

The Microscopic and Molecular Detections of Canine Ehrlichiosis

Orapin Ariyawutthiphan¹ Kaj Shokshai-utsaha¹ Thanida Sananmuang¹
Sudchit Chungpivat² Meena Sarikaputi³ Nareerat Viseshakul^{2*}

Abstract

The applications of molecular detection using nested PCR and the routine microscopic examination of ehrlichiosis were applied to 43 clinically affected dogs residing in Bangkok and Samutprakarn. This study, performed in 2005-2006, has determined that 79.5% (31/39) of the buffy coat smears were having the ehrlichial morulae plus initial bodies found in monocytes and lymphocytes. The nested PCR was used to confirm the occurrence of ehrlichiosis after tested by morulae identification. Both tests demonstrated that neither *Ehrlichia chaffeensis* nor *E. ewingii*, the zoonotic agents of human, were found in this area. However, the prevalence of *E. canis* alone detected by nested PCR and microscopy was at the rate of 65.12% (28/43). Having a lower number of microscopic morulae (33.33% or 13/39) than that of the initial bodies (72.09% or 31/43), the identification of initial bodies was subsequently taken into account for another factor for disease detection. The detection of initial body in the circulating blood of dogs had brought the sensitivity of the test to equalize the power of molecular identification. However, the nested PCR is still a method of choice to specifically determine ehrlichial infection and it has shown the strong association between thrombocytopenia and the infection of *E. canis*.

Keywords : nested PCR, ehrlichiosis, *E. canis*, Bangkok.

¹6th year student, academic year 2005, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

²The Unit of Parasitology, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

³The Unit of Biochemistry, Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

*Corresponding author

บทคัดย่อ

การตรวจวินิจฉัยโรคเออร์ลิคิโอซิสในสุนัขด้วยกล้องจุลทรรศน์แสงสว่าง และเทคนิคทางอณูชีววิทยา

อรพินท์ อริยะวุฒิพันธ์¹ กาญจนา โชคชัยอุตสาหะ¹ ธนิตา สนั่นเมือง¹ สุกจิตต์ จุ่งพิพัฒน์² มีนา สาริกะภูติ³ นาริรัตน์ วิเศษกุล^{2*}

รายงานฉบับนี้ เป็นการประยุกต์ใช้ปฏิกิริยาลูกโซ่แบบเนสต์ (nested PCR) ร่วมกับการตรวจด้วยกล้องจุลทรรศน์แสงสว่าง เพื่อวินิจฉัยโรคเออร์ลิคิโอซิส (Ehrlichiosis) ในสุนัขบ้าน 43 ตัว ที่อยู่ในเขตจังหวัดกรุงเทพมหานคร และสมุทรปราการ พบว่าในปี พ.ศ. 2548-2549 มีสุนัขบ้านร้อยละ 79.5 (31/38) ที่ตรวจพบลักษณะมอรูล (Morulae) และอินนิเซียลบอดี (Initial body) ในเม็ดเลือดขาวโมโนไซต์และลิมโฟไซต์ จากแผ่นฟิล์มเลือดบางของชั้นเม็ดเลือดขาว ร่วมกับการใช้เทคนิคปฏิกิริยาลูกโซ่แบบเนสต์เพื่อยืนยันการติดเชื้อ โดยทั้งสองวิธีให้ผลเหมือนกัน คือไม่พบเชื้อเออร์ลิเชียชนิด *Ehrlichia chaffeensis* และ *E. ewingii* ที่เป็นสาเหตุของโรคเออร์ลิคิโอซิสในคน อย่างไรก็ตามเชื้อ *E. canis* ยังเป็นเชื้อชนิดที่พบมากในประชากรสุนัข ที่นำมาศึกษาในครั้งนี้ คือ ร้อยละ 65.12 (38/43) เนื่องจากการทดสอบครั้งนี้ พบอินนิเซียลบอดีของเชื้อเออร์ลิเชีย มากกว่าจำนวนตัวอย่างที่มีลักษณะมอรูล จึงมีการใช้การตรวจวินิจฉัยอินนิเซียลบอดี ร่วมกับลักษณะมอรูล ในตัวอย่างดังกล่าว และพบว่า การวินิจฉัยอินนิเซียลบอดีมีความไวใกล้เคียงกับการใช้เทคนิคปฏิกิริยาลูกโซ่แบบเนสต์ อย่างไรก็ตามปฏิกิริยาลูกโซ่แบบเนสต์ มีความจำเพาะสูง และการทดลองนี้ยังพบความสัมพันธ์ของภาวะเกล็ดเลือดต่ำในสุนัข และการติดเชื้อ *E. canis* อีกด้วย

คำสำคัญ: ปฏิกิริยาลูกโซ่แบบเนสต์ โรคเออร์ลิคิโอซิส *E. canis* กรุงเทพมหานคร

¹นิสิตชั้นปีที่ 6 ปีการศึกษา 2548 คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

²หน่วยปรสิตวิทยา ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330

³หน่วยชีวเคมี ภาควิชาสรีรวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพฯ 10330

*ผู้รับผิดชอบบทความ

Introduction

Canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis* is one of the most important ehrlichiosis in mammals affecting wild and domesticated dogs of the family canidae worldwide. It appears that ehrlichiosis is caused by 2 genera of the Anaplasmataceae; *Ehrlichia* and *Anaplasma*, and both genera are the obligatory intracellular bacteria that could be found in human (Uilenburg et al., 2004; Taillardat-Bisch et al., 2003; Dumler et al., 2001). The clinical signs of CME in dogs have configured a high fever, bleeding tendencies particularly in lowering the platelet counts and in a severe chronic case, death may occur (Giger, 2005; Bulla et al., 2004; Harrus et al., 1999; Breitschwerdt et al., 1998). The brown dog tick, *Rhipicephalus sanguineus* is known for an important vector passing ehrlichiosis among dog populations, specifically in the cities as the metropolitan tick transmission (Rodriguez-vivas et al., 2005).

The diagnostic techniques of canine monocytic ehrlichiosis are based technically on at least 4 standard methods; the finding of morulae in stained buffy coat smears, the serological examination, the cell culture method

and the molecular based technique of polymerase chain reaction (PCR) (Mylonakis et al., 2003). The ehrlichial morulae findings using the method of stained blood smears could roughly differentiate ehrlichial species based on cell tropisms and restricted to an inadequate sensitivity (Bulla et al., 2004). The technique may produce both false positives and negatives in particular to the subclinical phase of the disease and it requires highly skilled diagnosticians to confirm the results. The immunological based techniques such as Enzyme linked immunosorbent assay (ELISA), Western blot analysis and Immunofluorescent assay (IFA) were used to detect pathogens indirectly and alternatively to observe the level of immune response in infected animals. The tests encountered cross reaction to a shared epitope found among species of *Ehrlichia*. Furthermore, the serological reactions could not differentiate the status of infection from the treated ones (Wen et al., 1997). The cell culture method was the most sensitive test, but required time, expenses and some difficulties applied as a true clinical application (Iqbal et al., 1994). The nested PCR was introduced to overcome the cell culture method and probably others hence used

in findings of the genetic material of ehrlichiae and it may be suitable for the clinical practices (Martin et al., 2005; Harrus et al., 1998; Murphy et al., 1998; Iqbal et al., 1994).

Ehrlichia canis is the most pathogenic ehrlichiosis in dogs causing a persistent infection. The pathogen could be detected in a long period of time after the initial infection (Harrus et al., 1998). Other ehrlichial pathogens such as *E. chaffeensis*, *E. ewingii* were reported earlier as the pathogens of humans found in dogs (Lee et al., 2005; Breitschwerdt et al., 1998; Murphy et al., 1998). *Anaplasma phagocytophilum* (*E. ewingii*) and *A. platys* are another ehrlichiae that are found worldwide. All mentioned species could be found in Thailand and they could be detected in dog populations (Suksawat et al., 2001). For the fact that, the nested PCR is widely used in determination of ehrlichial infections, in this report, we therefore performed a species identification of *Ehrlichia* found in Thailand using nested PCR (Branger et al., 2004, Harrus et al., 2004). We also determined the occurrence of *E. canis* in the city dogs using both microscopic and molecular methods previously described (Murphy et al., 1998). Subsequently, the initial body findings are introduced to confirm the ehrlichiosis cases and to increase the efficiency of microscopic determination. Forty-three dogs were included in this test as the preliminary report of canine ehrlichiosis in Thailand.

Materials and Method

The blood collection samples

Forty-three dogs from the teaching hospital of known conditions of ehrlichiosis were included in the tests. They were selected by having one of the clinical conditions; thrombocytopenia, fever ($>103.5^{\circ}\text{F}$), the history of bleeding tendencies, tick infestation, leucopenia and pancytopenia. The blood samples were collected to the amount of 0.5 ml preserved in EDTA as the anticoagulant. The sampled blood was kept at 4°C and within 48 hours the samples were processed with the buffy coat smears and DNA extraction.

The method of buffy coat smear

The buffy coat smears and Giemsa and Wright's Giemsa staining techniques were performed as the routine blood examination of ehrlichiosis. The thin film blood slides were examined for at least 500 oil immersion field (OIFs) (Mylonakis et al., 2003). Types of

white blood cells, for instance, granulocytic (neutrophil) cells, agranulocytic cells (monocyte and lymphocyte) and platelets were all examined. When morulae or initial bodies were found in at least one cell, ehrlichiosis will be recorded.

The DNA extraction and nested PCR

Total DNA was extracted from 200 μl blood of dogs with the standard phenol-chloroform extraction slightly modified from the previous reports (Murphy et al., 1998; Iqbal et al., 1994). The purified DNA was dissolved in sterile double distilled water and it was kept at -20°C until used.

The nested PCR was designed to target DNA in the region of 16S rRNA gene. The first round PCR was designated as the commonly primed PCR reaction. The reaction could amplify DNA from all possible species of *Ehrlichia* including *A. platys* and *A. phagocytophilum* by using Ehr-out-2 primer modified from the previous record (Murphy et al., 1998). We had selected the nested PCR to be specific to *E. canis* using ECAN5 and HE3, specific to *E. chaffeensis* using HE1 and HE3 and to *E. ewingii* using EE52 and HE3 (Murphy et al., 1998). DNA from dogs infected with *Babesia canis* and *Hepatozoon canis* were used as the negative controls.

The first round PCR was performed as follows; in the volume of 25 μl , there were 5 μl of template DNA, 1.0 μl of 10 mM forward primer, 1.0 μl of 10 mM reverse primer, 0.5 μl of 5U/ μl Taq Polymerase (Invitrogen[®], USA), 1.6 μl of 10 mM dNTPs (Intron[®], USA), 2.5 μl of 10x buffer (Intron[®], USA), 1.2 μl of 25 mM MgCl_2 (Intron[®], USA) and sterile ultrapure water was added to the volume of 25 μl . The GeneAmp System 2700 thermocycler was used and the PCR cycles were applied accordingly (Murphy et al., 1998). The nested PCR or the species specifically primed PCR reaction was performed in 25 μl reaction master mix like that of the first round PCR with 2 μl of the first round PCR product as DNA template. The PCR cycle program was similar to that of the first round except the annealing temperature that was raised to 55°C . To ensure that the PCR products obtained from the samples were not the contaminant, the full-length 16S rRNA gene of *E. canis* was cloned into pGEM-T plasmid (Promega[®], USA) and bidirectionally sequenced. The DNA sequence was submitted to GenBank database under the accession number: EU263991.

The statistic analysis

The Chi-square test for RxC (2x3 or 2x2) contingency table was used to analyze the relatedness between tests of nested PCR and the microscopic examination of *E. canis*, the test was described (Rosner, 2006). The observed and expected values were compared and the degree of freedom was tested with χ^2 when p -value < 0.05 then null hypothesis was determined. When there were no differences between tests, the null hypothesis was accepted.

Results

From 43 clinically affected dogs, there were 41.86%(18/43) males and 58.14%(25/43) females aging from 1 month to 17 years old. To these numbers, there were 65.12% (28/43) pure breeds and 34.88% (15/43) mixed breeds. All dogs were housed dogs located in Bangkok and Samutprakan (20 km to the east of Bangkok). The results of nested PCR and buffy coat smears in findings of morulae and initial bodies in these dogs were tabulated in Table 1.

The typical characteristics of morulae were examined in 39 samples from 43 dogs (ND=4). Morulae and initial bodies of *Ehrlichia* were depicted in Figs. 1A and B. In this study, no morulae were found without the presence of initial bodies. Morulae were detected at 33.33% (13/39, ND=4) while initial bodies were at 72.09% (31/43 or 79.48%, 31/39, ND=4). The appearance of only initial bodies without morulae was noticeable at a very high fraction, 46.15% (18/39) which was remarkably more than the presence of morulae in all samples. Furthermore, there were no granulocytic and platelet morulae in these samples. All ehrlichial morulae were found in the agranulocytic monocytes and lymphocytes. This indicated that *A. platys*, *E. ewingii* and *E. phagocytophilum*, ones that commonly found in platelets and the granulocytic leucocytes, could hardly exist in our samples. Our preliminary observation showed that the majority of dogs of all breeds, sexes and ages in Bangkok and Samutprakan were infected with only *E. canis*. There were 2 dogs having multiple infections of *E. canis* and *Babesia* spp.(Nos. 20 and 24). One was found with *E. canis* and *Hepatozoon canis* (No. 15).

The nested PCR reaction specific to *E. canis* using primers ECAN5 and HE3 produced the amplified DNA product of 389 bp. No cross-reaction was found when samples were infected with *Hepatozoon canis* or *Babesia canis* (Fig. 2). When 2 independent tests of

microscopy and nested PCR were compared, results in Table 2 showed the accuracy of the tests revealing the significantly true positives, 84.62% and true negatives 87.5%. Using the statistical analysis, Chi-square test for 2x3 contingency table, the fitness of accuracy of 2 independent variables showed no differences between tests whether it was ehrlichiosis with morulae (positives, 84.62%, false negatives, 15.38%) or ehrlichiosis with initial body (positives, 83.33%, false negatives, 16.67%) ($p<0.05$) (Table 2). However, the discrepancy between tests was found in one sample having a positive test for nested PCR while having a negative test for microscopy.

The association between thrombocytopenia and *E. canis* infection was obtained by this observation. In the total of 43 clinical affected samples, there were as high as 88.37% (38/43) thrombocytopenia cases (mean platelet counts, $79,585 \pm 83,365/\mu\text{l}$ and Table 3). Our results showed that as high as 68.75% of *E. canis* positive dogs were having the platelet counts less than 100,000 platelets/ μl . We also found that 5 of 6 cases of pancytopenic dogs also had *E. canis* infection.

Discussion

The sensitivity of nested PCR was tested previously to be as low as 0.2 pg of the total DNA and the sensitivity test was carried out in dogs infected with *E. canis* (Wen et al., 1997). We had adopted the sensitivity test and found a similar result (data not shown). We therefore applied the nested PCR to our observation. The specificity of the test of PCR was shown by the amplification of *Babesia* and *Hepatozoon* infected samples. The results showed no cross reaction in the samples (Fig. 2). The result also suggested the use of nested PCR in standard diagnosis of ehrlichiosis as previously described (Murphy et al., 1998).

Our observation was focused on *E. canis* occurred in the city dogs as the most pathogenic ehrlichiosis although *E. chaffeensis*, *E. ewingii*, *A. platys* and *A. phagocytophilum* could also be found and those ehrlichiae were reportedly in the US while anaplasmae were recored worldwide (Lee et al., 2005; Skotarczak 2003; Hildebrandt et al., 2002; Inokuma et al., 2001; Breitschwerdt et al., 1998; Murphy et al., 1998). In particular, *E. chaffeensis* was found in ticks collected from Vietnam (Parola et al., 2003). In Thailand, the example of *A. platys* infection in dogs and *A. phagocytophilum* in ticks were already reported (Parola et al., 2003; Suksawat et al., 2001). Furthermore,

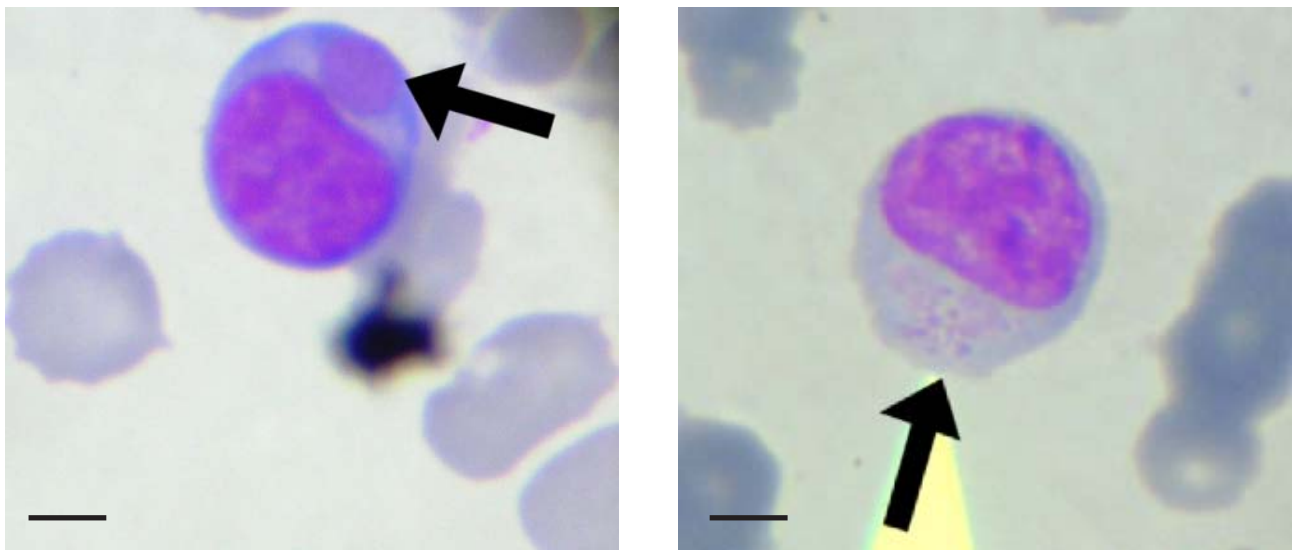


Figure 1 The buffy coat smears showing the characteristics of morulae (A) and initial bodies (B) stained with Wright's Giemsa. The presence of morulae and initial bodies were examined microscopically at 1000 x magnification. A: Morulae in a monocyte (arrow) from sample no. 27. B: Initial bodies in a monocyte (arrow) from sample no. 6, Scale bar = 2.5 μ m.

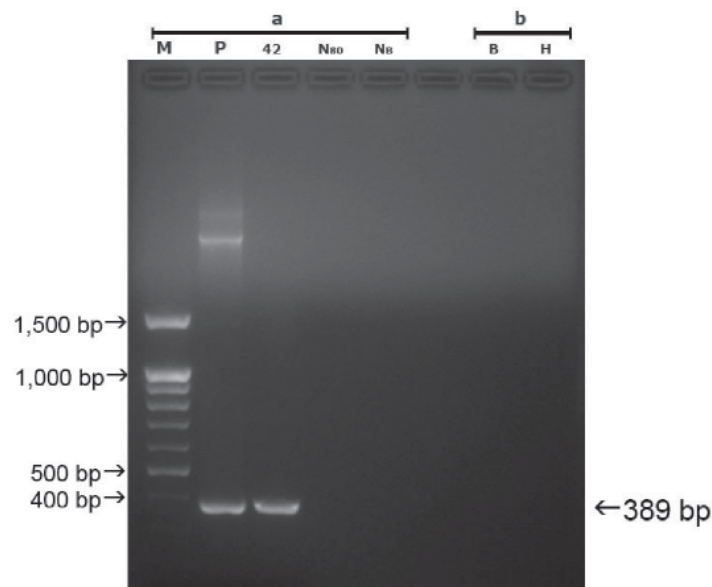


Figure 2 The electrophoresis analysis of nested PCR products. (a) The result of nested PCR using specific primer to *E. canis* (ECAN5 and HE3). The PCR product appeared at 389 bp was amplified from the sample no. 42 (M: 100 bp DNA ladder marker, P: positive control (*E. canis* DNA), N80: negative control (DNA from ehrlichial-free dog), NB: DNA-free negative control (sterile ultrapure H₂O), 42: Sample no. 42. (b) The result of the commonly primed PCR reaction indicated the genus specific reaction. The common primer, ECC and Ehr-Out-2 when used with DNA from B: *Babesia canis* infected dog DNA or H: *Hepatozoon canis*.

A. phagocytophilum, the agent that causes a disease in human and horses, was reported in dogs residing in Bangkok (Suksawat et al., 2001). We found that dogs exposed to ticks are most likely to be *E. canis* positive, supported by our results showing 72.72% (16/22) dogs infested by *R. sanguineus* became positive with

E. canis (Table 1). One possible reason that *E. chaffeensis* or *E. ewingii* were not found in our samples is that no wildlife reservoir hosts near our observation sites. In other country, the previous report showed that the wild deer of North America was the important reservoir of *E. chaffeensis* and *E. ewingii* (McQuiston et al., 2003).

Table 1 The historical examinations, clinical signs, hematology and the results of buffy coat smears and nested PCR in 43 dogs.

Samples	Buffy coat smears		Nested PCR	Historical Examinations		Hematology	
	Morulae	Morulae and IB		Bleeding tendencies	Ticks	Fever	Thrombocytopenia
43	13 (ND=4)	31 (1H, 1B)	27 (ND=1)	4	22	10	38
							6

Bleeding tendencies e.g. epistaxis, skin petichial hemorrhage IB: Initial body, ND = not determined; 1 H = *Hepatozoon canis*, 1B = *Babesia canis*

Table 2 The comparison between techniques of the buffy coat smear and nested PCR

Buffy coat smear		Nested PCR	
Nested PCR		(+) morulae (+) initial bodies n = 13	(-) morulae (-) initial bodies n = 18
(+) <i>E. canis</i>		11* (84.62%) ($p<0.05$)	15* (83.33%) ($p<0.05$)
(-) <i>E. canis</i>		2* (15.38%) ($p<0.05$)	3* (16.67%) ($p<0.05$)
Percentage		13/39 = 33.33%	18/39 = 46.15%
			8/39 = 20.51%

*: no differences, $p<0.05$ **: significantly different.

Table 3 The relationship between thrombocytopenia and nested PCR

Platelet counts		Thrombocytopenia	
Nested PCR		(< 150,000/ μ l) n = 38	(< 100,000/ μ l) n = 32
(+) <i>E. canis</i>		25 (65.79%)	22 (68.75%)
(-) <i>E. canis</i>		13 (34.21%)	10 (31.25%)

Our findings had not only resulted in the preliminarily epidemiologic study of ehrlichiosis in Bangkok and Samutprakarn but also for the comparison of the detection methods. The characteristics of morulae and initial bodies of *Ehrlichia canis* had been clearly described and it could be used as a defined diagnosis (Wen, et al., 1997). In the routine microscopic examination of ehrlichiosis particularly in our laboratory, there was the dispute in the detection of morulae and/or initial bodies. Our data demonstrated the presence of more initial bodies in monocytes than that of morulae. On the other hand, our observation suggested the initial body detection of ehrlichiae is so sensitive that gives no different results when compared between tests of nested PCR and microscopy. The findings suggested that initial body detection has given another mode of CME diagnosis. In some cases, such as the early infection or in the acute phase, the findings of initial bodies could be helpful in the demarcation of ehrlichiosis or in place of morulae. We suggested that the findings of initial bodies in monocytes should serve as a good mode of detection as the findings of morulae. In particular, the technique of initial body finding is required in the case that morulae were not found or in the subclinical infection.

Our study showed that 88.37% (38/43) of dogs with *E. canis* infection also had thrombocytopenia (<150,000/ μ l). Thrombocytopenia was shown earlier to be associated with *E. canis* infections (Rodriguez-Vivas et al., 2005). In the acute phase, thrombocytopenia is associated with platelet migration inhibition factors and anti-platelet antibodies (APA) leading to the destruction of platelets in spleen and liver and the distribution of platelets to the site of vascular inflammation. In case of chronic ehrlichiosis, thrombocytopenia occurred when bone marrow function was suppressed by CME (Harrus et al., 1999). We found 65.79% (25/38) of thrombocytopenia (<150,000/ μ l) had *E. canis* infection. A similar report was conducted in Brazil at the similar rate of 63.1% (Bulla et al., 2004). We also found that 2 of 3 dogs who were having a normal range of platelet counts also had *E. canis* infection. Furthermore, in this observation, there were 6 cases encountered pancytopenia and 5 samples were tested positive for *E. canis*. The pancytopenic condition was shown to have a strong association in the bleeding disorder in dogs and in *E. canis* infection (Gieger 2005). In conclusion, our study has revealed the significant occurrence of *E. canis* in the city dogs where *R. sanguineus* is endemic. The comparisons between

methods of diagnosis using morulae/initial body findings and the nested PCR are considerable in the disease detection in our laboratory. These methods are suggested as a routine diagnosis of *E. canis*. Finally, our study showed the observation of tick-borne disease in dogs has not only suggested a mode of the ehrlichiosis control in housed dogs but also shown a serious need in controlling tick population in the city.

Acknowledgements

We would like to thank the senior project of the undergraduate program, the small animal teaching hospital, the Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand and the Central Lab, Inc. in providing sources of canine ehrlichiosis. We are grateful to the helps of Silamon Choosaksaengthong, Suparin Jirasukprasert and Siriluk Jenchangkool for the technical supports. We would like to thank all staffs from the units of Parasitology and Biochemistry, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

References

- Branger, S., Rolain, J.M. and Raoult, D. 2004. Evaluation of antibiotics susceptibility of *Ehrlichia canis*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* by Real-Time PCR. Am. Soc. Microbiol. 48(12): 4822-4828.
- Breitschwerdt, E.B., Hegarty, B.C. and Hancock, S.I. 1998. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii* or *Bartonella vinsonii*. J. Clin. Microbiol. 36(9), 2645-2651.
- Bulla, C., Takahira, R.K., Araujo, J.R., Trinca, L.A., Lopes, R.S. and Wiedmeyer, C.E. 2004. The relationship between the degree of thrombocytopenia and infection with *Ehrlichia canis* in an endemic area. Vet. Res. 35: 141-146.
- Dumler, J.S., Barbet, A.F., Bekket, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and HGE agent as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Sys. Evo. Microbiol. 51: 2145-2165.

- Gieger, T. 2005. Bleeding disorders: epitaxis and hemoptysis. Clinical Manifestations of Disease. In Textbook of Veterinary Internal Medicine. Eds. S. J. Ettinger and E. C. Feldman. Philadelphia, W. B. Saunders. pp 225.
- Harrus, S., Kenny, M., Miara, L., Aizenberg, I., Waner, T. and Shaw, S. 2004. Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental *Ehrlichia canis* infection. Am. Soc. Microbiol. 48(11): 4488-4490.
- Harrus, S., Waner, T., Aizenberg, I., Foley, J.E., Poland, A.M. and Bark, H. 1998. Amplification of Ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis*. J. Clin. Microbiol. 36(1): 73-76.
- Harrus, S., Waner, T., Bark, H., Jongejan, F. and Cornelissen, A.W.C.A. 1999. Minireview: recent advances in determining the pathogenesis of canine monocytic ehrlichiosis. J. Clin. Microbiol. 37(9): 2745-2749.
- Hilderbrandt, A., Schmidt, K., Fingerle, V., Wilske, B. and Straube, E. 2002. Prevalence of granulocytic Ehrlichiae in *Ixodes ricinus* ticks in middle Germany (Thuringia) detected by PCR and sequencing of a 16S ribosomal DNA fragment. FEMS Microbiol. Lett. 211: 225-230.
- Inokuma, H., Parola, P., Raoult, D. and Brouqui, P. 2001. Molecular survey of *Ehrlichia* infection in ticks from animals in Yamaguchi Prefecture, Japan. Vet. Parasitol. 99: 335-339.
- Iqbal, Z., Chaichanasiriwithaya, Y. and Rikihisa, Y. 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. J. Clin. Microbiol. 32(7): 1658-1662.
- Lee, S., Na, D., Kim, C., Li, Y., Cho, Y., Park, J., Lee, J., Eo, S., Klein, T.A. and Chae, J. 2005. Identification and prevalence of *Ehrlichia chaffeensis* infection in *Haemaphysalis longicornis* ticks from Korea by PCR, sequencing and phylogenetic analysis based on 16S rRNA genes. J. Vet. Sci. 6(2): 151-155.
- Martin, A.R., Brown, G.K., Dunstan, R.H. and Roberts, T.K. 2005. *Anaplasma platys*: an improved PCR for its detection in dogs. Exp. Parasitol. 109: 176-180.
- McQuiston, J.H., McCall, C.L. and Nicholson, W.L. 2003. Zoonosis update: Ehrlichiosis and related infections. J. Am. Vet. Med. Assoc. 223(12): 1750-1755.
- Murphy, G.L., Ewing, S.A., Whitworth, L.C., Fox, J.C. and Kocan, A.A. 1998. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet. Parasitol. 79: 325-339.
- Mylonakis, M.E., Koutinas, A.F., Billinis, C., Leontides, L.S., Kontos, V., Papadopoulos, O., Rallis, T. and Fytainou, A. 2003. Evaluation of cytology in the diagnosis of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): a comparison between five methods. Vet. Microbiol. 91: 197-204.
- Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., Raoult, D., Telford III, S.R. and Wongsrichanalai, C. 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and Other Eubacteria in Ticks from the Thai-Myanmar Border and Vietnam. J. Clin. Microbiol. 41(4): 1600-1608.
- Rodriguez-Vivas, R.I., Albornoz, R.E.F. and Bolio, G.M.E. 2005. *Ehrlichia canis* in dogs in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors. Vet. Parasitol. 127: 75-79.
- Rosner, B. 2006. Fundamentals of Biostatistics. 6th ed. Thompson Co. USA.
- Skotarczak, B. 2003. Canine Ehrlichiosis. Ann. Agri. Env. Med. 10: 137-141.
- Suksawat, J., Pitulie, C., Arraga-Alvarado, C., Madrigal, K., Hancock, S.I. and Breitschwerdt, E.B. 2001. Coinfection with three Ehrlichia species in dogs from Thailand and Venezuela with emphasis on consideration of 16S ribosomal DNA secondary structure. J. Clin. Microbiol. 39(1): 90-93.
- Taillardat-Bisch, A.V., Raoult, D. and Drancourt, M. 2003. RNA polymerase beta-subunit-based phylogeny of *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp., and *Wolbachia pipientis*. Int. J. Sys. Evol. Microbiol. 53: 455-458.
- Uilenburg, G., Thaiaucourt, F. and Jongejan, F. 2004. On molecular taxonomy: what is in a name? Exp. Appl. Acarol. 32: 302-312.
- Wen, B., Rikihisa, Y., Mott, J.M., Greene, R., Kim, H.Y., Zhi, N., Couto, G.C., Unver, A. and Bartsch, R. 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J. Clin. Microbiol. 35(7): 1852-1855.