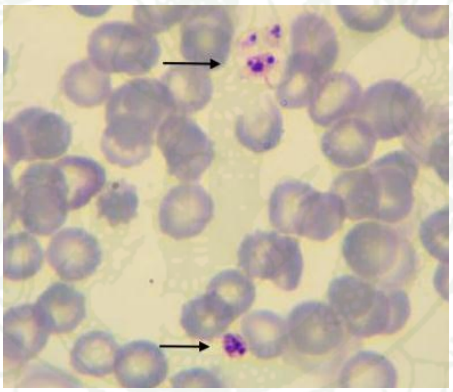

Figure 3 Morula stage of *A. platys* in platelet (arrows)

Source: De Tommasi (2014)

1.3 *Ehrlichia chaffeensis*

E. chaffeensis (Figure 4) is the etiologic agent of human monocytic ehrlichiosis (HME) (Anderson *et al.*, 1992), which is the most severe of the human ehrlichioses, and can also cause mild-to-severe disease in dogs (Dawson and Ewing, 1992; Breitschwerdt *et al.*, 1998). *E. chaffeensis* exhibits tropism for monocytes/macrophages. *E. chaffeensis* is maintained in nature in a zoonotic cycle

potentially involving many vertebrate species. However, the white-tailed deer appears to be the primary reservoir for *E. chaffeensis*, but dogs may also be a significant natural reservoir (Paddock and Childs, 2003). The primary vector is the lone star tick, *Amblyomma americanum* (Figure 5), which is distributed from west central Texas throughout the southeastern, south-central, and mid-Atlantic states (Paddock and Childs, 2003; Childs and Paddock, 2003).

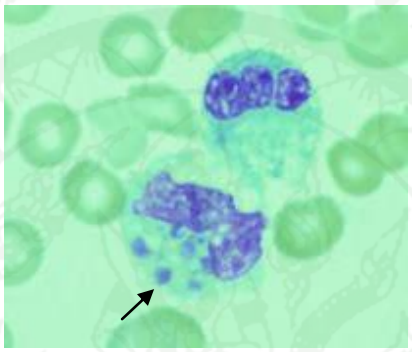

Figure 4 Morula stage of *E. chaffeensis* in cytoplasm of monocyte (arrow)

Source: Paddock and Childs (2003)

Figure 5 Adult stage of *A. americanum*

Source: Ismail *et al.* (2010)

1.4 *Ehrlichia ewingii*

Human granulocytic ehrlichiosis is caused by *E. ewingii* (Figure 6) and is the most recently described emerging human ehrlichiosis (Buller *et al.*, 1999). *E. ewingii* is also a veterinary pathogen first described in 1971 (Ewing *et al.*, 1971), and causes infections with two distinct clinical syndromes, anemia and polyarthritis in dogs (Goldman *et al.*, 1998). *E. ewingii* exhibits host cell tropism for granulocytes (neutrophils), and is transmitted by the lone star tick, *A. americanum* (Anziani *et al.*, 1990). Dogs are likely to be the main reservoir for *E. ewingii*, with many of the documented human cases reporting contact with dogs before onset of symptoms (Buller *et al.*, 1999). There is some serologic cross reactivity between *E. chaffeensis* and *E. ewingii*, but this is directed at higher molecular weight antigens and not the major outer membrane protein (*p28*) (Buller *et al.*, 1999).

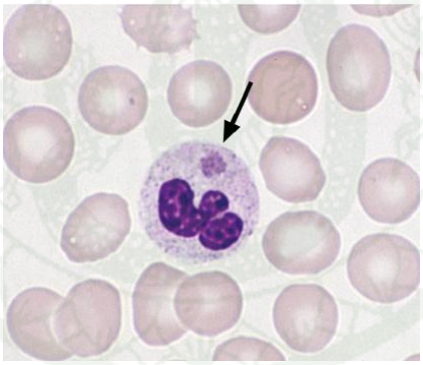

Figure 6 Morula stage of *E. ewingii* in cytoplasm of neutrophil (arrow)

Source: Hamilton (2004)

1.5 *Ehrlichia equi*

E. equi is a morphologically indistinguishable species that infect neutrophils. *E. equi* causes a febrile infection in horses and is also believed to be tick transmitted (Gribble, 1969). This disease is infrequently diagnosed but is thought to

occur widely throughout North and South America and perhaps may affect horses in Europe (Madigan *et al.*, 1990). Experimental transmission of *E. equi* from horses to other species including cattle, goats, sheep, and nonhuman primates is inefficient (Lewis, 1975). In spite of these experimental data, *E. equi* has been isolated by primary inoculation of blood from naturally infected dogs into susceptible horses (Madewell and Gribble, 1982), and high seroprevalence rates in dogs have been documented in some regions (Rodgers, 1989).

1.6 *Ehrlichia risticii*

E. risticii is a causative agent of equine monocytic ehrlichiosis (EME) and Potomac horse fever (PHF) was isolated, identified, and characterized in 1984 (Holland et al., 1985; Rikihisa and Perry, 1985) as the cause of an acute, fulminant diarrheal disease of horses first recognized in the Potomac River area in 1979 (Knowles *et al.*,1983).

2. Ocular disorders

2.1 Retinal detachment

Retinal detachment (Figure 7) characterized by separation of the retina from the underlying retinal pigment epithelium and is a leading cause of vision loss. Retinal detachment can be divided in three groups (D'Amico, 1994; Brinton and Wilkinson, 2009):

(1) Rhegmatogenous retinal detachment (RRD) develops due to a retinal break. Fluid from the vitreous cavity passes through the retinal break into the potential space under the retina, leading to separation of the retina from the underlying choroid.

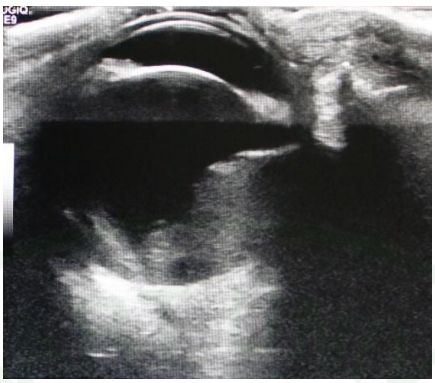

Figure 7 Retinal detachment

(2) Tractional retinal detachment (TRD) which occurs due to pre-retinal membrane formation and scarring that pulls the retina from its attachment. This may require surgery depending on the extent of the retinal detachment.

(3) Exudative retinal detachments (ERD) occur due to abnormalities in water transport across the bed of the retina (retinal pigment epithelium) or in its blood supply.

2.2 Keratoconjunctivitis sicca (KCS)

KCS results from lack of aqueous tearing that are many causes of poor tear production. In dogs, KCS is commonly characterized as an immune-mediated disorder, occasionally associated with systemic autoimmune conditions. Breeds most often affected with KCS include Shih Tzu, Bulldog, Cocker Spaniel, West Highland White Terrier, Boston Terrier, and Lhasa Apso (Kaswan and Salisbury, 1990).

2.3 Anterior uveitis

The uvea is the pigmented vascular tunic located between the fibrous and nervous tunics. It includes the iris, the ciliary body, and the choroid. Inflammation of

this tissue is known as uveitis. Uveitis can be classified into anterior uveitis, posterior uveitis, and panuveitis (Collins and Moore, 1999; Powell, 2002).

Anterior uveitis (Figure 8) is present when both the iris and ciliary body are inflamed that may have several etiologies such as trauma, extension of local infections, foreign bodies, neoplasm, or thermal trauma. Parasites and protozoa also may affect the uvea (Summers, 2007).

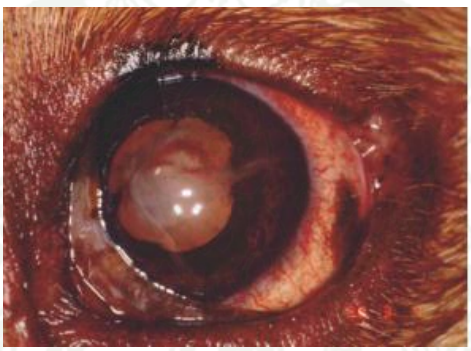

Figure 8 Anterior uveitis

Source: Sapienza *et al.* (2000)

3. Diagnosis

3.1 Blood smear evaluation

Demonstration of typical cytoplasmic *E. canis*-morulae in monocytes in blood smears by light microscopy strongly supports a diagnosis of CME. Morulae are membrane bound vacuoles which are usually densely packed, as seen by electron microscopy (Hildebrandt *et al.*, 1973). Unfortunately the search for morulae is difficult and time consuming and has been estimated to be successful in only about 4% of cases (Woody and Hoskins, 1991). It may be optimized by the examination of multiple buffy coat smears which significantly increases the chances of detecting *E. canis*-morulae. Sixty-six percent sensitivity was achieved after evaluating 1000 oil

immersion fields of buffy coat smears. The sensitivity of evaluating 1000 oil immersion fields of bone marrow was 34% (Mylonakis *et al.*, 2003).

3.2 Molecular detection

PCR and sequencing are sensitive methods for detecting and characterizing *E. canis* DNA. Detection of *E. canis* DNA can be achieved as early as 4–10 days post-inoculation (Iqbal *et al.*, 1994). Several assays are based on different target genes. However, the 16S rRNA and the *p30*-based PCR assays are most commonly used. The *p30*-based nested PCR assay has been shown to be more sensitive than the 16S rRNA-based nested PCR assay (Stich *et al.*, 2002). PCR performed on spleen samples is considered more sensitive for the evaluation of ehrlichial elimination when compared to blood and bone marrow samples (Harrus *et al.*, 1998b, 2004). PCR using serum samples may be useful when no blood samples are available.

3.3 Hematological findings

A complete blood count (CBC) is an essential basic in the diagnosis of CME. During the acute stage, moderate to severe thrombocytopenia is common (Grindem *et al*., 2002). Blood smear evaluation of platelet numbers is required in order to confirm the presence of a true thrombocytopenia. During the subclinical phase, a mild thrombocytopenia may be present in the absence of clinical findings. In experimentally infected dogs, reduced platelet counts by up to 42% have been observed with counts as low as 140,000/µL (Harrus *et al.*, 1998b; Waner *et al.*, 1997). Leukocyte and erythrocyte counts may also be reduced, although these changes may be relatively mild and difficult to notice in a clinical setting (Waner *et al.*, 1997). In the chronic phase, thrombocytopenia is usually severe and accompanied by marked anemia and leukopenia. Marked pancytopenia due to bone marrow hypoplasia is a hallmark of the chronic severe form (Harrus *et al.*, 1997).

MATERIALS AND METHODS

1. 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 two criteria of (1) having no history of ocular trauma or other systemic diseases, (2) having a tick infestation or a history of tick infestation. Blood samples were collected from the cephalic or saphenous vein and transferred into EDTA tubes for hematology, 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.

2. Detection of *Ehrlichia* by nested-PCR and DNA sequencing

Blood samples (100 µl each) were lysed in 600 µl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol, 0.5% (w/v) N-lauroylsarcosine) with shaking for 5 min. DNA was then extracted with phenol–chloroform extraction and precipitated in absolute ethanol as previously described (Sambrook and 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.

To identifying species of *Ehrlichia*, primers for amplification of *Ehrlichia* 16S rRNA gene were designed from the nucleotide sequences deposited in the GenBank database. All of the sequences were aligned for maximum homology by ClustalW Version 2.1. Conserved regions were selected and from these the outer EF- (5’-TTGT AGCTAACGCGTTAAGCACT-3’) and ER- (5’-AACTCGAAGCTGGTGYGCYAA CC-3’) primers, and the inner IEF- (5’-GTTCGGCTGGAYCTYRCACAGGT-3’) and IER- (5’-CTGMAACTCGAGAGCATGAAGTC-3’) primers were designed.

DNA was used as a template to amplify the 16S rRNA gene from *Ehrlichia* by nested-PCR and the amplicons were direct sequenced for species identification. In the first PCR, the EF- and ER-primer pair amplified a 551-bp region. In the second (nested) 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 pmol 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 transferred as the template to a new reaction (20 µl final), comprised as before except for using the IEF 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 72 °C for 10 min. The positive control consisted of DNA from a blood sample of a dog known to be infected by *A. platys*. The negative control used water in place of the DNA template. Both PCR reactions were processed in a MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). The second PCR products were added to 5 µl of 6X loading buffer and loaded in 1.5% agarose (SeaKem ME:FMC, USA) gel electrophoresis.

To identifying strain of *E. canis*, primers for amplification of *E. canis p30* gene were designed from the nucleotide sequences deposited in the GenBank database. All of the sequences were aligned for maximum homology by ClustalW Version 2.1. Conserved regions were selected and from these the outer OEP30F- (5'-AAGYGCRTTRATMTCAYTAATGTC-3') and OEP30R- (5'-GTAACTWAWRCC YAWTTTTCCTTG-3') primers, and the inner EP30F- (5'-TACATTAGTGCAAAGT AYAWKCCAAGT-3') and EP30R- (5'-AGGATTTKCAGGARSTATWGGTTACT C-3') were designed.

DNA was used as a template to amplify the *p30* gene from *E. canis* by nested-PCR and the amplicons were direct sequenced for species identification. In the first PCR, the OEP30F- and OEP30R-primer pair amplified a 648-bp region. In the second (nested) PCR, the EP30F- and EP30R-primer pair amplified a 220-bp region. For the

first and second 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), 1.5 mM $MgCl_2$, 1 pmol of each primer, 0.2 mM of each dNTP, and 1.5 U of *Taq* DNA polymerase. The thermal cycling conditions for the first PCR reactions were 95 °C for 5 min, 45 cycles of [95 °C for 20 sec, 57 °C for 20 sec, and 72 °C for 40 sec], and then a final 72 °C for 10 min. The thermal cycling conditions for the second PCR reactions were similar to the first PCR except annealing at 63ºC, 20 sec. Both of PCR protocols were processed in MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). Amplified PCR products (20 µl) were added to the 5 µl of 6X loading buffer and separated on 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.

3. Ophthalmic examination

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

4. Hematology and blood chemistry analysis

Total protein measurement and complete blood count 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 complete blood count from automated hematology analyzer were confirmed by

systematic analysis of blood smear stained with Modified Wright-Giemsa. Dogs with retinal detachment were divided into 2 groups depending on three factors: total protein levels (less than or equal to 7.8 g/dl, and more than 7.8 g/dl), or platelet numbers (less than 200 x 10^3/µl, and more than or equal to 200 x 10^3/µl), or PCV levels (less than 35%, and more than or equal to 35%).

5. Statistical analysis

Chi-square was calculated for association of *Ehrlichia* species and *E. canis* strain with ocular disorders. Chi-square and odds ratios (OR) with 95% confidence intervals (CI) were calculated for association of age or sex with retinal detachment and association of the hyperproteinemia, thrombocytopenia and anemia with retinal detachment. $p \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Results

The result showed 38/134 dogs (23 males and 15 females) were positive for *Ehrlichia* 16s rRNA gene. The average age of positive dogs was 10.08 years (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 significant differences of the relationship between the age or sex with retinal detachment ($p > 0.05$).

For 16s rRNA gene, the positive PCR products presented the DNA bands at 310 bp (Figure 9). The sequencing analysis presented that 34 dogs (89.47%) were positive for *E. canis* (99% similarity with EF139458) and 4 dogs (10.53%) were positive for *A. platys* (99% similarity with AF286699).

For *p30* gene, the results showed 34 dogs (89.47%) were positive for *E.canis p30* gene which showed the DNA bands at 220 bp (Figure 10). The sequencing analysis presented that 18 dogs (47.37%) were positive for *E. canis* strain Jaboticabal (more than 96% similarity with EU439942), 3 dogs (7.89%) were positive for *E. canis* strain Jake (more than 96% similarity with CP000107), and 13 dogs (34.21%) were positive for *E.canis* unidentified strain.

The chi-squared test did not show significant differences of the relationship between species of *Ehrlichia* or strain of *E. canis* with type of ocular disorders ($p > 0.05$).

The medical records showed 8 infected dogs were prescribed with doxycycline before the sample collection. The blood samples were collected at 3-10 days after treatment. Only one blood sample was collected on days 89 after treatment which still was positive by nested-PCR

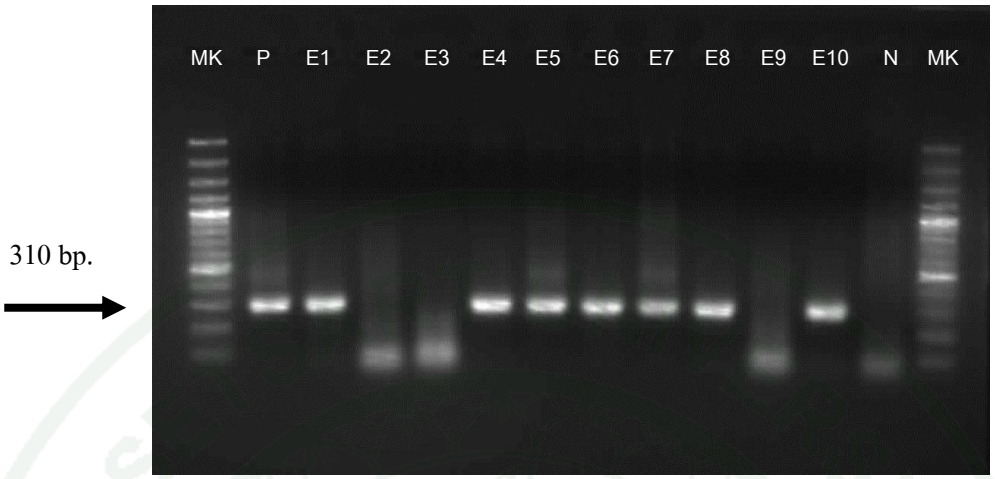

Figure 9 The positive DNA bands of *Ehrlichia* 16s rRNA gene presented at 310 bp. MK: DNA marker 100 bp plus, P: positive control, N: negative control, E1 – E10: DNA of samples No.1-10.

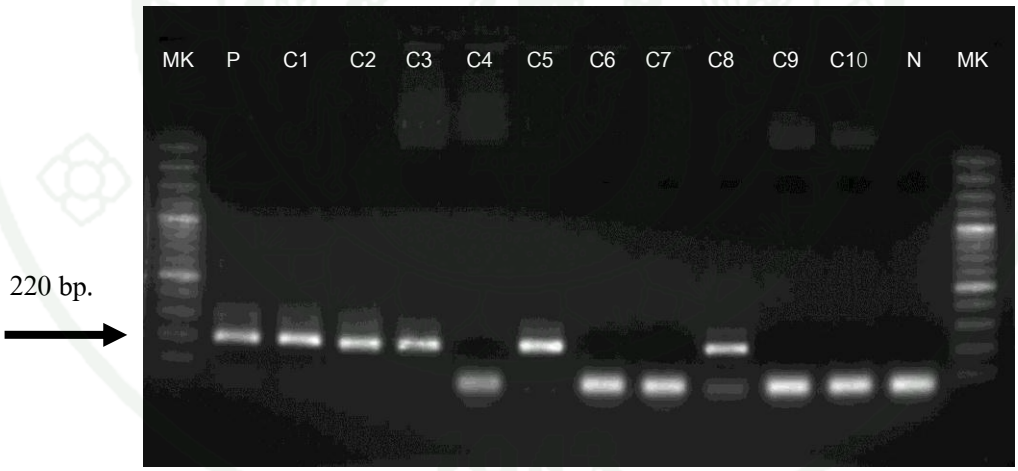

Figure 10 The positive DNA bands of *E. canis p30* gene presented at 220 bp. MK: DNA marker 100 bp plus, P: positive control of *Ehrlichia*, N: negative control, C1 – C10: DNA of samples No.1-10.

Ocular disorders in 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 dogs infected with *E. canis* were blindness (19/34, 55.88%), KCS (16/34, 47.06%), and retinal detachment (15/34, 44.12%). Ocular disorders in dogs infected with *A. platys* included KCS, anterior uveitis, panuveitis, hyphema, retinal hemorrhage, retinal detachment, and blindness. Bilateral ocular disorders were more than unilateral (Table1), especially for KCS (17/17, 100%), anterior uveitis (13/15, 86.67%), and retinal detachment (13/17, 76.47%).

Hematological disorders in 38 dogs included anemia in 29 dogs (mean of PCV= 23.4%, range = 12.0-33.0%), thrombocytopenia in 20 dogs (mean of platelet count = 89.53x10^3/µl, range = 1.12-160x10^3/µl), and hyperproteinemia in 23 dogs (mean of total protein = 9.4 g/dl, range = 8.0-12.0 g/dl). Twenty-three out of 34 dogs (67.65%) infected with *E. canis* presented with hyperproteinemia, 27/34 dogs (79.41%) presented with anemia, and 18/34 dogs (52.94%) presented with thrombocytopenia. Two out of 4 dogs infected with *A. platys* showed anemia (PCV = 25.6% and 31.9%), 2/4 dogs showed thrombocytopenia (platelet number = 1.12 x10^3/µl and 120 x10^3/µl) but all dogs infected with *A. platys* had total protein levels in 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 level (Table2). The chi-squared test showed significant differences of the relationship between the PCV levels or platelet numbers and the retinal detachment ($p \leq 0.05$). In addition, odd ratio showed thrombocytopenia and anemia were important factors for increased retinal detachment risk (odd ratio=14.3 and 8.0, respectively). Nevertheless, the chi-squared test did not show any significant differences of the relationship between hyperproteinemia and retinal detachment ($p > 0.05$).

Table 1 Ocular disorders in 34 dogs infected with *E. canis* and 4 dogs infected with *A. platys*

Ocular disorders	Number of infected dogs (%)			
	E. canis (n=34)		*A. platys* (n=4)	
	Unilateral	Bilateral	Unilateral	Bilateral
KCS		16 (47.06%)		1 (25.00%)
Keratitis	2 (5.88%)	5 (14.71%)		
Anterior uveitis	2 (5.88%)	12 (35.29%)		1 (25.00%)
Posterior uveitis	1 (2.94%)			
Panuveitis			1 (25.00%)	
Hyphema	7 (20.59%)	2 (5.88%)	1 (25.00%)	
Vitreous hemorrhage	2 (5.88%)	5 (14.71%)		
Retinal hemorrhage	6 (17.65%)	6 (17.65%)	1 (25.00%)	
Retinal detachment	3 (8.82%)*	12 (35.29%)	1 (25.00%)	1 (25.00%)
Blindness	4 (11.76%)	15 (44.12%)	1 (25.00%)	1 (25.00%)

* Phthisis bulbi on the left eye in 1 dog

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

Ocular disorder	Species of *Ehrlichia*	Number of dogs					
		Total protein level† (g/dl)		Platelet number¶ ($x10^3$/µl)		PCV ᵟ (%)	
		≤ 7.8	> 7.8	< 200	≥ 200	< 35	≥ 35
Retinal detachment	*E. canis*	3/7	11/23	13/18*	2/13*	16/27*	1/7*
	A. platys			1/2	1/2	1/2	1/2

† Normal range = 5.3-7.8 g/dl

¶ Normal range = 200-500 $x10^3$/µl

ᵟ Normal range = 35-55%

* Chi-square test showed significant differences ($p \leq 0.05$)

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 and 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* as a causative pathogen of both anterior and posterior uveitis.

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 (Shelah-Goraly *et al*., 2009) which thrombocytopenia increase the tendency of ocular bleeding leading to retinal detachment. However, it 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/d*l* (Carraro *et al.*, 2001). 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/d*l*) had retinal detachment. This tends to support that a high level of total protein could lead to retinal detachment in *Ehrlichia*-infected dogs. Nevertheless, *A. platys*-infected dogs with retinal detachment did not show hyperproteinemia. This is a possibility that hyperproteinemia does not relate to retinal detachment in *A. platys*-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.*, 1998c). 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 received a new *E. canis* infection.

To increase the chance for detection of other species of *Ehrlichia*, and clear information of the relationship of *Ehrlichia* infection and hematological factors, more blood samples should be collected. The more information will be benefit to surveillance serious ocular disorders cause by *Ehrlichia* infection in domestic dogs. Moreover, the information of this study that thrombocytopenia and anemia related with retinal detachment will encourage the veterinarians to be aware of these risk factors.

CONCLUSION

From the present study, Only *E. canis* and *A. platys* were found in infected dogs with ocular disorders in this study. Moreover, *A. platys* relate to cause both anterior and posterior uveitis in infected dogs in the clinical situation. Thrombocytopenia and anemia are the risk factors for retinal detachment.

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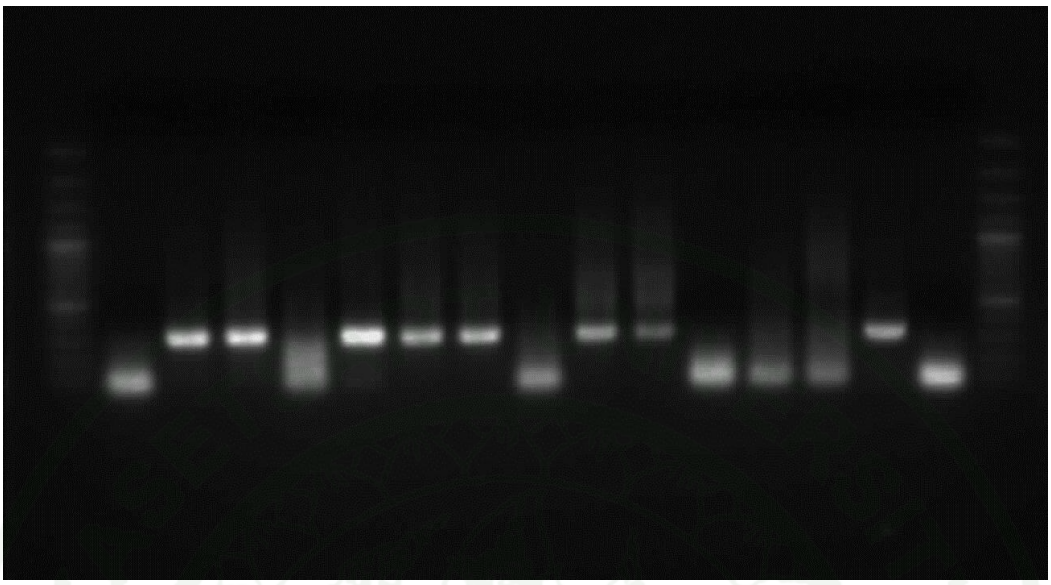
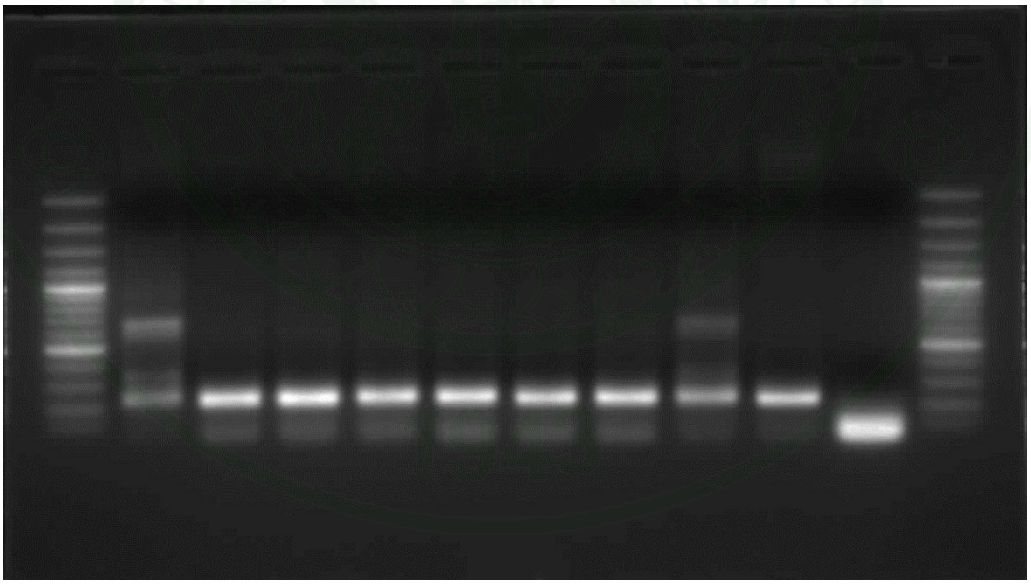
APPENDICES

Appendix A

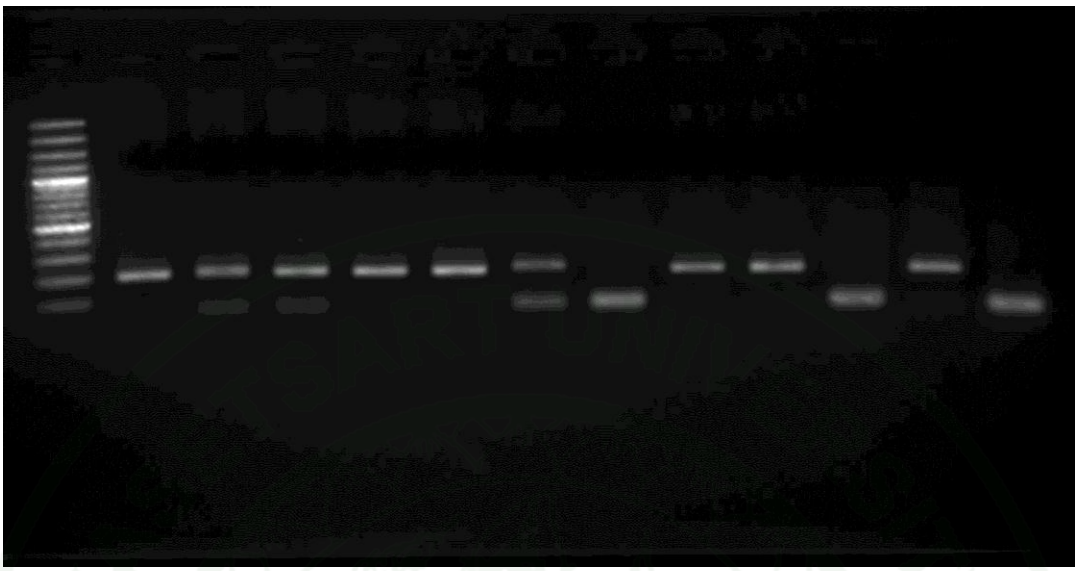
The figures of DNA bands of *Ehrlichia* 16s rRNA and *p30* gene



Appendix Figure A1 The DNA bands of *Ehrlichia* 16s rRNA gene presented at 310 bp.



Appendix Figure A2 The DNA bands of *Ehrlichia p30* gene presented at 220 bp.



Appendix Figure A3 The DNA bands of *Ehrlichia p30* gene presented at 220 bp.

Appendix B

The ocular disorders and hematological finding of 38 dogs infected with *Ehrlichia*

Appendix Table B1 The ocular disorders of 18 dogs infected with *E. canis* strain Jaboticabal

Number of dogs	KCS		Anterior Uveitis		Panuveitis		Keratitis		Chorio-retinitis		Hyphema		Vitrous hemorrhage		Retinal hemorrhage		Retinal detachment		Blindness	
	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.
1															/		/	/	/	/
2	/	/	/	/									/	/	/	/			/	/
3																	/	/		
4	/	/																	/	/
5	/	/	/									/		/		/	/	/	/	/
6																	/		/	/
7			/	/																
8																	/	/	/	/
9	/	/																		
10															/	/	/	/	/	/
11															/	/	/	/	/	/
12	/	/																		
13	/	/										/		/						/
14	/	/	/	/									/	/					/	/
15	/	/	/	/			/	/				/					/	/	/	/
16	/	/		/																
17									/											
18			/	/																

Rt. = Right eye

Lf. = Left eye

Appendix Table B2 Hematological finding of 18 dogs infected with *E. canis* strain Jaboticabal

Number of dogs	Total protein (g/dl)	PCV (%)	RBC ($x10^6$/cumm)	HGB (gm% or g/dl)	MCV (fL)	MCH	MCHC (gm%)	WBC ($x10^3$/cumm)	Platelet ($x10^3$/ul)
	(5.3-7.8)*	(35-55)*	(5-6)*	(10-18)*	(60-77)*		(32-36)*	(6-17)*	(200-500)*
1	6.0	31.5	4.71	10.20	66.90	21.60	32.30	21.50	135.00
2	8.8	37.0	1.28	2.80	77.00	21.80	28.30	8.10	200.00
3	8.2	37.6	5.46	12.30	68.90	22.50	32.70	10.60	65.20
4	8.0	26.0	4.63	9.53	65.87	20.58	31.25	18.90	280.00
5	11.0	15.1	2.18	4.59	69.27	21.06	30.40	23.50	80.00
6	8.0	15.2	1.88	4.70	81.10	25.00	30.80	8.34	50.00
7	n.d.	30.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	7.0	29.1	4.19	9.22	69.50	22.00	31.70	15.80	120.00
9	8.0	45.6	4.97	15.50	91.70	31.30	34.10	6.64	300.00
10	7.1	21.7	2.99	7.13	72.60	23.80	32.90	23.20	196.00
11	10.2	24.8	3.26	7.93	76.30	24.40	31.90	10.60	383.00
12	8.6	23.6	3.24	8.86	72.84	21.17	29.07	10.60	250.00
13	12.0	22.4	3.29	7.26	68.20	22.10	32.40	16.00	412.00
14	9.60	17.8	2.51	5.74	70.92	22.87	32.25	22.40	200.00
15	11.00	18.3	2.74	6.05	66.79	22.08	33.06	12.00	60.00
16	7.00	24.4	3.48	8.14	70.11	23.39	33.36	18.40	200.00
17	8.60	19.1	2.84	6.26	67.25	22.04	32.77	4.36	321.00
18	6.00	30.1	4.77	10.40	63.10	21.80	34.55	10.60	160.00

n.d. = No data

*Normal rang

Appendix Table B3 The ocular disorders of 3 dogs infected with *E. canis* strain Jake

Number of dogs	KCS		Anterior Uveitis		Panuveitis		Keratitis		Chorio-retinitis		Hyphema		Vitrous hemorrhage		Retinal hemorrhage		Retinal detachment		Blindness	
	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.
1							/													
2												/			/	/	/	/	/	/
3	/	/													/		/		/	

Rt. = Right eye

Lf. = Left eye

Appendix Table B4 Hematological finding of 3 dogs infected with *E. canis* strain Jake

Number of dogs	Total protein (g/dl)	PCV (%)	RBC ($x10^6$/cumm)	HGB (gm% or g/dl)	MCV (fL)	MCH	MCHC (gm%)	WBC ($x10^3$/cumm)	Platelet ($x10^3$/ul)
	(5.3-7.8)*	(35-55)*	(5-6)*	(10-18)*	(60-77)*		(32-36)*	(6-17)*	(200-500)*
1	8.2	50.9	7.29	15.60	69.80	21.40	30.60	16.80	78.00
2	10.4	20	3.36	8.11	76.70	24.10	31.40	15.40	800.00
3	9.0	27	4.08	8.38	66.30	20.50	31.00	1.65	3.74

n.d. = No data

*Normal rang

Appendix Table B5 The ocular disorders of 13 dogs infected with *E. canis* unidentified strain

Number of dogs	KCS		Anterior Uveitis		Panuveitis		Keratitis		Chorio-retinitis		Hyphema		Vitrous hemorrhage		Retinal hemorrhage		Retinal detachment		Blindness	
	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.
1			/	/							/	/	/		/				/	
2			/	/																
3	/	/					/	/												
4	/	/	/	/			/	/												
5			/	/																
6			/	/											/	/	/	/	/	/
7	/	/																		
8	/	/																		
9											/	/			/	/	/	/	/	/
10	/	/					/	/				/		/	/			/		/
11			/	/													/	/	/	/
12	/	/	/	/			/	/			/									
13							/				/		/	/		/	/	/	/	/

Rt. = Right eye

Lf. = Left eye

Appendix Table B6 Hematological finding of 13 dogs infected with *E. canis* unidentified strain

Number of dogs	Total protein (g/dl)	PCV (%)	RBC ($x10^6$/cumm)	HGB (gm% or g/dl)	MCV (fL)	MCH	MCHC (gm%)	WBC ($x10^3$/cumm)	Platelet ($x10^3$/ul)
	(5.3-7.8)*	(35-55)*	(5-6)*	(10-18)*	(60-77)*		(32-36)*	(6-17)*	(200-500)*
1	9.0	22.8	2.99	6.87	76.10	23.00	30.20	5.52	40.00
2	9.0	37.2	5.47	11.50	68.00	21.00	30.90	8.82	260.00
3	10.5	20.1	2.80	6.03	71.60	21.50	30.10	16.40	160.00
4	n.d.	12.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d.	33.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	6.0	21.0	3.16	7.20	66.50	22.80	34.30	26.70	180.00
7	8.0	35.7	5.16	12.20	69.10	23.60	34.20	5.05	200.00
8	7.0	26.4	4.06	9.03	65.02	22.24	34.20	20.80	1.57
9	10.0	32.5	5.11	12.40	63.60	24.27	38.15	7.37	130.00
10	10.60	23.3	3.99	7.79	58.40	19.52	33.43	4.14	50.00
11	11.20	13.7	1.87	4.46	73.26	23.85	32.55	13.90	20.00
12	7.8	47.9	7.12	16.5	67.28	23.17	34.45	10.80	336.00
13	8.00	20.00	2.79	6.51	71.68	23.33	32.55	26.00	140.00

n.d. = No data

*Normal rang

Appendix Table B7 The ocular disorders of 4 dogs infected with *A. platys*

Number of dogs	KCS		Anterior Uveitis		Panuveitis		Keratitis		Chorio-retinitis		Hyphema		Vitrous hemorrhage		Retinal hemorrhage		Retinal detachment		Blindness	
	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.	Rt.	Lf.
1					/						/				/		/		/	
2																	/	/	/	/
3	/	/	/	/																
4															/	/				

Rt. = Right eye

Lf. = Left eye

Appendix Table B8 Hematological finding of 4 dogs infected with *A. platys*

Number of dogs	Total protein (g/dl)	PCV (%)	RBC ($x10^6$/cumm)	HGB (gm% or g/dl)	MCV (fL)	MCH	MCHC (gm%)	WBC ($x10^3$/cumm)	Platelet ($x10^3$/ul)
	(5.3-7.8)*	(35-55)*	(5-6)*	(10-18)*	(60-77)*		(32-36)*	(6-17)*	(200-500)*
1	6.0	49.9	6.58	15.80	75.90	24.00	31.60	13.20	120.00
2	7.6	25.6	4.21	9.12	60.70	21.60	35.60	11.70	854.00
3	7.0	36.1	5.34	12.20	67.60	22.90	33.90	17.40	240.00
4	6.30	31.90	5.50	10.90	58.00	19.82	34.17	27.10	1.12

n.d. = No data

*Normal rang

Appendix C

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. Gel loading buffer (loading dye)

The loading dye buffer composed of 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol and 35 ml of ultrapure distilled water. The loading dye solution was kept at 4°C.

2. Tris acetate buffer (50x TAE)

Tris stock 50x TAE was prepared by dissolved 242 g of Tris-base in 500 ml of distilled water. After the ingredient was completely dissolved, 57.1 ml of concentrate glacial acetic acid and 100 ml of 0.5 M EDTA, pH 8.0, were added into the solution. The final volume was adjusted to 1,000 ml by distilled water. The 50x TAE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 50x TAE buffer with distilled water.

3. Working (0.5x TAE)

Twenty milliliter of 50x TAE was added to 1980 ml of UDW. This solution can be reused three times.

Appendix D

The phenol-chloroform DNA extraction method

The phenol-chloroform DNA extraction method

1. DNA was extracted from blood sample 100 µl mixed with denature solution 500 µl by shaken to 5-10 min.
2. Add chloroform 150 µl and phenol (pH 7.9) 150 µl (chloroform:phenol = 1:1), shaken for 10 min.
3. Centrifuge the mixture at 13,000 rpm for 5 min to separate the phases.
4. Collect the supernatant for 550-600 µl and transfer to the clean microtube (1.5 ml), carefully avoiding protein at the aqueous phenol interface at the last collecting.
5. Repeat the same protocol to clean the supernatant (step 2-4). In the second time, collect 400 µl of the supernatant and transfer to new microtube (1.5 µl).
6. Precipitat DNA by adding 1,000 µl (1 ml) of absolute ethanol (99.99%), invert gentally upside down and keep in -80^{o}C for 30 min or -20^{o}C for overnight.
7. Centrifuge at 13,000 rpm for 10 min. Remove the supernatant carefully.
8. Wash the DNA pallet with 75% ethanol. Centrifuge at 13,000 rpm for 5 min. Decant the supernatant, and dry the pallet by air.

Appendix E

The proceeding paper in the 52nd Kasetsart University Annual Conference

KASETSART UNIVERSITY

This is to certify that
the research report for

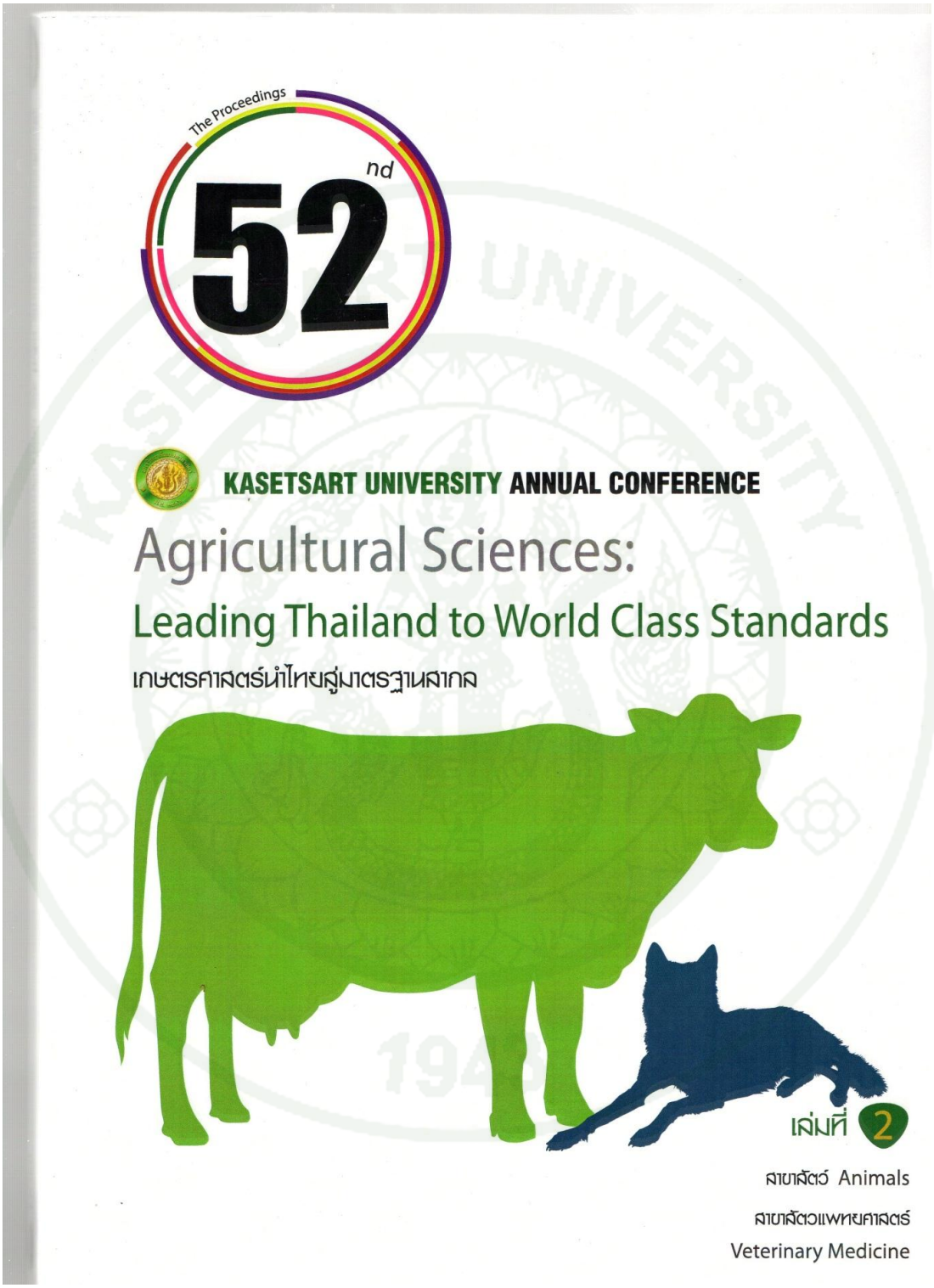
The Preliminary Study of *Ehrlichia* spp., a Causative Agent of Ocular Disorders in Dogs by Nested PCR

By

Kepalee Saeng-chuto Aree Thayananuphat
Kannika Siripattarapravat Natthanet Sritrakoon
Winyu Karntip Thanate Anusaksathien
Natapong Kraiwong Metita Sussadee
and Burin Nimsuphan

has been reviewed by the Veterinary Medicine Editorial Board
and was presented in the 52nd
of Kasetsart University Annual Conference, held during
February 4 – 7, 2014

(Associate Professor Dr. Siree Chaiseri)
Vice President for Academic Affairs
52nd Kasetsart University Annual Conference Committee Chairman



The Preliminary Study of *Ehrlichia* spp., a Causative Agent of Ocular Disorders in Dogs by Nested PCR

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ABSTRACT

Canine Ehrlichiosis is a tick-borne disease caused by bacteria in family *Anaplasmataceae* such as *Ehrlichia canis* and other species of *Ehrlichia.* This disease is manifested by a wide variety of ocular signs. The aim of this study was to study the relationship between species of *Ehrlichia* and ocular disorders in infected dogs in Thailand. Samples from 98 dogs that showed clinical signs of unilateral or bilateral ocular disorders and positive result with *Ehrlichia* infection either were diagnosed by blood smear or SNAP 4Dx test. All samples were to amplified for 16s rRNA gene for *Ehrlichia* by nested PCR (nPCR). The result showed 28 samples (27.44%) were positive for *Ehrlichia*. Twenty-seven samples (26.46%) were positive for *E. canis* and 1 sample (0.98%) was positive for *Anaplasma platys*. The most common ocular disorders in dogs infected with *E. canis* were keratoconjunctivitis sicca (KCS) (11/27, 40.74%), and retinal detachment (11/27, 40.74%). One dog infected with *A. platys* showed sign of anterior uveitis. However, the chi-square test did not show any significant differences between species of *Ehrlichia* and ocular disorders.

Keywords: ehrlichiosis, *Ehrlichia* spp., ocular disorders, dog, nested PCR

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INTRODUCTION

Ehrlichia species are obligative intracellular parasites residing within the cytoplasmic vacuoles of monocytes, granulocytes, or platelets of humans and animals. *Ehrlichia* is a causative agent of canine ehrlichiosis transmitted by tick in family *Ixodidae*. The disease from *E. canis* infection appears in three stages, including acute, subclinical, and chronic infections (Woody and Hoskins 1991). Infected animals present several clinical signs depending on the stage of the disease. The most frequent symptoms consist of high fever, anorexia, emaciation, hepatomegaly, splenomegaly, lymphadenopathy, and ocular changes. Dogs can be infected with several species of *Ehrlichia*, including *E. canis*, *E. chaffeensis*, *E. equi*, *E. ewingii*, *E. risticii*, and *Anaplasma platys.* However, only *E. canis* and *A. platys* were reported as the etiologic agents of ocular diseases in dogs. *E. canis* has been reported to cause several ocular lesions such as conjunctivitis, conjunctival or iridal petechiae and ecchymoses, corneal edema, panuveitis, hyphema, retinal haemorrhage and detachment (Collins and Moore, 1991) episcleral congestion, anterior chamber flare, rubeosis iridis, miosis, corneal opacitation, keratic precipitates, hypopyon, and reduced intra-ocular pressure (Glaze and Gaunt, 1986; Clerc and Laforge, 1999; Stiles, 2000). Inflammatory debris can obstruct the trabecular meshwork to induce secondary glaucoma, and annular posterior synechiae can result in iris bombé formation (Collins and Moore, 1999). Posterior segment lesions also include papilledema and chorioretinitis (Glaze and Gaunt, 1986). However, there was only one report stated that *A. platys* was a cause of uveitis in infected dog (Glaze and Gaunt, 1986).

In Thailand, *Ehrlichia* (*E. canis, E. chaffeensis*, *E. equi* and *A. platys*) infection were common in dogs (Suksawat *et al*., 2001; Parola *et al*., 2003; Pinyoowong *et al*., 2008). However, the relationship between species of *Ehrlichia* and ocular disorders in infected dogs has never been studied in Thailand. Thus, the aim of this study was to study the relationship between species of *Ehrlichia* in Thailand and ocular disorders in infected dogs.

MATERIALS AND METHODS

Blood samples collection

Blood samples were collected from 98 crossbred and purebred dogs of both sexes that were visited the Ophthalmology Clinic, Veterinary Teaching Hospital, Kasetsart University. All dogs showed clinical signs of unilateral or bilateral ocular disorders. The criteria for sample collection were dogs positive result with *Ehrlichia* infection either diagnosed by blood smear or SNAP 4Dx test (IDEXX Laboratories, Westbrook, ME), tick infestation, or thrombocytopenia. Blood samples were collected from cephalic vein or saphenous vein and kept into EDTA tubes for buffy coat smear and DNA extraction.

Buffy coat smear examination

Buffy coat smear was prepared by fixing smeared slides with absolute methanol for 3 minutes. The slides were dried and stained with Modified Wright-Giemsa for 20 minutes, dried and immersed in buffer for 10 minutes. The slides were then examined under light microscope for the detection of *Ehrlichia* morulae.

DNA extraction

Blood samples (100 µl each) were lysed with 600 µl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.1 M 2-mercaptoethanol, 0.5% N-lauroylsarcosine), and shaken for 5 minute. DNA was extracted with phenol–chloroform extraction and precipitated in absolute ethanol as previously described (Sambrook and 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 designed for 16S rRNA amplification

Primers for amplification of *Ehrlichia* 16S rRNA gene were designed from nucleotide sequences deposited in GenBank database (EU143636, HQ290362, EU123923, EU781686, EU781688, EF195134, EU781690, EU781694, DQ460714, M73226, M73221, EF139458, EF011111, EU439944, GQ395381, AF373612, EU781689, AF162860, AY394465, EU7816 92, EU263991, EU178797, EU139493, EF424612, EF195135, GQ395378, EU781691, AB28 7435, AF536827, EU567025, DQ915970, U54805, AF373613, AF156784, AF303467, AY07 7619, AF536828, AY530806, AF286699, AF287153, M82801, U23503, U96436, AF295573 and DQ342324). All of the sequences were aligned for the maximum homology by ClustalW Version 2.1. Conserved regions were selected and outer primers named EF-primer (5'-TTGTAGCTAACGCGTTAAGCACT-3') and ER-primer (5'-AACTCGA AGCTGGTGYGCYAACC-3'), and inner primers named IEF-primer (5'-GTTCGGC TGGAYCTYRCACAGG T-3') and IER-primer (5'-CTGMAACTCGAGAGCATGA AGTC-3') were derived.

Nested-PCR and DNA Sequencing

DNA products that were isolated from blood samples were used as a template to amplify the 16S rRNA gene from *Ehrlichia* by nested-PCR. The first PCR, primers EF-primer and ER-primer amplify a 551-bp region. The second PCR, primers IEF-primer and IER-primer amplify a 310-bp region of the 16s rRNA gene. In the first PCR, 2 µl of DNA template were added to the PCR mixture to give a final volume of 18 µl (total 20 µl per reaction). Reaction mixture contained 1x buffer (10 mM Tris–HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 2.0 mM $MgCl_2$, 1 pmol 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 transferred to a new tube containing 18 µl of PCR mixture. Reaction mixture was similar to the first PCR except using 1.5 mM $MgCl_2$ for this step. The PCR condition was pre-denaturation at 94ºC for 5 minutes, then 45 cycles of denaturation at 94ºC for 20 seconds, annealing at 57ºC for 20

seconds, and extension at 72ºC for 40 seconds, and followed by final extension at 72°C for 10 minutes.

Both of PCR protocols were processed in MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). Twenty microliter of amplified PCR product was added to the 5 µl of 6X loading buffer and loaded in 1.5% agarose (SeaKem ME:FMC, USA) gel electrophoresis. Amplicons were processed with UltraCleanTM GelSpin DNA purification Kit (MO BIO LABORATORIES Inc, CA, USA) and submitted for sequencing (1st Base Laboratory, Malaysia). DNA sequences were compared the similarity of nucleotide base with the database in Genbank by using BLAST algorithm.

Statistical Analysis

Chi-square and Number Cruncher Statistical System (NCSS) ver. 2000 (Kaysville, UT) programs were used to assess the relationship of the speicies of *Ehrlichia* and ocular lesions. P-Value ≤ 0.05 was considered as statistical significance.

RESULTS AND DISCUSSIONS

The buffy coat smear examination showed the negative result in all samples. Twenty-eight samples (27.44%) were positive to *Ehrlichia* by PCR technique. The size of positive DNA target was 310 bp as shown in figure1. The sequencing analysis presented 27 samples (26.46%) were positive to *E. canis* (99% similarity with KC479024) and 1 sample (0.98%) was positive to *A. platys* (99% similarity with JQ396431).

The most common of ocular disorders in 27 dogs infected with *E. canis* included keratoconjunctivitis sicca (KCS) 11/27 cases (40.74%), and retinal detachment 11/27 cases (40.74%), and anterior uveitis 8/27 cases (29.63%). The result was according to the study of Komnenou *et al.*, 2007 that anterior uveitis was the most common ophthalmic disorders in infected dogs. Only 1 dog infected with *A.*

platys showed anterior uveitis, panuveitis, and hyphema (Table 1). However, the chi-square test did not show any significant differences between species of *Ehrlichia* and ocular disorders.

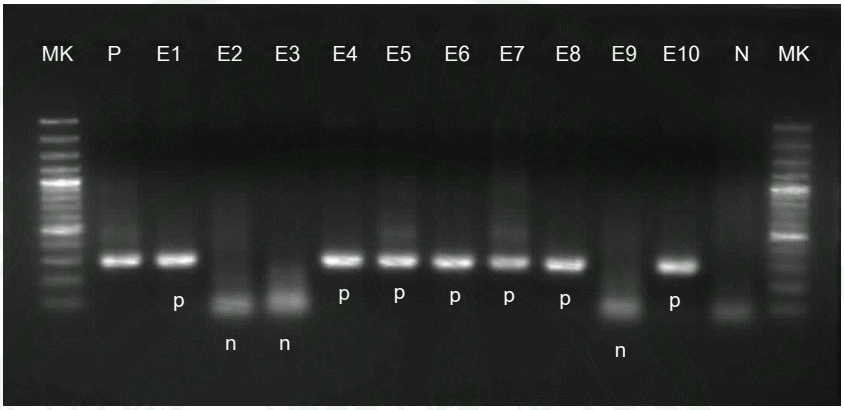

Figure 1 The positive DNA bands of *Ehrlichia* 16s rRNA gene presented at 310 bp. MK: DNA marker 100 bp plus, P: positive control of *Ehrlichia*, N: negative control, E1 – E10: DNA of sample No.1-10, p: positive samples, n: negative samples.

Although, dogs can be infected with *E.canis*, *A. platys*, *E. chaffeensis*, *E. equi*, *E. ewingii* and *E. risticii*, the result of this study showed that only *E. canis* and *A. platys* were found to cause of ocular disorders in the infected dogs. This information accords with the previous study in 2001 about the relationship between the infection of 4 species of *Ehrlicia* including *E. canis*, *E. ewingii*, *E. chaffeensis* and *E. equi*, and ocular diseases in experimental dogs which only *E. canis* was able to cause ocular disorders (Panciera *et al.*, 2001). Furthermore, the ocular disorders from *A. platys* infection in this study was correlated to the study by Glaze and Gount (1986) which published *A. platys* was the causative pathogen of uveitis.

The infections with *E. canis* and *A. platys* can cause thrombocytopenia (Harvey *et al*., 1978; Troy and Forrester, 1990; Gaunt *et al.*, 1990; Chang *et al.*, 1996; Bradfield *et al.*, 1996; Bulla *et al.*, 2004). Thrombocytopenia is one of a cause of ocular disorders such as retinal and vitreal hemorrhage, retinal detachment,

papilledema, and disc neovascularization (Black and Terry, 1911). In addition, the previous studies published *E. chafeensis*, *E. ewingii*, and *E. equi* can also cause of thrombocytopenia (Anziani *et al.*, 1990; Goodman *et al.*, 2003; Zhang *et al.*, 2003; Baneth, 2010; Yabsley *et al.*, 2011; Eberts *et al.*, 2011). However, *Ehrlichia* in Thailand were found only *E. canis, A. platys*, *E. equi* and *E. chaffeensis* but in the present study only *E. canis* and *A. platys* were found in infected dogs with ocular disorders. One of the possible reason is the infected dogs with *E. equi* or *E. chaffeensis* did not show the ocular disorders or dogs with low grade of ocular disorders might be missed the ocular examination which all of them were not referred to the Ophthalmology Clinic. The major clinical signs of the dogs infected with *E. equi* are fever, lethargy, weakness, anorexia, depression, polyarthritis, limb edema, and neurological signs (Baneth, 2010) and the dogs infected with *E. chaffeensis* did not show clinical signs (Zhang *et al.*, 2003). However, parasites might be not a cause of ocular disorders.

To increase the chance for detection of other species of *Ehrlichia*, more blood samples will be collected in the future. The more information will be benefit to prevent the ocular disorders cause by *Ehrichia* infection in domestic dogs. The ocular examination of infected dogs might be help to reduce the severity of ocular disorders, particularly retinal detachment leading to acute blindness. Strain of *E. canis* could be one possible cause of the difference in ocular disorders in this study. In the future, DNA amplification with the other genes will be studies to identify the strain of *E. canis*.

ACKNOWLEDGMENTS

This study was supported by a research grant from Faculty of Veterinary Medicine, Kasetsart University.

Table 1 The ocular disorders of twenty-eight dogs infected with *Ehrlichia*

Sample No.	KCS	Conjunctivitis	Keratitis	Corneal edema	Corneal rupture	Keratic precipitate	Cataract	Nuclear sclerosis	Anterior Uveitis	Panuveitis	Hyphema	Vitrous hemorrhage	2° Glaucoma	Subretinal hemorrhage	Retinal hemorrhage	Retinal detachment	Purulent discharge	Phthisis bulbi	*Ehrlichia* spp.
01	(+,+)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(+,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
02	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,-)	(-,+)	(-,-)	(-,-)	*E. canis*
03	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
04	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
05	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
06	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
07	(-,-)	(-,+)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
08	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
09	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
10	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
11	(-,-)	(-,-)	(+,-)	(-,-)	(-,-)	(-,-)	(-,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
12	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
13	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
14	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,-)	(-,-)	(-,+)	(-,+)	(-,+)	(-,+)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
15	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,-)	(-,-)	(-,+)	*E. canis*
16	(-,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,-)	(+,-)	(-,-)	(-,-)	*E. canis*
17	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,-)	(+,-)	(+,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*A. platys*
18	(+,+)	(+,+)	(-,+)	(-,+)	(+,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
19	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
20	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
21	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
22	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
23	(+,+)	(-,-)	(-,-)	(+,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
24	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(+,+)	(-,-)	(-,-)	*E. canis*
25	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(+,+)	(-,-)	(-,-)	*E. canis*
26	(+,+)	(-,-)	(+,+)	(+,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
27	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(+,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	*E. canis*
28	(+,+)	(-,+)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,-)	(-,+)	(-,-)	*E. canis*

(+,+): (Right eye positive for ocular disorders, Left eye positive for ocular disorders)
(+,-): (Right eye positive for ocular disorders, Left eye negative for ocular disorders)
(-,+): (Right eye negative for ocular disorders, Left eye positive for ocular disorders)
(-,-): (Right eye negative for ocular disorders, Left eye negative for ocular disorders)

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