การสำรวจปรสิตในเลือดสุนัขที่มีพาหะนำโรคในเขตพื้นที่ราบของประเทศกัมพูชา

นางสาวกิมเฮียง งวน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพยาธิชีววิทยาทางสัตวแพทย์ ภาควิชาพยาธิวิทยา
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

A SURVEY OF CANINE VECTOR-BORNE BLOOD PARASITES IN THE PLAIN REGIONS OF CAMBODIA

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2014

Thesis Title	A SURVEY OF CANINE VECTOR-BORNE BLOOD PARASITES IN THE PLAIN REGIONS OF CAMBODIA
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กิมเฮียง งวน : การสำรวจปรสิตในเลือดสุนัขที่มีพาหะนำโรคในเขตพื้นที่ราบของประเทศกัมพูชา (A SURVEY OF CANINE VECTOR-BORNE BLOOD PARASITES IN THE PLAIN REGIONS OF CAMBODIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ปิยนันท์ ทวีถาวรสวัสดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: สุดจิตต์ จุ่งพิวัฒน์, 109 หน้า.

โรคปรสิตในเลือดของสุนัขเป็นโรคที่มีความสำคัญโรคหนึ่งโดยมีแมลงเป็นพาหะนำโรค จุดประสงค์การศึกษาครั้งนี้เพื่อสำรวจการติดปรสิตในเลือดของสุนัขที่มีแมลงและเห็บเป็นพาหะนำโรคในเขตพื้นที่ราบต่ำ ประเทศกัมพูชา โดยตรวจวินิจฉัยด้วยวิธีที่นิยมใช้โดยทั่วไป และตรวจวินิจฉัยยืนยันด้วยวิธีทางอณูชีวโมเลกุล ทำการเก็บตัวอย่างเลือดสุนัขระหว่าง เดือนพฤศจิกายน พ.ศ.2557 ถึง เดือนกุมภาพันธ์ พ.ศ. 2558 จำนวนทั้งสิ้น 444 ตัวอย่างในเขตอำเภอตะแกว กัมปุงสะปือ กัมปุงชะหนัง เปรเหวง สวายเรียง และคลินิกสัตวแพทยท์ในกรุงพนมเปญ ผลการสำรวจพบสุนัขติดปรสิตในเลือดจำนวน 5 ตัวอย่างคิดเป็น 1.13% ได้แก่ *Dirofilaria immitis* 0.45%, *Brugia pahangi* 0.22% ในจังหวัด เปรเหวง และกำปงชะหนัง ตามลำดับ ซึ่งตรวจโดยวิธีการทำแผ่นเลือดบางจากชั้นบัฟฟี่ โค้ด ย้อมด้วยสี 10% ยิมซ่า และตรวจวินิจฉัยยืนยันด้วยการย้อมเอมไซม์ แอซิด ฟอสฟาเตส *Babesia canis* 0.22% จากคลินิกสัตว์ในกรุงพนมเปญ และการติดเชื้อร่วมระหว่าง *Ehrlichia canis* และ *Anaplasma platys* 0.22%.จากสุนัขในจังหวัดตะแกว โดยการตรวจโดยวิธีวิธีการทำแผ่นเลือดบางจากชั้นบัฟฟี่ โค้ด ย้อมสี และตรวจวินิจฉัยยืนยันด้วยวิธีทางอณูชีวโมเลกุล นอกจากนี้ยังตรวจพบพาหะนำโรค ได้แก่ หมัดสุนัข (*Ctenocephalides canis*) เห็บสุนัข (*Rhipicephalus sanguineus*) และ เหาสุนัข (*Hetrodoxus spiniger*) คิดเป็น 62.8%, 18.5% และ 12.2% ตามลำดับ โดยจะพบหมัดสุนัขมากกว่าเห็บสุนัข และเหาสุนัข จากผลศึกษาในครั้งนี้จะเห็นว่าสุนัขติดเชื้อปรสิตในเลือดค่อนข้างน้อย ถ้าหากมีการสำรวจการติดโรคปรสิตในเลือดสุนัขด้วยการตรวจวินิจฉัยด้วยวิธีอื่นที่มีความไว และจำเพาะมากกว่า เช่นวิธีทางอณูชีวโมเลกุล อาจพบอัตราการติดโรคเปลี่ยนแปลงไปก็ได้ อย่างไรก็ตามการศึกษาในครั้งนี้เป็นการศึกษาครั้งแรกเกี่ยวกับโรคปรสิตในเลือดที่มีพาหะนำโรคในประเทศกัมพูชาซึ่งผลการศึกษาที่ได้สามารถนำไปใช้ในการพัฒนาวิธีการตรวจวินิจฉัย และเสริมสร้างองค์ความรู้ ให้แก่ผู้เลี้ยงสัตว์ สัตวแพทย์ ให้มีความตระหนักถึงโรคปรสิตในเลือดของสุนัขในประเทศกัมพูชา

ภาควิชา	พยาธิวิทยา	ลายมือชื่อนิสิต	.....
สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2557	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

5675332731 : MAJOR VETERINARY PATHOBIOLOGY
KEYWORDS: CANINE BLOOD PARASITES, CAMBODIA

KOEMSEANG NHUONG: A SURVEY OF CANINE VECTOR-BORNE BLOOD PARASITES IN THE PLAIN REGIONS OF CAMBODIA. ADVISOR: ASST. PROF. DR. PIYANAN TAWEETHAVONSAWAT, CO-ADVISOR: MRS. SUDCHIT CHUNGPIVAT, 109 pp.

Canine vector-borne blood parasites (CVBBPs) are considered as blood parasitic infection in dogs which is transmitted by vectors. The objective of this study is to survey blood parasites of dogs in the plain region Cambodia by using conventional and molecular methods to confirm intracellular parasites from the suspected samples. The study was carried out from November 2014 to February 2015. The total of 444 dogs was found only 5 infected dogs (1.13%) with a low parasitemia and low parasitic infection including *Dirofilaria immitis* (0.45%), *Brugia pahangi* (0.22%) were found in Prey Veng and Kompong Chhnang province by Buffy coat stained blood smear with giemsa staining and acid phosphatage activity confirmation, and followed by *B. canis* (0.22%) was collected from a private clinic, Phnom Penh, and co-infection of *Ehrlichia canis* with *Anaplasma platys* (0.22%) was indicated in Takeo by Buffy coat stained blood smear staining and PCR confirmation. Moreover, three vectors, *Ctenocephalides canis, Rhipicephalus sanguineus* and *Heterodoxus spiniger*, affecting dogs were also found in 62.8%, 18.5% and 12.2%, respectively. *C. canis* was the most common presence than tick and lice in our areas. Therefore, this result may indicate that there is low parasitemia or low of tick transmission. Certainly, the prevalence of parasitic case infections may change if other diagnostic methods with a higher sensitivity, specificity and accuracy, e.g. molecular methods. However, it is the first investigation of CVBBPs. It is expected to develop a better understanding in diagnostic techniques and improving knowledge the pet owners, veterinarians to beware of accretion regarding CVBBPs infection in Cambodia.

Department:	Veterinary Pathology	Student's Signature .....
Field of Study:	Veterinary Pathobiology	Advisor's Signature .....
Academic Year:	2014	Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to deeply thank to the financial support from CU Graduate Scholarship Program for 26 months providing and sincerely thank the research grant that was supported by the 90th Anniversary CU, Rachadapisek Sompote Fund in Thailand.

Second, I would like to express my special thanks to my principle advisor Asst. Prof. Dr. Piyanan Taweethavonsawat and co-advisor Mrs. Sudchit Chungpivat from the bottle of my heart for their highly patience, strongly support, special guide, advice, encouragement, assistance, and kindness to me over two years. I gratefully thank Dean of the Faculty of Veterinary Science, Prof. Dr. Roongroje Thanawongnuwech, and all committee members: Assoc. Prof. Dr. Theerayut Kaewamatawong is a chairman of thesis final examination and Prof. Dr. Pewpan Maleewong from Department of Parasitology, Faculty of Medicine, Khonkaen University, is an external committee for recommendation and advice.

Third, I would like to thank with my great appreciation to instructors namely: Asst. Prof. Dr. Sonthaya Tiawsirisup, Dr. Woraporn Sukhumavasi, Dr. Morakot Kaewthamasorn, Assoc. Prof. Dr. Supol Luengyosluechakul, Dr. Chatree Khatiworavage and other instructors and thank to staffs of Vet Central Lab and staffs in parasitology unit: Miss Sirasa Sukpisan, Mr. Nutpiphat Khrutkham, Mrs. Thurien Keawthong and friends: Mr. Samuth Sum, Miss Nam, and Miss Ruangurai Kitchodok for their friendship, helpful, kindness within my awkwardness.

Fourth, I am very thanks to Prof. Dr. Meas Sothy for allowance me using his laboratory equipment and my juniors: Mr Sela Ros, Mr. Vet Chren, Mr. Minh Vin, Mr. Samorn Sok, authorities, heads of villagers, veterinary surveillances, dog owners and clinician for their collaboration, and allowance.

Finally, I deeply thank to my beloved families and relatives, who highly support, inspire and encourage me. Again, I would like to gratefully thank you all from the bottle of my heart, without all of you I wouldn't present in this day.

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LIST OF ABBREVIATIONS

CVBBPs	Canine vector-borne blood parasites
CVBDs	Canine vector-borne diseases
APA	Acid phosphatase activity
BCTBS	Buffy coat thin blood smear
PCR	Polymerase chain reaction
MKT	Modify knott'test
PCV	Packed cell volume
ELISA	Enzyme-linked immunosorbent assay
CD4	Cluster difference 4
IFN-γ	Interferon γ
NK	Natural killer
T cell	Thymus-derived cell
RBCs	Red blood cells
WBCs	White blood cells
IgG	Immunoglobulin G
B. canis	*Babesia canis*
A. platys	*Anaplasma platys*
E. canis	*Ehrlichia canis*
D. immitis	*Dirofilaria immitis*
B. pahangi	*Brugia pahangi*
H. canis	*Hapatozoon canis*
R. sanguineus	*Rhipicephalus sanguineus*
H. spiniger	*Heterodoxus spiniger*
C. canis	*Ctenocephalides canis*
EDTA	Ethylenediaminetetraacetic acid

DNA	Deoxyribonucleic acid
NF	Not found
Ecto-p	Ectoparasites
m	Month
y	Year
MB	Mixed Breed
SD	Stay dog
M	Male
F	Female
GC	Good care
FL	Free living
T	Tick
F	Flea
L	Lice

CHAPTER I

INTRODUCTION

Importance and Rational

Canine vector-borne blood parasites (CVBBPs) are associated with a group of infectious disease which is transmitted by blood sucking arthropod vectors or blood-feeding ectoparasites including ticks, fleas, lice, mosquitoes and tabanids. In addition, CVBBPs are caused by various of pathogens such as protozoa, filariae, viruses and parasitic bacteria (Dantas-Torres, 2008; Otranto et al., 2009b). Dogs are a pet animal which live in close contact to cat as well as humans and eventually consider as reservoirs hosts of pathogens of zoonotic concern and also can serve as a source of blood-feeding of arthropods (Dantas-Torres, 2007; Menn et al., 2010). Therefore, this contact between dog and human can enable to spread the diseases and can increase the risk of pathogens transmissions (Otranto et al., 2009b). In addition, since there are increasing of the movements of people, environmental changes, increasing pet animal travel, goods or material for animal using, canine vector-borne diseases have become a global issue and pose public interesting (Baneth et al., 2012). Therefore, the CVBBDs may play a potential role for emerging and re-emerging disease with establishment of novel vector species and pathogens (Shaw and Day, 2005; Otranto et al., 2009a). For example, in current years canine vector-borne diseases have become increasingly important in Germany, Mediterranean and South-East European countries due to the extent of companion animal movements over last two decades that caused many dogs were infected with parasites and death (Hamel et al., 2011).

Recently, researchers have suggested that the several vector-borne diseases in domestic dogs are commonly widespread via many regions in worldwide especially a tropical and subtropical regions. For instance, each region has its own risks of infection.

The diseases can causes by different pathogens including *Babesia, Hepatozoon, Ehrlichia, Anaplasma, Mycoplasma, Dirofilaria* and *Trypanosoma*. Particularly, some of which can infect both animals and humans when an infectious disease is delivered between varied species. Importantly, the pathogens can pose severe life threatening of dogs and cause zoonotic disease such as *Brugia pahangi* (Palmieri et al., 1985; Tan et al., 2011; Muslim et al., 2013b), *Ehrlichia canis* (Perez et al., 1996; Perez et al., 2006), *Anaplasma phagocytophilum*, *Leishmania infantum*, *Dirofilaria immitis*, and *Dirofilaria repens*, *Bartonella* spp., and *Rickettsia* spp (Dantas-Torres, 2007; Otranto et al., 2009b; Otranto et al., 2010b). However, the transmission of multiple pathogens or co-infection by one vector has a high attention of the difficulties in clinical diagnosis and the complex interaction of different infectious agents throughout the world. Pathogens are characteristic differences in genotype, vector distribution, geographic distribution, pathogenicity, and vector specificity. Through morbidity and mortality of dogs continue occurring because of various factors such as less of animal care, diagnostic tool, and a broader distribution (travel with their pets, and commercial trade of pet dogs), the CVBBPs should be concerned or controlled by reducing a minimum of ectoparasides with acaricides or/and insecticides for an inactive spreading of the enzootic transmission cycles of these pathogens and vectors (Bowman et al., 2009). Molecular assays, serological test and cytological test are the most frequently used for CVBBPs diagnosis (Otranto et al., 2010a). By contrast, the understanding of CVBBPs in Cambodia is seemed to be limit due to lack of information, reporting and surveys of these pathogens.

Cambodia is a one of Southeast Asia, surrounded by uplands and low mountains including the Tonle Sap Lake and the upper reaches of the Mekong River delta. The climate is controlled by tropical monsoons and has two different seasons; rainy season from May to October and dry season from November to April (http://en.wikipedia.org/wiki/Geography_of_Cambodia#Climate). Temperature is range

from 21 to 35 ℃ (69.8 to 95.0 °F) and fairly similar throughout the Tonlé Sap Basin that the average annual means of around 25 ℃ (77.0 °F); with this, the maximum mean is about 28.0 ℃ (82.4 °F) and the minimum mean is about 22.98 ℃ (73.36 °F). The climate conditions in this country seem to be suitable conditions for vectors and pathogen infections. This country is bordered by Thailand to the north and west, Laos to the northeast, Vietnam to the east, and Gulf of Thailand to southeast. Additionally, according to the report has been published by the Ministry of Environment, Cambodia is naturally divided into four classification regions including the plain region, Tonle Sap lake region, Coastal region, and Plateau and Mountainous region (http://geodata.rrcap.unep.org/all_reports/cambodia_081010.pdf). The plain regions or the lower part region is located to the east and southeast of the country nearby the border of Vietnam. There are several provinces including Phnom Penh (a main capital city), Kandal, Kompong Chhnang, Kompong Cham, Kompong Speu, Svay Rieng, Prey Veng, and Takeo province. The population is about 6 million with the density 235/km^2. The populations of these regions have combined with many nationalities and have also reported that the populations are higher than other areas. Nowadays, Cambodia, the observation of pet animals (dogs) seems to be increasing from year to year due to the dogs migrate from other countries, thus, the capacity of pathogens or vectors may bring together with the dog that is the main concern in the future. Therefore, the importance of public health in both animals and humans are needed to be considered, for instance the prevalence, epidemiology, diagnosis, prevention, management, treatment and all relevant problem studies on CVBBPs. The CVBBPs are seemed to be limited and poor understanding to students, veterinarians, researchers and public knowledge gaps in Cambodia. With these knowledge gaps, the plain areas are suitable areas for this primary study and investigation with a topic on “A survey of Canine vector-borne blood parasites in the plain regions of Cambodia”. Furthermore, the expectation of this study will be useful for better understanding in term of CVBBPs.

Objective of study

To survey blood parasites of dog in the plain regions of Cambodia.

Hypothesis

Dogs can be infected with various types of CVBBPs in the plain regions of Cambodia.

CHAPTER II
LITERATURE REVIEW

1. Babesiosis

Babesiosis or piroplasmosis is a tick-borne disease of animals which caused by intraerythrocytic protozoa of the genera *Babesia* and *Theileria* (Order: Piroplasmida) (Irwin, 2009; Vannier and Krause, 2009). Babesiosis is a disease that reported in canine domestic and wild animals worldwide which mainly caused by anemia in case of a high prevalence of parasitic infection. The pathogen was found by the Romanian scientist Victor Babes, who was investigated febrile hemoglobinuria in cattle at the end of the 19th century (Babes, 1888) and was followed by first description in dog in Italy. *Babesia* in canine is divided into two forms (small and large form) by morphology of intraerythrocytic form (intraerythrocytic merozoite stage measures in the diameter of a red blood cell). Small forms tend to be 1 to 3 μm in length such as *Babesia* (*B*) *gibsoni* and *Babesia annae*; whereas, large forms tend to be 3 to 7 μm in length and it was categorized into three subspecies such as *Babesai canis rossi* (*B. rossi*)*, Babesia canis canis* (*B. canis*)*,* and *B canis vogeli* (*B. vogeli*). These three subspecies are differentiated by the basis of cross immunity, serological testing, vector specificity, molecular phylogeny and geographic distribution (Uilenberg et al., 1989; Birkenheuer, 2012). Another fourth unknown of large *Babesia sp*. has been defined recently in amount of dogs with clinical signs and hematological parameter consistent with babesiosis in North Carolina, USA (Birkenheuer et al., 2004; Lehtinen et al., 2008). In most parts of the world, the variety of ticks are the most important way of pathogenic transmission including *Rhipicephalus sanguineus* (*R. sanguineus*)*, Haemaphysalis* spp.*, and Dermacentor* spp. (Shaw et al., 2001) that known as 'vector'. In general *Babesia* spp. completes its lifecycle in an invertebrate host (ixodid ticks) and a vertebrate host.

With the transmission, *babesia* can get into the host via the saliva gland of the tick (transovarial transmission) during the blood feeding. However, some researchers have published that *B. gibsoni* can transmit via transfusion (Stegeman et al., 2003), transplacental transmission (Fukumoto et al., 2005), and direct transmission (Jefferies et al., 2007), for instance American Pit Bull Terrier-type dogs were reported with *B. gibsoni* infection due to biting and fighting between infected and non-infected dogs (Birkenheuer et al., 2005).

Canine babesiosis is caused by *B. canis* and *B. gibsoni* that shows a hemolytic anemia and thrombocytopenia disease. *B. gibsoni* infection seems to be more pathogenic and hardly treatment than *B. canis* infection as a reason of its virulence (Boozer and Macintire, 2003; Sakuma et al., 2012). The severe clinical signs of canine babesiosis are depended on the *babesia* species; for instead, this disease has been reported in France with a high prevalence of *B. canis* which is the main agents; whereas, *B. vogeli* has been reported in Europe and nearby the Mediterranean Sea (Rene et al., 2012). In addition, the acute phase of *B. canis* infection is showed by a mild to severe disease, in which parasitemia is often low and not necessarily correlated with the severity of clinical illness (Uilenberg et al., 1989). The main acute clinical signs are dehydration, lethargy, anorexia and fever. At initial clinical examination, the majority of dogs present with mild to severe thrombocytopenia, mild to moderate anemia, hemolysis and neutropenia (Solano-Gallego et al., 2008). Although, the acute of phase can show fever, anemia, hemoglobinuria, jaundice, thrombocytopenia, lethargy and anorexia; whereas, the chronic of phase is asymptomatic (Birkenheuer, 2012; Schnittger et al., 2012). Infrequently, owners can recognize some clinical sings such as jaundice, pale mucous membranes or discoloration of the urine caused by bilirubinuria or hemoglobinuria (Birkenheuer, 2012).

1. 1 Life cycle

The life cycle occur when infected ticks take blood meal of vertebrate host then sporozoites are released from the saliva grand of the tick and go through the bloodstream then invade into erythrocytes. Sporozoites are developed into first merozoites and trophozoites form. Within the erythrocytes, the organisms develop themselves to many forms such as amoeboid form, binary fission, pyriform, and cruciform (asexual reproduction or asexual form of schizogony) and finally infected morozoite are ruptured from the erythrocyte cells. After that some morozoite are infected other new erythrocyte cells and become a second-infected merozoits and the other merozoites are transformed into gametocytes. Even though, the transformation form from merozoite to gamete is unclear whether it start in the host or in the tick while some researchers have reported that trophozoites develop into gametocytes which can initiate infection in the tick vector. In the sexual phase of tick midgut, the organisms have developed into macro- and micro-gamonts in the erythrocytes and then rupture and fuse to form a zygote. The zygotes migrated to various organs of the tick including the epithelial cell and then to the ovary and the final in the saliva gland tissue. In the saliva gland tissue, the organisms form to sporoblast and develop new sporozoites in the saliva gland where they take part in transstadial and transovarial transmission. Finally, when the infective ticks bite the host then it becomes a life cycle (Birkenheuer, 2012). The incubation period of canine babesiosis changes from 10– 21 days for *B. canis* and 14–28 days for *B. gibsoni*. The female ticks feed on their host for about one week only and have left the host by the time disease develops (Schoeman, 2009).

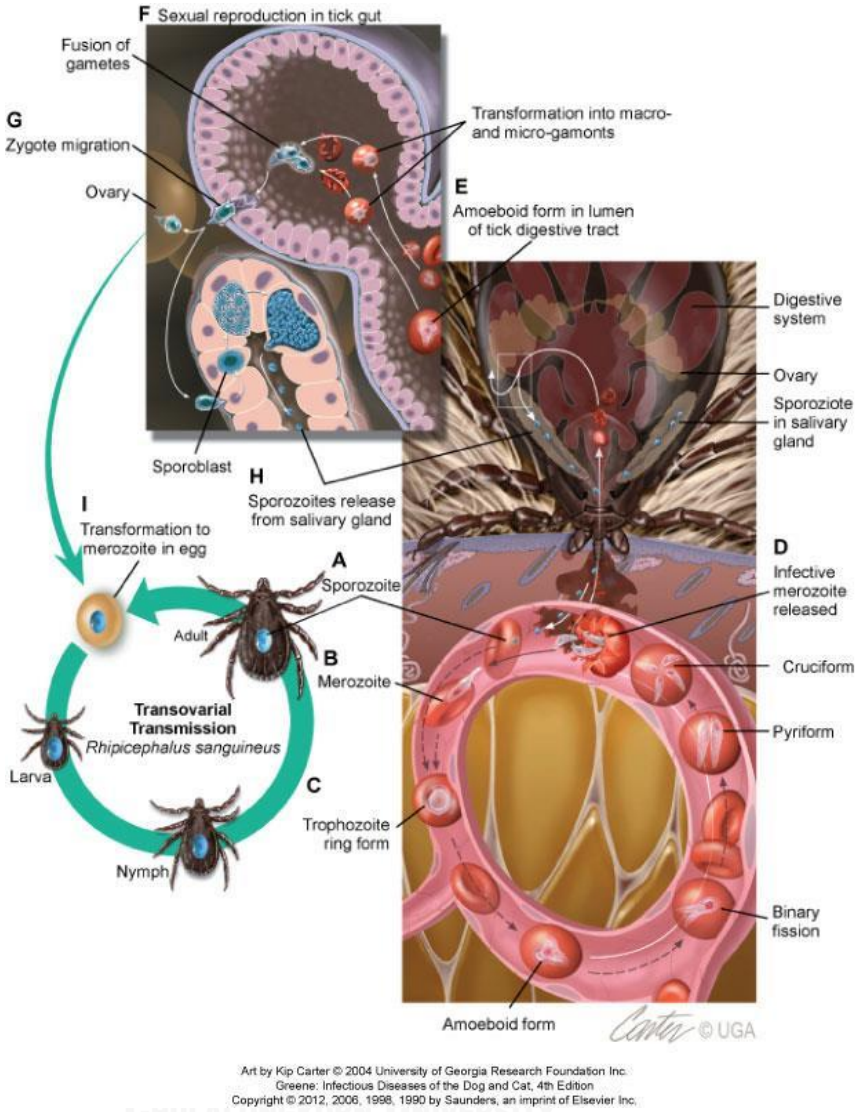

Figure 1: The life cycle of *Babesia* in the invertebrate host (ixodid ticks) and the vertebrate host. Graig EG, 2011, Infectious Disease of the Dog and Cat, 4th ed. p 774. (Art by Kip Carter © 2004 University of Georgia Research Foundation Inc.)

1. 2 Pathogenesis

The pathogenicity of *Babesia* spp. is discovered by species and strain involved (Uilenberg et al., 1989; Schetters et al., 1995). The importance of the host factors are age and the immunologic response producing to eliminate the organism or vector (Vannier et al., 2004). In hosts, infected erythrocytes induce organism antigens to their surface and the host induces antibody to remove the infected erythrocytes by

mononuclear-phagocyte system. Moreover, parasite antigen may stick to the surface of some non-infected red blood cell and platelets and destroy them as a result of hemolytic anemia and thrombocytopenia (Birkenheuer, 2012). However, both humoral immunity (antibody-mediated immune system) and cellular factor are complicated in immunity of babesiosis. Humoral responses are the kind of the adaptive immune system which presently considered as an importance protecting against babesia infections. The first stage of the infection with babesia, immune serums are displayed that antibodies in the serum stop effect babesial sporozoites or merozoites while they are freeing in plasma in the bloodstream or extracellular stage (Abdalla et al., 1978; Reduker et al., 1989; Winger et al., 1989). In this stage, immunoglobulin G (IgG) antibodies play a main role in averting or preventing by binding sporozoites before they invade into the red blood cells. Certainly, the effect of antibodies in the extracellular parasites is better than in the intraerythocytes (Winger et al., 1989). As a result of the quickly invade cell, the protective action of antibodies seems to be limited between the time that the parasite gets into the bloodstream and the time that it invade inside cells. Other stage begins when the babesia organisms start invading the red blood cells, and then the merozoites start increasing and destroying the infected red blood cell. After destroyed cells, those organisms free living in the bloodstream again and invade other new cells for several rounds of cycle. This stage, the parasitemia level becomes large in amount and acute disease can exist such as anemia, hemogobinuria, jaundice, lethargy and anorexia. Cells of the innate immune system are a duty for controlling the growth level of the organisms and developing of parasitemia. Particularly, macrophages and natural killer cells are the anti-babesia activity. The stop actions of organisms appear to be depending on the production of soluble factors such as interferon by natural killer cells and tumor necrosis factor alpha, nitric oxide, and reactive oxygen species by macrophages. Although, there are unclear about these molecules can be against with the rise of the parasite inner the

erythrocyte. The other stage, the high level of parasitemia inside the erythrocytes begin lower and lower around 10 days after infection due to cell-mediated responses which consisted in the spleen are removed (Zwart and Brocklesby, 1979). The spleen is a big lymphoid organ which contained such as NK cells T cells, B cells and macrophages. In this spleen, intraerythrocytic killing stage, T-cells are directly responded for producing the subtype of $CD4^+$ IFN-γ to protect against the intraerythrocytic parasites (Igarashi et al., 1994).

2. Hepatozoonosis

Hepatozoon spp is a protozoon parasite in the white blood cell of mammal including in dogs which caused by apicomplexan parasite, which transmitted by tick (Baneth et al., 2003) and have up to 300 *Hepatozoon* spp from different groups and 46 of *Hepatozoon* spp have found in reptile, anurans, and birds (Smith, 1996). Two species of canine hepatozoon such as *H. canis* and *H. americanum* are caused distinguished clinical sigh (Baneth et al., 2003). The main vector of *H. canis* is the brown dog tick, *R. sanguineus,* although the other tick species are also able to transmit such as *Amblyoma ovale* were found in Brazil (Rubini et al., 2009) and the suspected tick of *Haemaphysalis* spp. in Japan (Murata et al., 1995); whereas the vector of *H. americanum* is transmitted by *Amblyoma maculatum* ticks in North America (Little et al., 2009). In addition, hosts can infected with *H. americanum* via eating the cystozoites in muscle of other infected animal such as wild rodents, coyotes, squirrels, rabbits or vertebrates canids have been determined as the capable of natural hosts (Ewing and Panciera, 2003). *H. canis* infection was first documented from India in 1905 cited by (Otranto et al., 2009b). The prevalence of *H. canis* are widespread in the worldwide such as Italy (Gavazza et al., 2003), in Southern Europe, Middle East, South America (Vojta et al., 2009), in Africa and in Asia including Thailand (Jittapalapong et al., 2006),

Singapore, the Philippines, Japan (Inokuma et al., 2002) and Malaysia were cited by (Baneth, 2012). A comparative study of *H. canis* by using blood smear detection were positive 10.6% , 25,8% by PCR and 36.8% by indirect fluorescent antibody testing in Turkey (Karagenc et al., 2006). Moreover, the survey by using blood smear evaluation in Brazil were showed by 39% (O'Dwyer et al., 2001), 22% of dog surveyed in Nigeria (Ezeokoli et al., 1983) and 1.2 % in Malaysia (Rajamanickam et al., 1985) were cited by (Baneth, 2012). In addition, the studies on the seroprevalences of *H. canis* infection has showed a difference of percentages such as 33% in Israel (Baneth et al., 1996), 4.2% in Japan (Inokuma et al., 1999) and 2.6% in Thailand by microscopy (Jittapalapong et al., 2006). In addition, the highly data of canine hepatozoonosis is depended on the presence of *R. sanguineus* tick infection which is the main role of epidemiological cycle (Hornok et al., 2013). The clinical signs in dogs that infected with *H. americanum* can cause a severe disease such as lethargy, myalgia, lameness and mucopurulent ocular discharge; whereas *H. canis* is an asymptomatic with a normal or develop mild disease to sever clinical disease such as fever, lethargy and emaciation. The organism can pass to many tissues such as spleen, liver, bone marrow, and lymph nodes (Baneth et al., 2003). In the blood smears, the present of gamonts stage in neutrophil are seldom differentiated (Ewing and Panciera, 2003). The gamonts observation by microscopy in stained blood smears seem to be less sensitive than detection of *Hepatozoon* DNA by polymerase chain reaction (PCR) in blood (Karagenc et al., 2006). In the infected blood can find the co-infection with *H. canis* such as *Ehrlichia, Mycoplasma, Anaplasma* and *Leishmania* (Mylonakis et al., 2004).

2. 1 Life cycle

Life cycle of *H. canis* in domestic dogs, within the dogs ingest the infected ticks, *R. sanguineus* that contain mature oocysts. Oocysts are quickly ruptured to release sporocysts. The sporozoites are released from the sporocyst and penetrate to the gut

wall of the dog's intestine and then pass via the blood circulation to the hemolymphatic target tissues. Within this target tissue, merogony or schizonts develop and produce a small number of mature meronts containing macromerozoites and micromerozoits which can be found in leucocytes. The macromerozoites can make free from the meronts to invade new cells to form secondary meronts, whereas micromerozoites invade other cells to form gamonts that surrounded by a capsule in a neutrophil of the blood (Baneth, 2012). Also, these gamonts show asexual phase and no change until ingested by the tick (Baneth et al., 2007). During taking a blood meal, gamonts in the host are transported by the tick and passed through the tick's gut then leaved from leucocyte of the tick by differentiation into macro and microgametes. Sexual stages, macro and microgametes fertilize to produce a zygote. Zygote penetrates the gut epithelium and enters the haemocoel of the tick where it grows to become large oocyst which envelops numerous sporocysts. Oocyst measured about 260 × 300 mm and has about 10 to 26 infective sporozoites (Mathew et al., 1999). The life cycle will be completed when the infected tick is ingested by the dog. According to an experimental transmission, the life cycle can be completed during 81 days including both tick and host (Baneth et al., 2007).

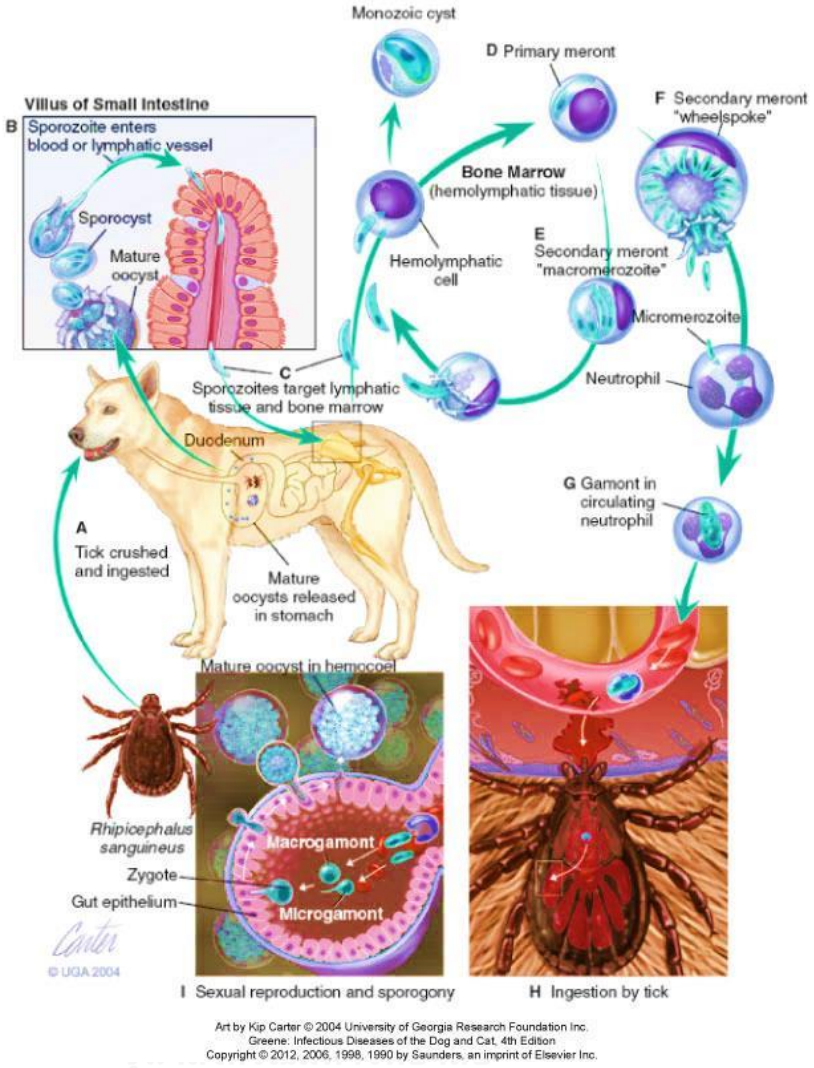

Figure 2: Stages in life cycle of *Hepatozoon canis* in the invertebrate host (ixodid ticks) and the vertebrate host. Graig EG, 2011, Infectious Disease of the Dog and Cat, 4th ed. p 753. (Art by Kip Carter © 2004 University of Georgia Research Foundation Inc.).

2. 2 Pathogenesis

The pathogenesis of *H. canis* infection is affected by immunodeficient conditions and an immature immune system in animals. These conditions weaken the immune responses increase and easily cause the infection reactivate. Once the host ingests the infected tick, *H. canis* sporozoites are released and penetrated to the gut wall of the host. The sporozoites invade mononuclear cell and spread via the lymph to hemolymphatic target organs such as bone marrow, spleen, lymph nodes and other

organs including the liver, kidney, and lungs. The clinical sign of dogs are often normal or can develop only mild disease. By contrast, in cases of co-infection with other organisms or with high parasitemia, dogs with *H. canis* may start mild to severe clinical disease characterized by fever, lethargy, loss of weight, and loss of appetite, hepatitis (Baneth, 2012). In addition, glomerulonephritis, and pneumonitis can develop (Baneth, 2012). Infection with *H. canis* produces a different humoral immune response within the current stages of the disease, while cellular response to *H. canis* infection has no information (Baneth et al., 2001). Although, because of *H. canis* is an intracellular organism, cell-mediated immunity probably plays a main role in the immune mechanism increased by the host against *H. canis* infection (Baneth, 2012).

3. Ehrlichiosis

Canine monocytotropic ehrlichiosis is caused by intracellular organism of the genus *Ehrlichia* (family Anaplasmataceae). According to literature of pathogen, dogs can be infected with several *Ehrlichia* species, including *E. canis* (Donatein, 1935), *E. chaffeensis* (Dawson et al., 1996), *E. equi* (Madewell and Gribble, 1982), *E. risticii* (Kakoma et al., 1994), *E. platys* (Harvey et al., 1978a), and *E. ewingii* (Ewing et al., 1971) were cited by (Suksawat et al., 2001a). However, *E. canis* is the most common presence in many countries. *E. canis* is gram-negative bacterium appears in monocytes and macrophages (Harrus et al., 2012). In group of organism is called morulae form. Vertebrate hosts include many different canine species, the domestic dog is considered the definitive host of this bacteria. Importantly, according to the first report in Lara, Venezuela, *E. canis* have been given that the human infection with *E. canis* was infected asymptomatic human (Perez et al., 1996). Lately, in 2006 it was designated again in human patients with clinical signs of human monocytic ehrlichiosis (HME) and 30 % (6/20) patients were positive for *E. canis* 16s rRNA on gene-specific PCR (Perez et al., 2006). The vector of *E. canis* is transmitted by the brown tick, *R. sanguineus* and

also *Dermacentor variabilis* via experiment transmission in California (Johnson et al., 1998; Harrus et al., 2012). This organism was firstly report in Algeria in 1935 (de Morais et al., 2003). *E. canis* is established as a result of morbidity and mortality in dog in many regions in worldwide including Africa, Europe, the Americas, and Asia. For instance, the detection of *E. canis* antibodies were reported 19.8% from Brazil (Labarthe et al., 2003). In Malaysia, by using molecular detection techniques were confirmed of *E. canis* with a prevalence rate of 2.0% in naturally infected dogs (Nazari et al., 2013). Also, in Thailand were showered by immunofluorescence antibody (IFA) testing was most prevalent to *E chaffeensis* 74% and *E canis* 71% antigens, followed by *E equi* 58% (Suksawat et al., 2001b) and by nested PCR and microscopy was found 65,12% (28/43) of *E. canis* infection in dogs (Ariyawatthiphan et al, 2008). *E. canis* infections can be acute, subclinical, or chronic in dogs. Common clinical signs include depression, lethargy, anorexia, weight loss, and hemorrhagic tendencies. Because the organism is transmitted by *R. sanguineus*, the disease may be involved by co-infection with other pathogens such as *Babesia* and *Hepatozoon* which transmitted by the same vector (Ramos et al., 2010). The morular detection of *E. canis* can use a buffy coat smears technique of the blood (Fehr et al., 2010).

3. 1 Life cycle

The brown dog tick, *R. sanguineus* is considered as the primary vector of *E. canis* to transport the pathogen to hosts during taking blood meals, while dogs are considered as reservoir hosts for this pathogen. During the tick takes blood meals from the infected host, the *E. canis* are carried into the midgut and salivary gland of the tick. After infection, the *E. canis* is stored till the next 2 life stages of tick in both the nymph and adult stage in transstadial transmission. The life cycle is completed involving both in tick and in host. Although, the organisms can infect a new host through saliva gland during blood feeding (Bowman et al., 2009).

3. 2 Pathogenesis

During the *E. canis* entry into monocyte and macrophage of the host, *E. canis* multiplies itself by binary fission in the mononuclear phagocyte system and spread via the lymphatic system such as the spleen and liver. These reasons can cause a sign of an abnormal increasing in white blood cell and number, known as hyperplasia. Because of *E. canis* cell are lacks enzymes for the biosynthesis of peptidoglycan and lipopolysaccharide (LPS) that supply strength to the outer membrane (Lin and Rikihisa, 2003). The host's immune system is able to respond to the presence of LPS and therefore the absence of LPS can provide the organism avoid and survive from antibodies of the host (Mavromatis et al., 2006). Also, the macrophages or neutrophils use Toll-like receptors to bind molecules with protected pathogen-associated molecular patterns such as LPS or peptidoglycan (Lin and Rikihisa, 2003; Harrus et al., 2012). As a result of replication of *E. canis* can be spread to infect new cells of the host cell membrane with the stage morulae form that eventually develop to anemia and an increase in platelet count (Thomas et al., 2010). In addition, the infected monocytes bind to vascular endothelial cells and initiate a vasculitis and subendothelial cell infection with the acute stage of disease. Furthermore, the thrombocytopenia is due to the increased consumption of platelets, sequestration of platelets in the spleen, immune-mediated destruction of platelets, decreased bone marrow production of platelets, or some combination of these mechanisms. Overall, however, the basis for ehrlichial thrombocytopenia remains unclear. Several signs of ehrlichiosis include thrombocytopaenia, pyrexia, reduction in the packed cell volume and the presence of *E. canis* in mononuclear cells. If the infection is not cured, the disease can develop to a chronic sign because bone marrows will dysfunction well which cause anemia and also make the host more easily to get other infections. The

dog will recover poorly from treatment at this stage and the will soon die because of hemorrhage (Mavromatis et al., 2006).

4. Anaplasmosis

Anaplasma species is belonged to the order Rickettsiales, the family of Anaplasmataceae, genus *Anaplasma* (Dumler et al., 2001), and also a formerly of *Ehrlichia* that was first identified and described in 1978 by Giemsa-stained blood smears from Florida, USA. Infectious cyclic thrombocytopenia of dogs is caused by intracellular bacterial agents, *A. platys*, a small rickettsial parasite of platelets infection which described in dogs (Harvey et al., 1978b). This organism is caused a thrombocytopenia. Platelets are important for blood clotting. The vector of *A. platys* is the brown tick, *R. sanguineus* (Inokuma et al., 2000; de Caprariis et al., 2011). Also, *Dermacentor* spp (Parola et al., 2003), *Haemaphysalis* spp and *Ixodes persulcatus* tick collected from wild-caught mammals and the environment in Korea (Kim et al., 2006). *A. platys* have been reported in the worldwide distribution such as in Europe, USA, Australia, Africa and Asia (Hua et al., 2000) including Thailand (Chungpivat and Taweethavonsawat, 2008; Pinyoowong et al., 2008). Dogs are the most common mammalian host with this agent. *A. platys* organisms characterize as dark blue in platelets when blood smear are stained with Giemsa or new methylene blue, the organisms range from 0.35 μm to 1.25 μm in diameter, are round-, oval- or bean-shaped, and are surrounded by a double membrane (Harrus et al., 2012). Most dogs with naturally occurring infection have mild clinical disease, although more severe clinical signs of fever, lethargy, pale mucous membranes, petechial hemorrhages, epistaxis, and lymphadenopathy can occur (Kontos et al., 1991; Amano et al., 1997). As other tick-transmitted diseases as, co-infection of *A. platys* with other infectious organisms such as *E. canis* and *B. canis* species can result in more severe clinical manifestations (Kordick et al., 1999).

4. 1 Pathogenesis

The natural path of transmission has not been demonstrated conclusively, although it seems to be linked to a tick vector. According to an experimental observation in dogs, the highest percentage of parasitized platelets appears between eight and fourteen days after inoculation and usually no longer seen. The organism appears in platelets circulation, resulting in a severe thrombocytopenia, typically < 20,000/µl. Platelet counts usually remain below 20,000/µl. After the organisms disappear rapidly from the peripheral blood, and then platelet counts rise quickly, reaching a normal value in three or four days (Hoskins et al., 1988). Subsequently, organisms will reappear in one to two weeks, resulting in a cyclic thrombocytopenia which link to a breeding in dogs. The organism can go through a complete lifecycle in the tick gut. The disease causes severe anemia and wasting in infected adult dog. Young dog will not show clinical signs but may serve as carriers (Harrus et al., 2012).

5. Heartworm disease

Heartworm disease is endemic in tropical and subtropical countries where the arthropod vectors are commonly located in those areas. Heartworm is caused by *Dirofilaria (D) immitis* (McCall et al., 2008). In United States, more than 70 species mosquitoes are able to transmit heartworm (Nelson, 2012). Although, the most common vectors of transmission are caused by mosquito, including *Culex* and *Aedes* (Brown et al., 2012). Canine dirofilaria is now spread in South America, Southern Europe, Australia, and in Asia including Japan, Indonesia (Vezzani, Carbajo et al., 2011), China (Hou et al., 2011) and Thailand (Tiawsirisup et al., 2010). Laboratory studies have showed that the temperature is very importance for the development of microfilaria to the infected stage (Ledesma et al., 2011). Worms live in the right ventricle and pulmonary artery of the heart and are known as canine heartworm disease. The size of *D. immitis* female has about 25-30 cm in length, whereas male has approximately

15 to 18cm in length within 5 to 7 years (Kotani and Powers, 1982; Nelson, 2012). According to the detection of microfilaria by using parasitological methods was showed 10% of the stay dog in Bangkok (Tiawsirisup et al., 2010) and 94.8% of a retrospective study in Chulalongkorn University from January 2001 to December 2003 (Niwetpathomwat et al., 2006). In Indian, two surveys of the prevalence of microfilaria in dog are found 7% and 21%, respectively. Additionally, the risk of microfilariae infection are found in dog upper one year of the age since the L3 need five to seven months to mature into adult heartworms after infection. Due to worms die from either natural case or drug using, heartworms can cause a congested capillary tube and pulmonary arteries in heart which are the main function to supply the blood to other organs of the body, particularly blood flow to the lungs, liver and kidneys. Many dogs seldom show or no clinical signs, because dogs can accept heartworm very well, particularly if worms are not very active. Even though, the most common clinical signs of heartworm disease are mild coughing, weakness, shortness of breath, poor condition and anemia. In addition, it may die suddenly in case of severe infection (Nelson, 2012).

5. 1 Life Cycle

The life cycle of *D. immitis* go throughout several stages in both mosquito vector and vertebrate host to complete their life cycle. When mosquitos take a bloodsucking meal from the infected dog, the microfilariae or pre-larvae migrate to the proboscis of the mosquito (Ledesma et al., 2011). After those microfilariae develop into L1, L2 in malpighian tubule of the mosquito midgut, and then the infected stage (L3). L3 migrate to the proboscis of the mosquito where they are waiting for infection other hosts. The duration of microfilariae development bases on the average ambient temperature. Once the host is bitten by the infected mosquitos, the L3 that contains in proboscis are leased and deposited under the host's skin and enter into the subcutaneous tissue then they grow for 7 days or 14 days to molt to fourth-stage

larvae (L4). Then, the L4 migrates in the subcutaneous tissue and muscle toward the thorax and resides there about up to two months before molt to a fifth-stage (immature or juvenile worms). Approximately three months after infection, the L5 penetrates muscle tissue and enters the blood circulation then passes through peripheral vein to the heart until finally arrive in the main pulmonary artery (Kotani and Powers, 1982; Ferasin, 2004; Nelson, 2012). Juvenile worms have about 2.5 to 3.5 cm in length and increase their size to become larger and larger as a result of congestive heart. Finally, worms become mature and mating occur in the pulmonary vessels and then produce new microfilariae. The microfilariae enter to the bloodstream and wait for the mosquito bite to complete their life cycles. Approximately, the completion of life cycle is around 180 to 210 days post-inoculation (Nelson, 2012).

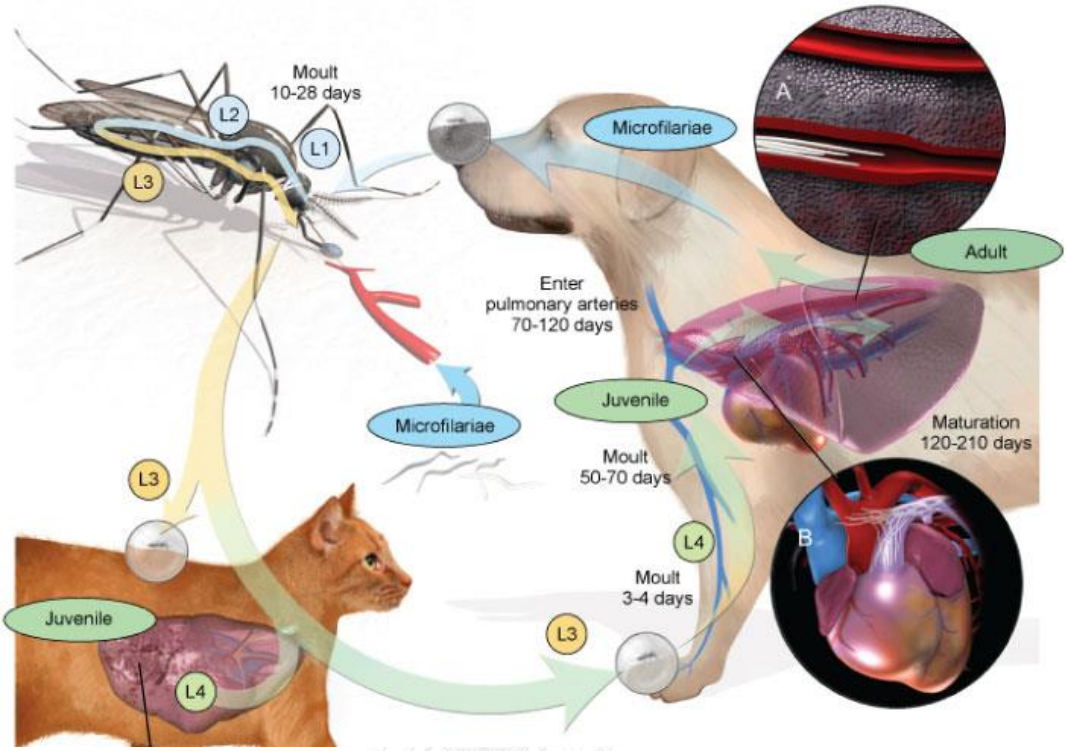

Figure 3: The life cycle of *D. immitis* in the dog and mosquito. Graig EG, 2011, Infectious Disease of the Dog and Cat, 4th ed. p 867. (Art by Brad Gilleland © 2010 University of Georgia Research Foundation Inc.)

5. 2 Pathogenesis

Heartworm disease can affect the severity of cardiopulmonary in dogs and its clinical sign are depended on the number of adult worm presence, host immune response, duration of infection, the location of the worms and the level of damage. During adult worm in the heart cause endothelial damage, villous proliferation and inflammatory cells which the result of pulmonary hypertension (Nelson, 2012). After infection, lesions of the endothelial damage of pulmonary arteries become swollen which is the sign of the first step in the pathogenesis. After the endothelial damage, leukocytes invade the wall and smooth muscle cells and migrate toward the endovascular surface as response to growth factor release. Unexpectedly, death worm

causes inflammation and platelet aggregation with the result in pulmonary thromboembolism because of the capillary beds are blocked the blood flow by its small worm fragments (Ferasin, 2004). Additionally, pulmonary hemorrhage and fibrosis can also occur while the capillaries are broke (Nelson, 2012). Besides that, the endosymbiotic bacteria *Wolbachia* pipiens which lives inside the worms are also considered in the pathogenesis of filarial diseases. Recent studies have demonstrated that a primary surface protein of *Wolbachia* (WSP) induces a specific IgG response in hosts infected by *D. immitis*. In severe cases, worms can move to the right ventricle, right atrium and caudal vena cava of the heart with signs of right-sided heart failure (Nelson, 2012). By the blood flow through the plenty of parasites formed that can also cause intravascular haemolysis and with the result haemoglobinaemia. Beside the infection of the pulmonary arteries, immature parasites can also migrate to another site such as the central nervous system, eye and subcutaneous tissues (Ferasin, 2004).

6. Lymphatic filariasis

Lymphatic filariasis is the major impact in numerous regions especially in tropical and subtropical areas. It is a disease of microfilaria nematode which caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* in human (Weil and Ramzy, 2007). Filaria nematode has also caused in cats, dogs and other wild animals with several species of filarial nematodes such as *Brugia* (*B*) *pahangi* and *Dirofilaria* (*D*) *immitis*. Particularly, *B. pahangi* and *B. malayi* are very close related species and they are a lymphatic filarial worm of domestic cats, dogs and human (Denham and McGreevy, 1977). The vectors of pathogen are transmitted by mosquito including *Culex, Aedes, Anopheles, Mansonia and Coquilletidia spp.* (Muslim et al., 2013a; Yokmek et al., 2013). The parasite is known to infect the lymph vessels of host. For the epidemiology has been reported in Southeast Asia (Simón et al., 2012), Malaysia (Muslim et al., 2013a), the Philippines, Indonesia (Palmieri et al., 1985), Pacific islands,

Vietnam (Meyrowitsch et al., 1998) and Thailand (Chungpivat and Taweethavonsawat, 2008; Thanchomnang et al., 2013). The species of *B. pahangi* and *B. malayi* are very closely related in mammals like dog and cats (Denham and McGreevy, 1977). So far, *B. pahangi* has not been known to cause in human in natural environment although there was a reported of *B. pahangi* by morphology of adult worm in human blood via acid phosphatase activity method in South Kalimantan (Borneo), Indonesia (Palmieri et al., 1985). In addition, according to the experimental inoculation of *B. pahangi* lavae, there was only one human volunteer was found microfilaria infection in blood (Edeson et al., 1960). Moreover, in the recent study of zoonotic *B. pahangi* in Kuala Lumpur City, Malaysia, was also reported that two infections from five patients were infected with *B. pahangi* based on clinical findings, serology results and PCR confirmation (Tan et al., 2011). Shortly after that, in 2010, Malaysia was reported a case of human eye infection by *B. pahangi* (Muslim et al., 2013b). Lately, *B. pahangi* was reported again by mosquitos confirmation with species of *Armigeres subalbatus* as the vector of the zoonotic infectious diseases (Muslim et al., 2013a). The disease presents as a broad range of clinical and subclinical symptoms, including fever, acute and chronic inflammation, lymphadenopathy and lymphedema (Miller et al., 1991). While many researchers have focused on the disease caused by infection with *Brugia* or Wuchereria, the mechanism underlying the pathogenesis of lymphatic filariasis has not been clearly defined. In both acute and chronic infections, these mechanisms probably involve a different range of inflammatory reactions attributable to the parasite, host inflammatory responses, and opportunistic infections.

6. 1 Life cycle

The life cycle of filarial nematodes is required two hosts to complete the life cycle: an intermediate host which serves as the vector, mosquito, and a vertebrate host serve as a final host. The life cycle of *B. papangi* is similar to *B. immitis* life cycle, but the difference is that the adult worms of *B. pahangi* are found in lymph nodes and lymphatic vessels. The length of female worms is probably 80-100 mm and male worm is about 40 mm.

CHAPTER III

MATERIALS AND METHODS

1. Population size for blood sample collection

According to the animal data have never been reported, the dog population was estimated by according to the population of the household from the preliminary results of the 2008 General Population Census of Cambodia, approximately 1410507 households in the plain regions. In addition, the dog population was selected randomly by walking from household to household. The total samples were calculated by the formula of Yamane (1967:886) as the following:

$$n = \frac{N}{1 + N\,(e)^2}$$

n = the sample size

N = the population size of the household

e = the acceptable sampling error or level of precision or 95% confidence level

According to the sample size formula as showed above, the blood samples in this study were calculated as below:

$$n = \frac{1410507}{1 + 1410507\,(0.05)^2} = 399 \text{ dogs}$$

From the calculation of the total sample size, the result was demonstrated that 399 blood samples were collected in this study including Takeo, Prey Veng, Svay Rieng,

Kompong Chhnang, Kompong Speu and a private clinic in the city (Phnom Penh). However, the sample size was collected as much as possible.

2. Criteria of sample collection

Regions of study

The total samples2 of the dogs were accumulated randomly from households to households of dog owners which conducted in Takeo, Kompong Speu, Kompong Chhnang, Prey Veng, and Svay Rieng province and a private clinic in Phnom Penh, of the plain regions in Cambodia (Fig 4). These areas are located in the plain regions, so the distance from Phnom Penh to each province is about 100-200 km that it is not difficult to get those areas compared to Tonle Sap lake region, Coastal region and Mountainous region. The sample collection was carried on from November 2014 to February 2015. The study was conducted during cool and dry season which is the best weather such a kind of warm days, clear skies, no rain, light breeze and cool evening. The average temperature ranged from 20 ^{0}C at mid-day and sometime dipping below 20 ^{0}C at night. Additionally, this season farmers were relaxed at home after their harvest from fields or farms and animals were also home with their owners that was easy to collect blood samples.

Figure 4: Map of sample collection in the plain regions of Cambodia

Information from history taking

The information of dogs was asked for an agreement and an allowance from dog owners with collaboration to handle the dogs during blood taking and recorded by questionnaire including the bread, sex, and age of each dog via grouping as puppy (< 6 months old), juvenile (6 months – 1 year old), adult (1-7 years) and geriatric (> 7 years). The suspicion of clinical sign including anemia, fever, depression, anorexia, and weight loss and skin problem were recorded (Fig 5). In addition, a head of authorities, villagers and veterinarian surveillances were also asked for their permission to guide the blood collection term to dog-raising-houses. During the blood collection was collected by walking from house to house.

Figure 5: The pale gum of a suspected dog with the clinical sign of anemia

Ectoparasites searching

The infestation of vectors was also noticed by searching on the skin, hair coat or other locations of each dog (Fig 6) for 1-2 minute to collect vectors and stored in 70% ethanol solution tube to laboratory identification of genus. In each genus were used with main keys of each vector. With this, ticks were identified with keys from basis capituli, scutum, ventral and dorsal view; fleas were examined with head, thorax, abdomen and leg; lice were looked for head and body parts with special key of genus by following Key book Richard, 2001.

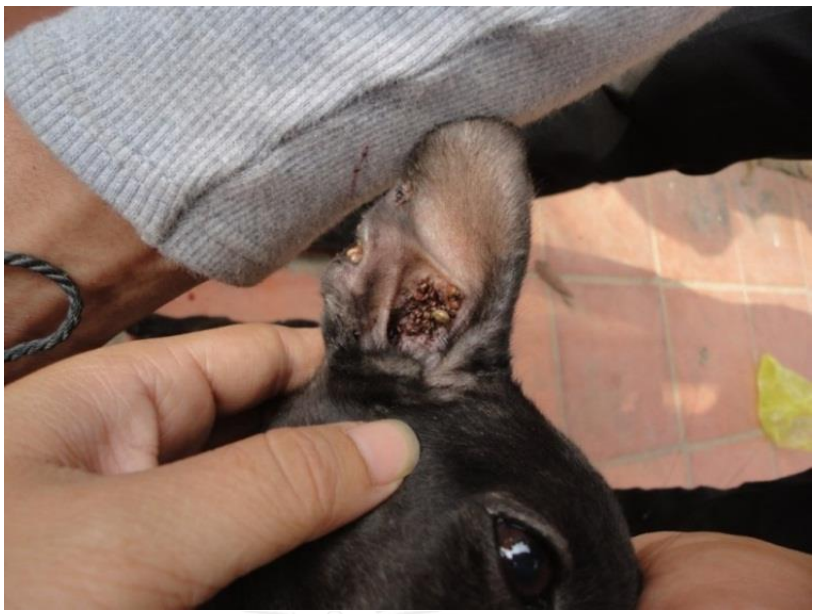

Figure 6: The presence of ectoparasite, tick, collections from the ear of dog in a province in the plain regions of Cambodia

3. Blood preparation

The total 444 blood samples were drawn about 4 ml from the cephalic vein of each dog (Fig. 7) and placed into two Ethylenediaminetetraacetic acid (EDTA) tubes with storing in the ice box. About 2 ml of blood was stored at 4 ℃ and performed within 12- 48 hours for laboratory examination throughout conventional techniques including Fresh blood smear, Buffy coat thin blood smear, and Modify knott's test and another about 2 ml of blood was kept at - 20 ℃ for molecular technique (PCR) and Acid Phosphatase Activity to confirm the suspected blood samples.

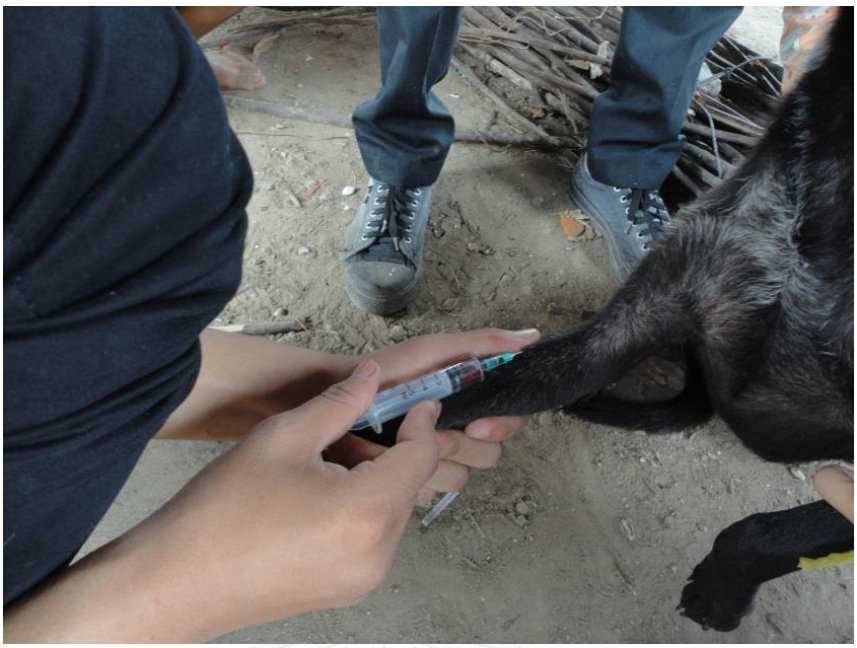

Figure 7: The activity of blood sample was drawn about 4 ml from the cephalic vein path of dog from fields

Hematologic feature

All blood samples were performed immediately after returned. It was inserted into glass capillary tubes and centrifuged for measurement of hematocrit level and check hemolysis of the blood. According to hematocrit measurement value, the blood samples were classified into two groups: group of abnormal of pack cell volume (PCV) has less than 25 % and group of normal of PCV has more than 26%.

4. Conventional techniques

Direct blood smears examination

All blood samples were prepared on a glass slide within 12-24 hours after collection and scanned for extracellular parasites. A single drop of the blood was placed in the center of the glass slide and covered by a coverslip (18x 18mm) and then looked under light microscope with 10x and 40x lens to observe a movement of microfilaria and trypanosome. To avoid false negative each blood sample was used for duplicate slides. This laboratory procedure was followed by the diagnostic Parasitology for Veterinary Technicians, of Parasitology unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

Modify knott's test

The blood samples were used approximately 1 ml of blood with 9 ml of 2% formalin in a centrifuge tube then fitted with the stopper of the tube and rocked it backwards and forwards for 1-2 minutes until the mixture became clear red. After that centrifuged the tube at 1,500 rpm for 5 minutes then poured off the liquid supernatant with leaving the sediment at the bottom of the tube. Transferred a drop of the sediment onto duplicate glass slides and applied a coverslip then examined under light microscope. This procedure was followed by Diagnostic Parasitology for Veterinary Technicians book of the laboratory of Parasitology unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand. This technique was used for microfilaria count/ 1 ml of whole blood.

Buffy coat thin blood smears with 10% Giemsa staining

A total 444 blood samples were used about 60 µl of the blood to insert into hematocrit tube and sealed at the end of the tube with plasticine and then centrifuged

with heamatocrit centrifuge machine for 15.000 rpm in 5 minutes. Hematocrit blood parameter was measured for packed cell volume. The buffy coat part was taped onto the microscopic slide and made a thin blood smear then air dry the slid. After that fix with absolute methanol (99%) about 1-2 minute and air dry again. Poured 10% giemsa about 4ml on the slide and kept 30-45 minutes after that washed with tape water for several times next kept the slide dry. The blood stained-slides were looked under light microscopy 10x, 40x and 100x lens with a drop of immersion oil.

Note: 10% giemsa equal 1ml of giemsa with 9ml of buffy

Buffy coat thin blood smears with Wright giemsa staining

For the first several steps of wright giemsa staining was performed as same as 10% giemsa staining but the difference is after making a thin blood smear then air dry the slides. Poured wright giemsa on the blood smeared- slides and kept for 5 minutes. Next, pour wright giemsa buffy slowly on the slide and kept for 10 minutes then washed several times with tape water and let it dry. The blood stained-slides were examined under light microscope 10x, 40x and 100x lens with a drop of immersion oil.

The protocol of 10% giemsa and wright giemsa were followed by laboratory guideline of Parasitology unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

5. Special technique

Acid Phosphatase Activity (APA)

Positive blood of microfilariae was prepared for blood smear then fixed in cold acetone for 1 minute before incubation in the incubating medium (see in the appendix) within 60 minute at 37°C. The slide blood smear was rinsed in distilled water for 2 or 3 times and then examined under light microscope for the precipitated red azo dye

indicatine acide phosphatase activity. This laboratory procedure was identified in Parasitology unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

Polymerase Chain Reaction (PCR)

DNA extraction

DNA was extracted from 200 µl of whole suspected blood samples by according to the manufacturer's instructions of DNA extraction commercial kit (Macherey-Nagel, Germany). DNA was kept at -20 °C until PCR procedure. To detect *E. canis* and *A. platys* were used 16S rRNA gene and others, *B. canis* and *H. canis* was used 18S rRNA gene as fragments of DNA. The primer detection of pathogens was followed by In House primer from a private diagnostic laboratory of Vet Central Lab, Thailand.

Mix reaction of *E. canis* and *A. platys:*

The primers of *E. canis* from BioDesign were used to amplify 357 base pairs (bp) fragment as below:

No: OS101029/9252, F.ED, 5'-GGTACCYACAGAAGAAGTCC-3';

No: OS101029/9253, R.ED, 5'TAGCACTCATCGTTTACAGC-3'

The primers of *A. platys* from BioDesign were used to amplify the 678 bp fragment of 16S rRNA gene as below:

No: OS101029/9250, F.As, 5'GGATTTTTGTCGTAGCTTGC-3'

No: OS101029/9251, R.As, 5'GTTTGCTCCCCACGCTTT-3'

Mix reaction of *B. canis* and *H. canis*:

The primer of *B. canis* from BioDesign was used to amplify the fragment of 321 bp of *B. canis* DNA as below:

No: OS101103/9392, F.Bca, 5'-AAATTACCCAATCCTGACACAG-3'

No: OS101103/9393, R.Bca, 5'-CCATGCTGAAGTATTCAAGACAA-3')

The primer of *H. canis* from BioDesign was used to amplify 556 bp of 18S rRNA gene as bellowed:

No: OS101103/9394, F-Hc, 5'-GCTAATACATGAGCAAAATCTCAA-3'

No: OS101103/9395, R-Hc 5'-GGACATCATTGCTAAATACACACC-3')

PCR reaction

According the Multiplex PCR protocol, the amount of reaction was contained of 12.5 μl of PCR Master Mix, 6.5 μl of sterile water, 1 μl of primers (Forward and Reward) and 1 μl of DNA. Those reactions were mixed together for the final volume 23 μl. The PCR was performed in amplification 40 cycles in Thermal cycler (Bioer, Little Genius in China) including initial denaturation (94 °C for 5 min), denaturation (94 °C for 30 s), annealing (60 °C for 30 s), extension (72 °C for 30 s) and final extension (72 °C for 30 s).

The PCR products were separated by electrophoresis in concentration from 0.8-3% of agarose gel solution with RedSafe™ Nucleic Acid Staining Solution (20,000X) (Cat. No. 21141) and detected the bands under UV illumination. RedSafe™ Nucleic Acid Staining Solution (20,000X) allows visualization of DNA (>50 ng) in the agarose gel.

6. Statistical analysis

The results of convention methods and animals' information were interpreted by descriptive statistical analysis. The percent of the positive results were calculated by the total positive numbers multiplied 100 and divided the total number of tested dogs.

CHAPTER IV

RESULTS

Animal status

The information of 444 dogs from rural and urban locations was obtained based on the observation of the clinical sign, questionnaires recording and laboratory processing. The frequency of age groupings were mostly adult, juvenile, puppy and geriatric group (55.18%, 17.12%, 14.86% and 12.84%), respectively. Gender of male were 52.47% and female were 47.97% of the total dogs. The majority of dog bread in rural areas was the local breed and some of which are cross breeds. The clinical signs at the time of sample collection were mostly showed the normal and healthy condition in rural areas but some of which were presented an anemia, weight loss, lethargy, loss of appetite and so on (Fig 8, 9). For the living condition, the dogs were free eating and living as stay dogs even they have their owner. Hematocrit valume was rang between 6-45% and the mean of hematocrit valume was 25.18%. Additionally, the hematocrit between 37- 45% was considered as a normal condition, and another less then 37% was determined as an abnormal (Table 1).

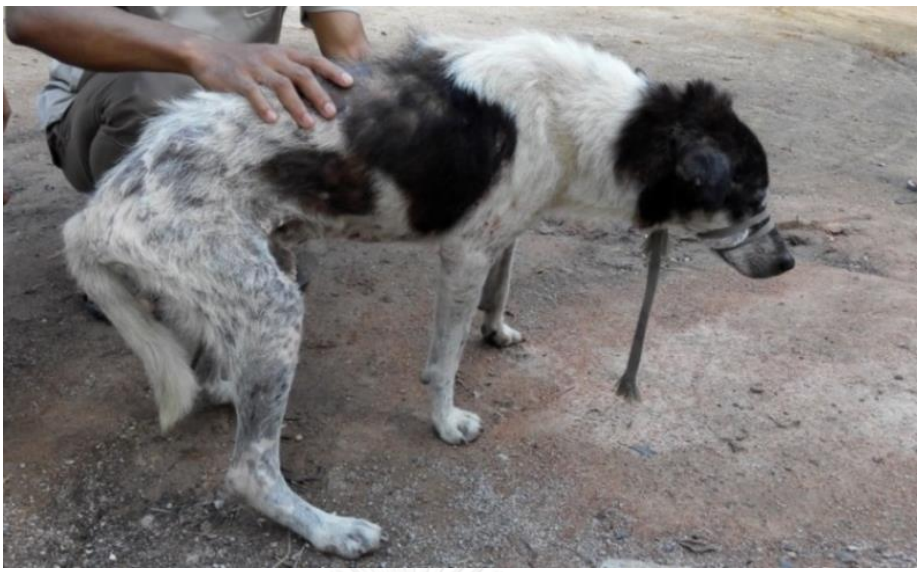

Figure 8: The physical examination of the dog during sample collection in rural areas showed the clinical sign of weight loss and loss of appetite.

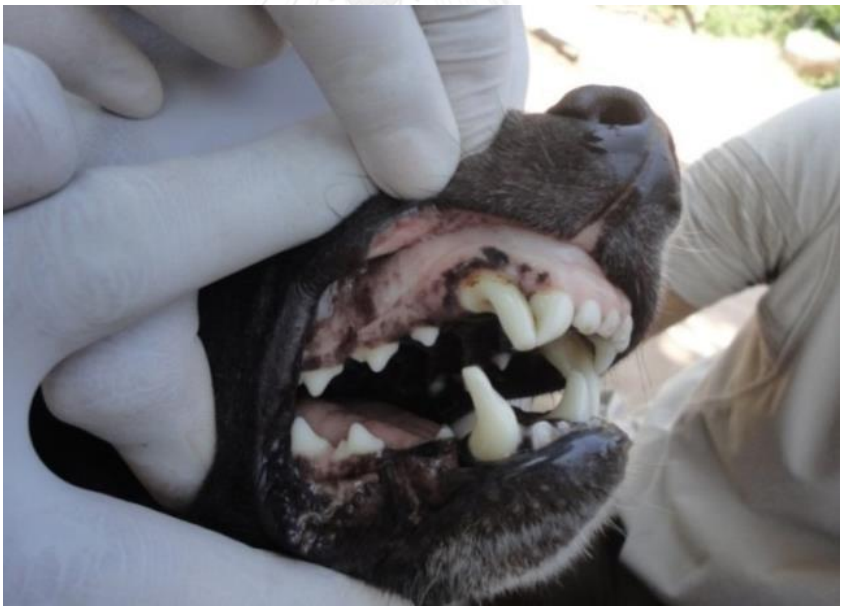

Figure 9: Checking dog during sample collection in rural areas showed the clinical sign of pale gum.

Laboratory examination of convensional methods

Modify knott's test

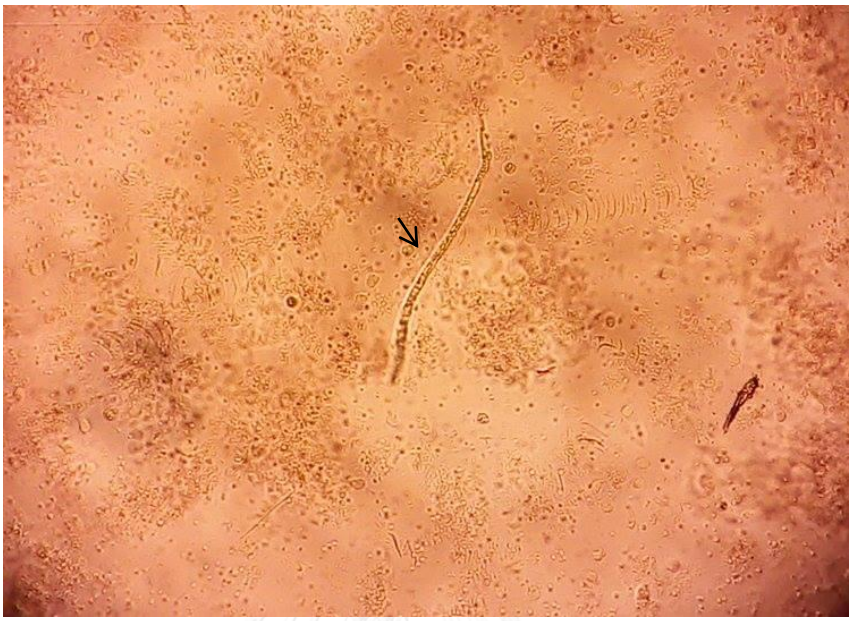

Figure 10: A positive blood sample of microfilaria under microscope with 10x lens was observed by using modify knott's test. The characterization showed a long slender of microfilaria with the head blunt curve and the sharp tail.

Buffy coat thin blood smears

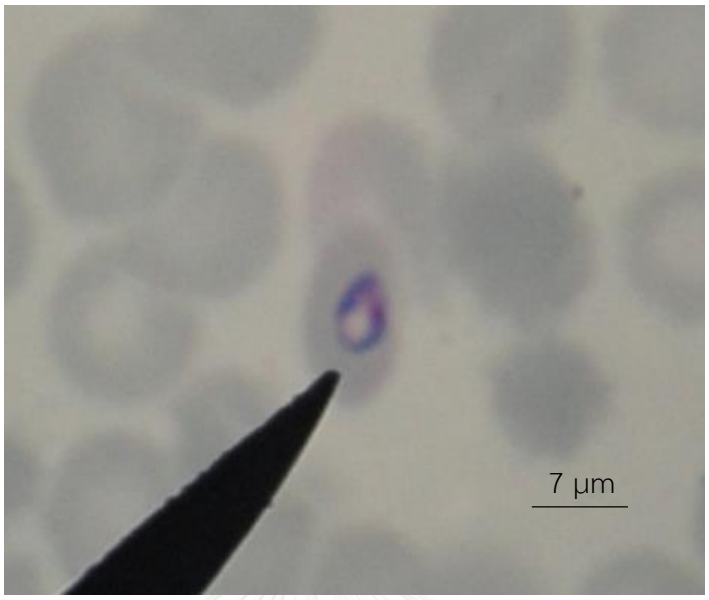

Figure 11: A positive sample showed a ring form of *Babesia canis* inside the erythrocyte by staining (black pointer) under 100x lens with immersion oil.

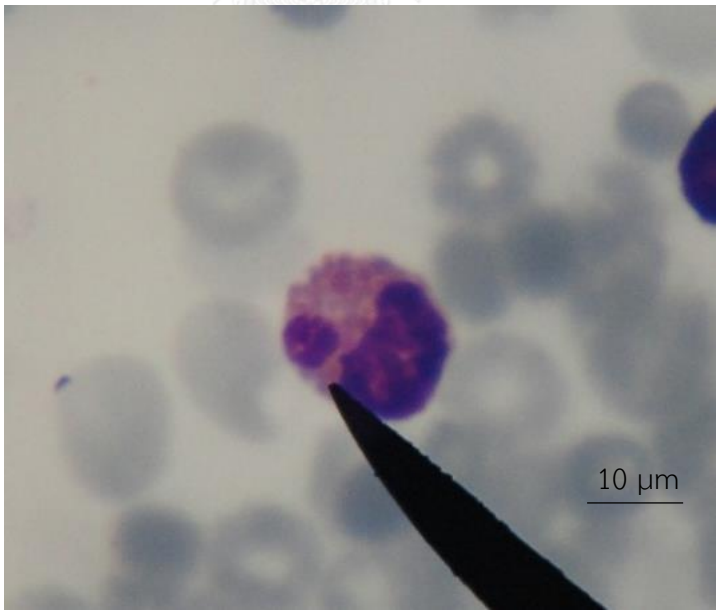

Figure 12: Under light microscopic examination was showed the morulae form of *Ehrlichia canis* (black pointer) infection in monocyte of white blood cell under 100x lens with a drop of immersion oil.

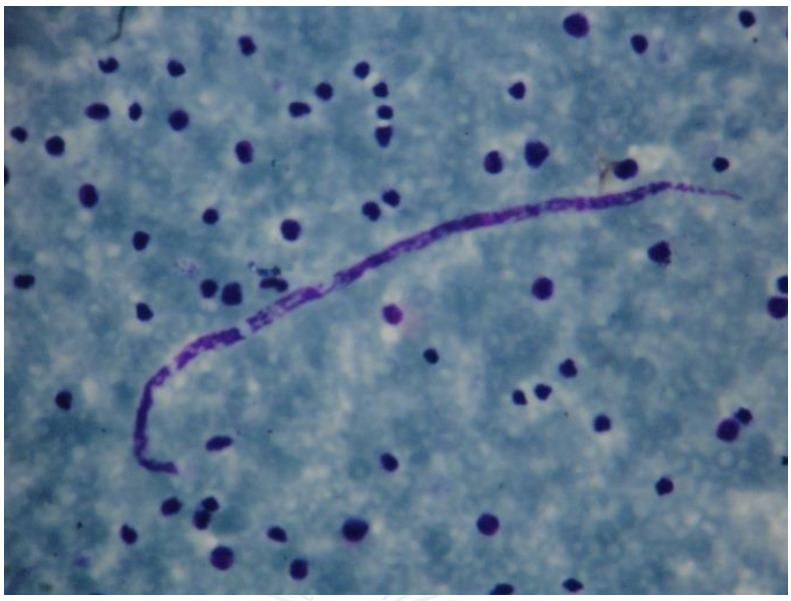

Figure 13: Unsheathed microfilaria of *Dirofilaria immitis* was observed under 10x lens of light microscope by using wright giemsa staining.

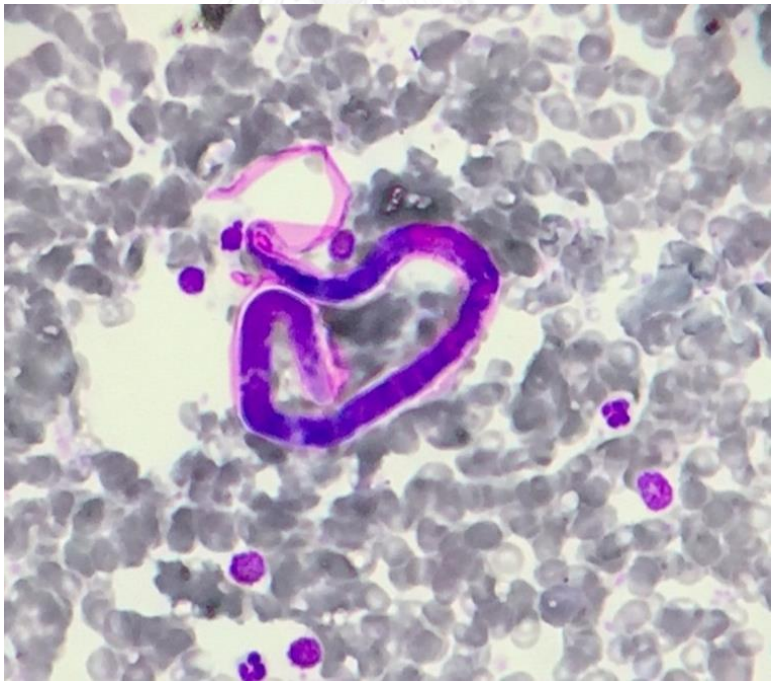

Figure 14: The sheathed microfilaria of *Brugia sp* were found in buffy coat thin blood smear staining with wright giemsa staining under 100x lens of light microscope.

Acid Phosphatase Activity (APA)

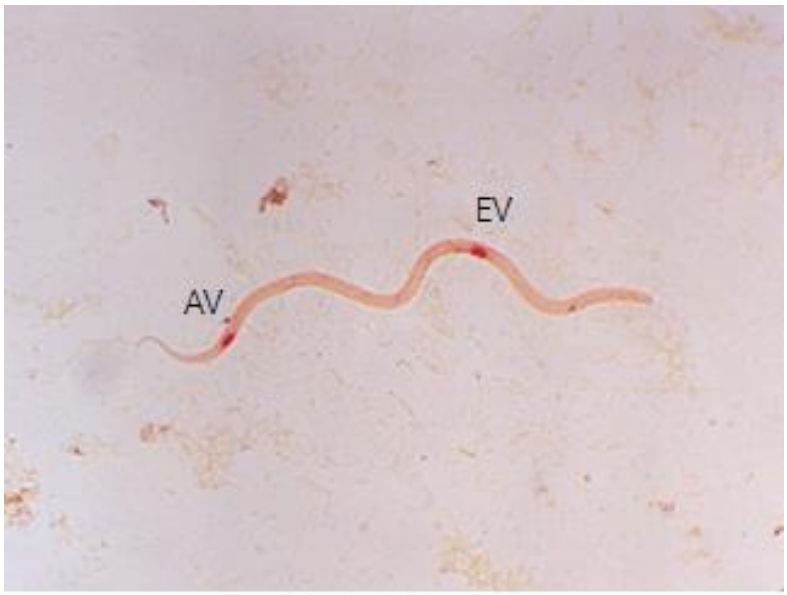

Figure 15: Microfilaria of *Dirofilaria immitis* confirmation was stained by APA which demonstrated two red spots, Excretory Vesicle (EV) and Anal Vesicle (AV) in the body of microfilaria under light microscopic examination.

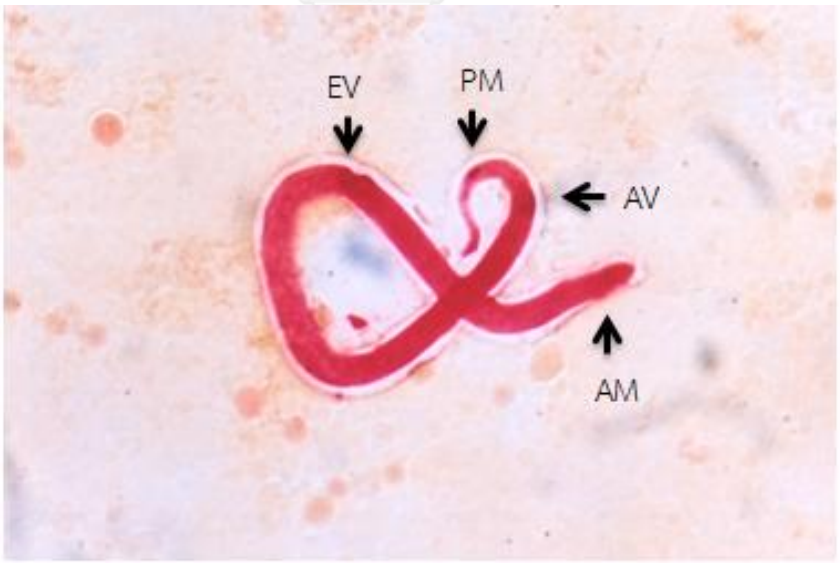

Figure 16: Microfilaria of *Brugia pahangi* confirmation was stained by APA which demonstrated four red spots, Amphids (Am), Excretory Vesicle (EV), Anal Vesicle (AV) and Phasmids (PM) and the red color along entire body.

Polymerase Chain Reaction (PCR) result confirmation

Electrophoresis display

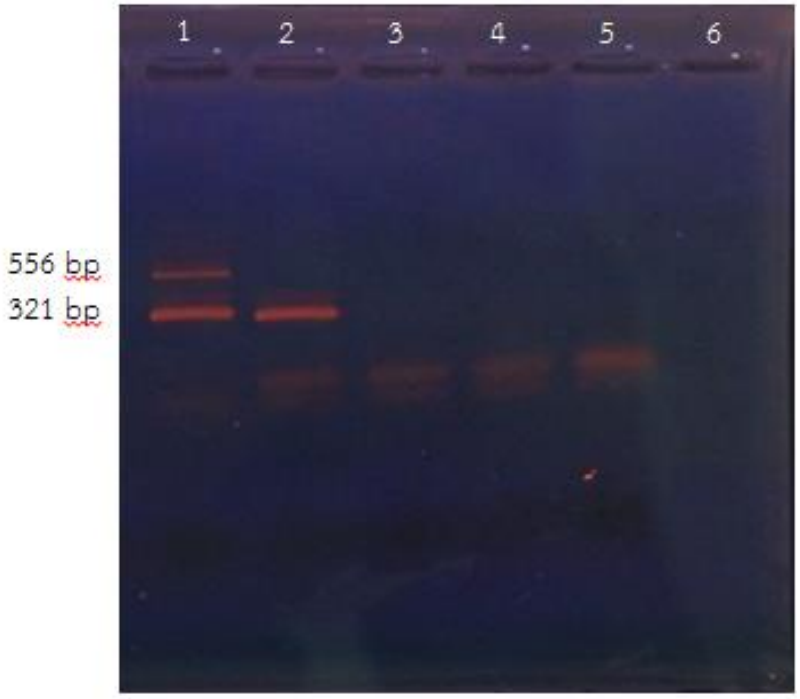

Figure 17: Lane No.1 is *B. canis* and *H. canis* double band positive control. Lane No. 2 is a positive sample of *B. canis* only with suspected band approximately 321 bp and *H. canis* DNA band is not showed an infected result. Lane No. 3, 4 and 5 are non-infected blood samples which no compatible band of DNA product was presented. Lane No.6 is negative control which is no evidence of contamination.

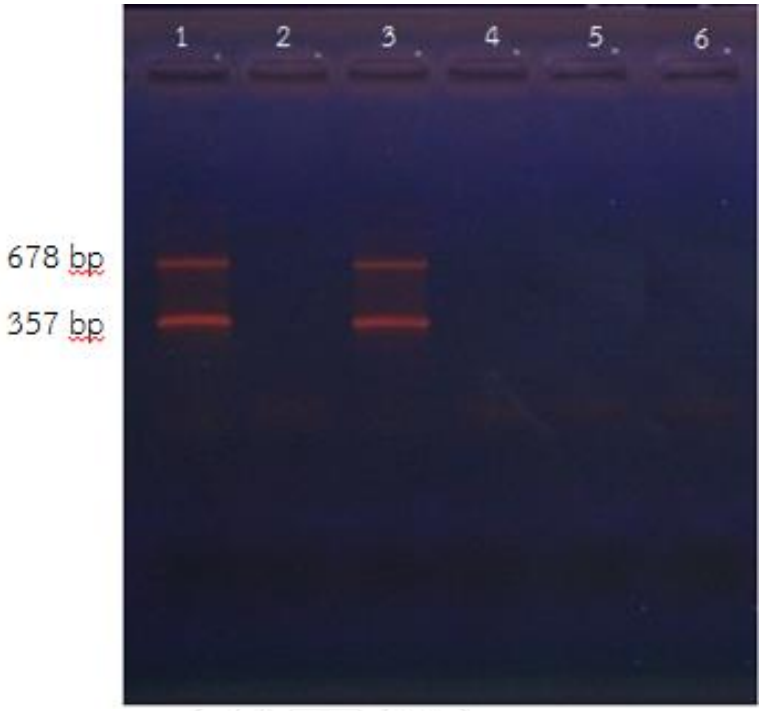

Figure 18: Lane No.1 is *E. canis* and *A. platys* double band positive control. Lane No.3 is positive sample for *E. canis* with showed in the suspected band approximately 357 bp and *A. platys* represented in the suspected band approximately 678 bp of DNA product. Lane No. 2, 4 and 5 are non-infected blood samples. Lane No.6 is negative control which is no evidence of contamination.

Blood-feeding ectoparasites

Figure 19: the morphology of adult *Ctenocephalides canis* has a bluntly rounded of head (A) and a short stout dorsal incrassation (B). The first genal comb (1st spine) (C) is shorter than the second comb.

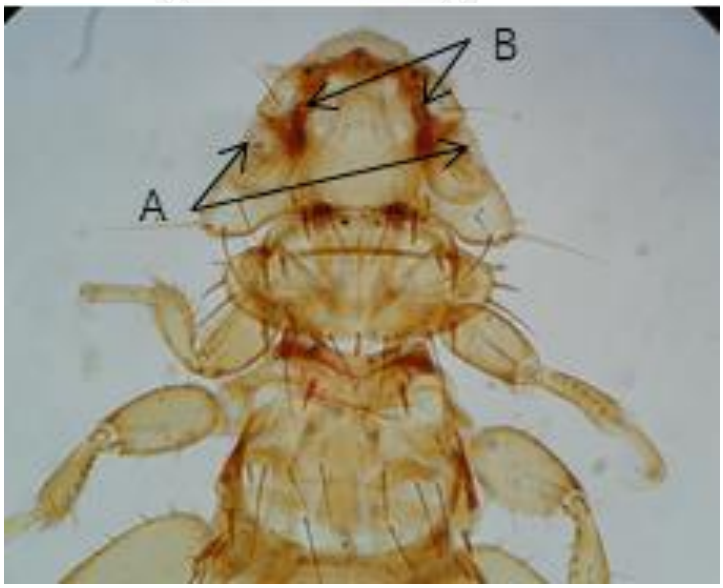

Figure 20: The anterior end of *H. spiniger* is roughly triangular shape of head that has a pair of antennae (A) and a pair of spinelike process (B).

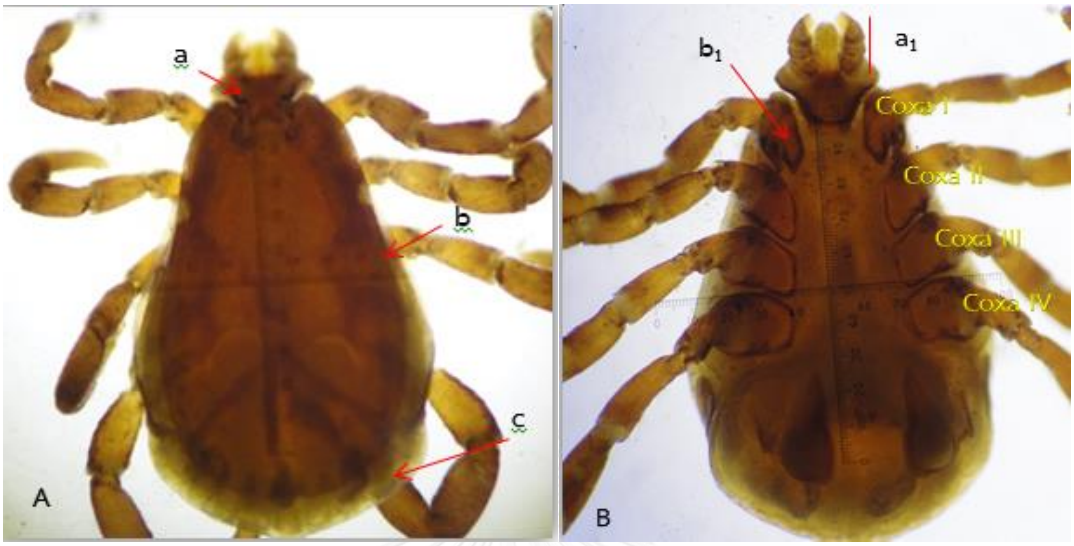

Figure 21: The morphology of adult male tick, *Rhipicephalus sanguineus,* showed in dorsal view (A), at the anterior of basis capituli demonstrated a hexagonal in sharp (a), Lateral groove is connected together (b), Festoons present (c). In ventral view (B) has short mouthparts (a_1), Coxa I is larger than, distinct, equally paired internal and external spurs (b_1). The external spurs of Coxa II-IV are smaller than internal spur.

A survey of Canine vector-bone blood parasites (CVBBPs) in the regions

In this study, the total number of 444 blood samples were colleted and examined for CVBBPs by using Direct fresh blood smear, Modify knott's test, Buffy coat thin blood smear (giemsa and wright giemsa staining) and the positive samples were confirmed by special techniques such as APA and PCR. The percentage positive samples of CVBBPs were showed in (Table 2) and the results of these methods were showed in (Table 3).

CVBBPs of dog in the plain regions of Cambodia were found only 5 samples (1.13%) including *Babesia (B) canis* which found in one sample from a private clinic in Phnom Penh city. The parasite of *B. canis* was performed by Buffy coat thin blood smear with staining both giemsa and wright giemsa as demonstrated *B. canis* form in red blood cell (Fig. 11) and confirmed by PCR with suspected band approximately 321 bp of DNA produce (Fig. 17) and one sample was found with co-infection of *Ehrlichia (E) canis* and *Anaplasma (A) platys* as *E. canis* was appeared in morulae form of monocyte by wright giemsa staining (Fig. 12) and confirmed by PCR with suspected band approximately 357 bp of DNA produce (Fig. 18), while *A. platys* was not found in platelet with staining but it was confirmed by PCR technique with suspected band approximately 678 bp of DNA product (Fig. 18). Other three samples were infected with microfilaria worm. Microfilariae were found by using Modify knott's test (Fig. 10). Species identification was used by buffy coat thin blood smear with giemsa and wright giemsa staining and comfirmed by APA. The result showed that two positive samples were found *Dirofilaria (D) immitis* in Prey Veng province and another one sample was found *Brugia (B) pahangi* in Kompong Chhnang province. *D. immitis* showed unsheathed microfilaria and has one nucleus in cephalic space from giemsa and wright giemsa staining (Fig. 13), while APA staining demonstrated only two areas of the red spot at excretory vesicle (EV) and anal vesicle (AV) (Fig. 15). Microfilaria of *B. pahangi*

showed sheathed microfilaria and two terminal nucleuses from buffy coat stain blood smear technique (Fig. 14), while APA staining showed the red spot at amphids (AM), excretory vesicle (EV), anal vesicle (AV) and phasmids (PM) and more intense red color along entire body (Fig. 17). With this, the prevalence of total positive sample of CVBBPs were demonstrated that there were three areas including Prey Veng province (0.45%), Takeo province (0.22%), Kompong Chhnang province (0.22%), and a private clinic (0.22%) were found different pathogens in each place, while Kompong Speu and Svay Rieng province were not found the parasitic infection in the plain regions (Table 2). Furthermore, the percentage of *B. canis, D. immitis, B. pahangi, and E. canis with A. platys* in each location were presented 7.69%, 2.3%, 1.3% and 1.03% (Table 2) and (Fig 22).

Table 1: Animal characteristics by recording of age, gender, breed, and hematocrit measurement of dogs in the plain regions of Cambodia

Locations	Age	Samples (n)	Sex		PCV (%)	
			M	F	Rang	$\bar{x}$
Takeo	< 6m	21	8	13	12-45	25.73
	6m- 1y	17	7	10		
	1y-7y	43	18	25		
	> 7 y	16	12	4		
	Total	**97**	**45**	**52**		
Kompong Speu	< 6m	7	3	4	8-45	24.8
	6m- 1y	15	12	3		
	1y -7y	66	40	26		
	> 7y	12	10	2		
	Total	**100**	**65**	**35**		

Kompong Chhnang	< 6 m	9	6	3		
	6m- 1y	16	11	5		
	1y -7y	39	21	18	9-37	24.19
	> 7y	13	6	9		
	Total	**77**	**44**	**35**		
Prey Veng	< 6m	17	10	7		
	6m- 1y	16	9	7		
	1y -7y	49	25	24	6-42	25.91
	> 7y	5	3	2		
	Total	**87**	**47**	**40**		
Svay Rieng	< 6m	7	2	5		
	6m- 1y	12	6	6		
	1y -7y	43	15	28	15-42	24.91
	> 7y	8	4	4		
	Total	**70**	**27**	**43**		
Clinic	< 6m	5	2	3		
	6m- 1y	0	0	0		
	1y -7y	5	1	4	10-38	26.62
	> 7y	3	2	1		
	Total	**13**	**5**	**8**		

Note: $\bar{x}$ is the mean of hematocrit measurement of blood sample

Table 2: The percentage of CVBBPs infection in each location from the plain regions of Cambodia

Parasites	Takeo (n = 97)	Kompong Speu (n=100)	Kompong Chhnang (n= 77)	Prey Veng (n= 87)	Svay Rieng (n= 70)	Clinic (n= 13)	Total (N=444)
B. canis	-	-	-	-	-	7.69	0.22
E. canis+ A. platys	-	-	-	-	-	-	0.22
D. mimitis	-	-	-	2.3	-	-	0.45
B. pahangi	-	-	1.3	-	-	-	0.22
Total	1.03	-	1.3	2.3	-	7.69	1.13

Note: n = the number of dog samples size in each location, N = The total number of dog samples size in the plain regions

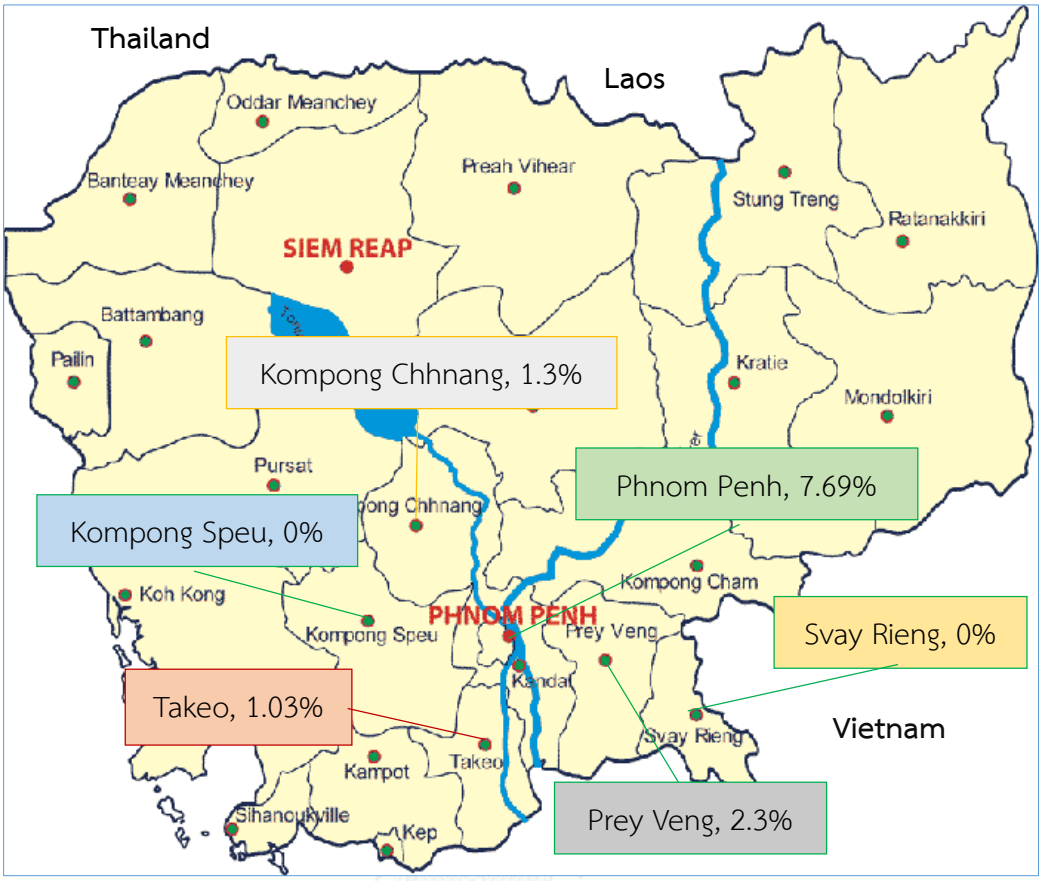

Figure 22: The summary results of CVBBPs infection in each region of Cambodia by using conventional methods and confirmed by special methods.

The among three methods of detection: Fresh blood smear, Buffy coat thin blood smear and Modify Knott's test were uesed for detection of parasites and other two methods were used to confirm the suspected parasite infection in the dog. They were suspected positive samples by Buffy coat thin blood smear with wright giemsa staining and 10% giemsa staining were found 5.4% (24/444) and Modify Knott's test was presented 0.67% (3/444), whereas Fresh blood smear was not found any parasitic infection. With these positive results, *B. canis* and co-infection of *E. canis* with *A. platys* were positiv only 2 samples from 26 suspected positive samples by PCR and 3 out of 444 total samples were presented *D. immitis* and *B. pahangi* which confirmed by Acid Phosphatase Activity confirmation (Table 3).

Table 3: The total 444 of canine blood samples from the plain regions of Cambodia was examined by using routine methods and special method confirmation

Pathogens	Methods detection (N = 444)			Methods confirmation	
	Fresh Blood Smear	Buffy Coat Smear	Modify Knott's Test	APA	PCR
Microfilaria	-	-	(1) 0.22%	(1) 0.22%	-
D immitis	-	(2) 0.45%	-	(2) 0.45%	-
B. pahangi	-	(1) 0.22%	-	(1) 0.22%	-
B. canis	-	(1) 0.22%	-	-	(1) 0.22%
E. canis	-	(1) 0.22%	-	-	(1) 0.22%
A. platys	-	-	-	-	(1) 0.22%

The dogs' information of positive samples was recorded during sample collections, the infected dog with *B. canis* was taken from the city which lived in good condition, but the other infected dogs were lived in rural areas as a free eating and traveling althought they have the owners. For the hematocrit measurement volume was determined as a normal group only in the dog who was infected with *D. immitis*, while the other animals infection of parasites were recorded an abnormal measurement of blood. However, the only one from 5 infected dogs with *B. canis* was showed the clinical sign of loss of appetite, weight loss, fever and anemia. Beside, the vectors of the ectoparasitic searching were presented only flea infestation, while ticks and lice have no presented at the time of blood collection (Table 4).

Table 4: Characterisation of animal and risk factors of 5 positive pathogens

Pathogens	Positive (N)	Age	Bread	Sex	Living	Vectors	PCV
B. canis	1	3y	MB	M	GC	-	Abnormal
E. canis+ *A. platys*	1	3m	SD	M	FL	Flea	Abnormal
D. immitis	2	4y	SD	F	FL	-	Abnormal
		4y	SD	M	FL	Flea	Normal
B. pahangi	1	6y	SD	M	FL	-	Abnormal

Note: y = year, m = month, MB= Mixed Breed, SD = Stay dog, M = Male, F = Female, GC = good care, FL = Free living

Ectoparasitic searching

The prevalence of ectoparasitic searching in dogs recorded including one kind of vector infestation was found flea 9.0%, lice 47.5%, and ticks 4.3% of 444 speciments; the two kinds of vector infestation such as ticks with fleas 10.1%, ticks with lice 2.7% and fleas with lice 3.6%, and the three kind infestation ticks ,fleas and lice 1.6%. The study showed that the one infestation was higher than two and three infestation of vectors. In this investigation was noticed fleas are the most common presence in each area followed by ticks and lice (Table 5).

Table 5: The prevalence of ectoparasitic searching from the dogs in each location

Locations	Ectoparasitic infestation							Total
	One (%)			Two (%)			Three (%)	
	T	F	L	T+F	T+L	F+L	T+F+L	
Takeo	9.3	60.8	-	28.9	-	-	-	97
Kompong Speu	25.0	14.0	8.0	6	12	12	3	100
Kompong Chhnang	6.5	37.7	2.6	1.3	-	2.6	-	77
Prey Veng	-	66.7	9.2	-	-	-	-	87
Svay Rieng	-	72.9	1.4	14.3	-	2.9	5.7	70
Clinic	7.7	-	-	-	-	-	-	13
Total	9.0	47.5	4.3	10.1	2.7	3.6	1.6	444

Note: T= Tick, F= Flea, L= Lice

After ectoparasitic examination under stereomicroscope (Nikon SMZ745) and identified using key book with morphology, the only three species of ectoparasitic were found including *Ctenocephalides (C) canis, Rhipicephalus (R) sanguineus* and *Heterodoxus (H) spiniger. C. canis* has a bluntly rounded of head and a short stout dorsal incrassation. The first genal comb is shorter than the second comb that is the characterization under light microscope (Fig. 19). This *C. canis* was the most common present in the plain regions which infested until 62.8% and followed by *R. sanguineus* (18.7%), and *H. spiniger* (12.2%). The morphology of *R. sanguineus* vector showed in dorsal view (A) at the anterior of basis capituli demonstrated a hexagonal in sharp and in ventral view has short mouthparts (Fig. 21) and the species of *H. spiniger* was

identified through the anterior end which has roughly triangular sharp with a pair of antennae and a pair of spinelike process (Fig. 20).

According to the presence of these three vectors was demonstrated that the total prevalence of *C. canis* (62.8%) was higher than *R. sanguineus* (18.7%) and *H. spiniger* (12.2%), but in each location *C. canis* was not found in clinic, whereas *R. sanguineus* was not found in PreVeng and *H.* spiniger was not presented in Takeo province and in clinic (Table 6).

Table 6: The prevalence of ectoparasitic species infestation in dogs from each location

Provinces	Takeo	Kompong Speu	Koppong Chhnang	Prey Veng	Svay Rieng	Clinic	Total
Vectors	(n=97)	(n =100)	(n= 77)	(n = 87)	(n=70)	(n =13)	(N=444)
R. sanguineus	38.1	46.0	7.8	-	20.0	7.7	18.7
C. canis	89.7	35.0	41.6	66.7	95.7	-	62.8
H. spiniger	-	35.0	5.2	9.2	10.0	-	12.2

CHAPTER V
DISCUSSIONS AND CONCLUSIONS

Discussions

In this study is the first investigation of CVBBPs in Cambodia by using conventional methods detecting and molecular methods to confirm intracellular parasites from the suspected samples of conventional methods. In Cambodia, the information on the importance of CVBBPs was little known so far. Consequently, the revealing prevalence of CVBBPs infection among dogs living in the plain regions of Cambodia was presented with a low parasitemia and low parasitic infection. Positive blood samples were found only 1.13% (N= 444) by using routine and special technique confirmations. Certainly, the prevalence of parasitic case infections may change if other diagnostic methods with a higher sensitivity, specificity and accuracy, e.g serological and molecular methods, were chosen in particular purpose. As the result of this study, flesh blood smear method was not discovered any parasitic infection. This method was used only a drop of blood to look for extracellular, so if the dog has low parasitimia infection, the priority of positive sample has also low or misdiagsnostic detection with this methods. By contrast, if the dog has high infection of parasites in blood, the case of infection might probably showed high prevalence. Therefore, from 444 dogs could not find the parasites with this method. In the screening methods, Modify knott's test is the one of standard method for detection microfilaria in the blood circulation. The only one positive sample of microfilaria was found with very low parasitemia in the blood from 5-year-old of female dog, while fresh blood smear could not find the positive microfilaria from the same total numbers of blood. Therefore, this technique was found better than fresh blood smear since it used about 1 ml of blood compared to fresh blood smear which was used approximately 0.5 µl

or a drop of blood. However, it is realized that these two methods are not expensive for their laboratory equipment and fast examination if compare to molecular methods. Another is buffy coat thin blood smear that was used both intra- and extracellular parasites detection. Form this method was found only two infected dog for *D. immitis* (0.45%) and other three infected dog were found for *B. pahangi* (0.22%), *B. canis* (0.22%), and *E. canis* (0.22%) by the morphology of parasites in the blood with staining and another one is *A. platys* was found as a co-infection with *B. canis* by PCR. It is thought that buffy coat smear has higher abilities to search for parasite than Fresh blood smear and Modify knott's test even it used about 60 µl of blood. Surprisingly, *H. canis* infection was not demonstrated in this study. The absence of *H. canis* may probably cause by the presence of tick vectors or in the plain regions are clean areas with this pathogen. As it is believed that the path of *H. canis* transmission is different from *E. canis, A. platys* and *B. canis* or other pathogens. However, buffy coat thin blood smear may have less sensitivity detection of pathogens compared to molecular PCR assay (Watts et al., 1999). In addition, microscopic examination for identification of parasites was more likely to give a false negative result by using this observation alone. Supportively, one infected dog was positive for *E. canis* with *A. platys* as a co-infection was proved by PCR. As the same way of confirmation, Acid phosphatase activity considered as the best quality to confirm the species of microfilaria infection with the indication of red azo dye via acid phosphatase activity reaction. However, in this study the positive case infections have low sensitivity results from conventional methods of parasitic finding. Just to give the example of affinity affair, in many countries have been reported a rather prevalence of CVBDs infection including Malaysia was found *D. immitis* 9.6%, *H. canis* 1.2%, *B. canis* 1.1%, and *E. canis* 0.2% by PCR (Rajamanickam et al., 1985). In Thailand, there were demonstrated for *B. canis* 3.07 %, *H. canis* 4.54 %, and *E. canis* 2.32 % (Salakij et al., 1999). In India, *H. canis* 30%, *E. canis* 20.6%, and *A. platys* 6.5% were observed by using PCR detection and only *H. canis* gamonts 2.3%

(N=525) were observed by microscopy examination in blood smear (Rani et al., 2011). In German, PCR was proved for *E. canis* 34%, *A. platys* 10%, *B. vogeli* 8%, and *H. canis* 7.5% in blood samples by (Menn et al., 2010). Other PCR studied on 73 dogs from Grenada showed that *A. platys* 19%, *B. canis* 7%, *Bartonella* spp. 1%, *E. canis* 25% and *H. canis* 7% were report (Yabsley et al., 2008; Kelly et al., 2013); while a study on 348 dogs from Trinidad have proved that dogs were infected with *E. canis* 14% and *B. canis vogeli* 7% (Georges et al., 2008). In the serological and PCR evidence of infected dogs with *E. canis* (27%; 46/170), *Babesia* spp. (24%; 90/372), *B. canis vogeli* (12%; 43/372), *B. gibsoni* (10%; 36/372), *A. platys* (11%; 17/157) and *H. canis* (6%; 15/266) were also published (Kelly et al., 2013). Although, the result from this primary survey study in dog will discuss more as below.

The infected blood with Babesia was detected in Wright giemsa and 10% Giemsa staining/ buffy coat thin blood. The dog was presented a clinical sign of fever with high temperature, appetite, and anemia as linked to PCV of blood was showed only 17%. It is thought that this abnormal of PCV may have affected for *B. canis*. Depending on the disease of *Babesia* spp. pose mainly in young dogs of age, although dogs of all ages can be affected pathogen (Schoeman, 2009) as well as in this study, dog was recorded a 3 year male dog with mixed bread. Moreover, other serological study of *B. canis* was showed that the amount of different age groups have not significant difference from each other (Imre et al., 2013). Despite, previous results have been reported by alternative authors demonstrated that highest seroprevalence in the age group of 3.1-5 year (Hornok et al., 2006), and of 6 to 10 year (Cabannes et al., 2002; Imre et al., 2013). Since the results have been already mentioned, one infected dog out of 13 dogs (7.69%) was taken from a private clinic in Phnom Penh city was considered as the higher prevalence or case of parasitic infection compared to other areas in this study. Thus, if the blood was more collected from the clinic, it may

probably have more parasitic infection. With this infected dog may be posed by other infected travel dogs from other countries.

Up to date, *E. canis* and *A. platys* have no information related to these pathogens in Cambodia. *E. canis* was detected by wright giemsa and 10% giemsa staining/ buffy coat thin blood smear and only morulae form of *E. canis* was found in monocytes of white blood cells. According to PCV was showed only 28% of the whole blood. In a way that agrees with light microscopy is difficult to search for *E. canis* in monocytes in blood smear as well as *A. platys* in platelet and need time consuming. Importantly, according to the first report in Lara, Venezuela, *E. canis* have been given that the human infection with *E. canis* was infected asymptomatic human (Perez et al., 1996). Lately, in 2006 it was designated again in human patients with clinical signs of human monocytic ehrlichiosis (HME) and 30 % (6/20) patients were positive for *E. canis* 16s rRNA on gene-specific PCR (Perez et al., 2006). Therefore, in this study, the only one male dog in 3-month-old of the age from 12 suspected dogs was presented with *E. canis* infection and flea infestations were presented during of blood collection. As the fact that the false positive of *A. platys* infection was failed in the result by conventional methods, it is believed that infection with *A. platys* was difficult to detect and not able to evident in this study as similar as the unclear demonstration of this pathogen in Crotia (Dyachenko et al., 2012). Luckily, PCR was performed to confirm *E. canis* and it was found that in this dog was also infected with *A. platys* as a result of co-infection together. Thus, the only microscopy examination is enough to prove the pathogenic infection. In this regarding a complete blood count (CBC) should be used since it is a necessary constituent linked to the diagnosis of Canine Monocytic Ehrlichiosis. As limited time, in this study did not perform CBC. In a similar way to a study which released that there were no specific clinical signs, thrombocytopenia, anemia and other symptoms (Dyachenko et al., 2012). The co-infection together should

have more serious sign, despite the fact that the dog may have slightly infected with these two pathogens at the time of blood collection from this present study. To our knowledge, this is the first case of a co-infection with *E. canis* and *A. platys* of CVBBPs in a dog without typical symptom in Cambodia.

Canine dirofilariosis is known as filarial worm of parasitic infectious disease which has been found worldwide. In this preliminary study, microscope examination was used to identify parasitic species with confirmation of APA technique through parasitic morphology. The only 0.45% of the overall 444 specimens was denoted with very low parasitemia, 1-2 microfilarias in the blood smear per slide, in Phea Rang district, Prey Veng province. In Thailand, the prevalence of 18.2% (N= 589) was revealed a high risk for dirofilariasis in dogs in the Chiang Mai province (Boonyapakorn et al., 2008). In China, the prevalence of *D. immitis* was indicated by two methods, microscopic examination 16.6% and PCR 24.0% of the total 886 specimens, with a high risk in that region (Hou et al., 2011) and also in wild animals in Chengdu zoo, 2.26% (N= 177) was affected in China (Bo et al., 2009), In Spain, the prevalence decreasing of *D. immitis,* 30.19% to 19.36%, was observed by circulating *D. immitis* antigens (Montoya-Alonso et al., 2010). In South Korea, 20.9% (N= 81) was existed by ELISA method (Song et al., 2010). Regarding to two infected dogs was showed asymptomatic clinical sign of disease, although an abnormal (24%) and a normal (37%) of haematocrit measurement was observed in the age of 4-year-old and 5-year-old, respectively. Considering to the relevance of the ages, *D. immitis* infection was associated with the ages up to 7 months depending on the life cycle and endemic area of *D. immitis* infection. However, the length period of age, older dogs, may have more time and more chance to increase the risk factor of infection than younger dogs since older dogs exposure to the mosquito bites (Fan et al., 2001; Boonyapakorn et al., 2008; Hou et al., 2011). Unsurprisingly, the highly infected rate of indoor dogs may reduce the ability

risk of pathogens transmission though mosquitos than outdoor dogs (Walters et al., 1995; Theis et al., 1999). Also, in Thailand, the result was indicated that the dog in rural areas were obtained the higher risk infection than the dog in urban areas (Boonyapakorn et al., 2008). As supportively, in this study, the dogs were presented for *D. immitis* only in rural areas, while other areas were absent of *D. immitis* in a Phnom Penh city.

B. pahangi and *B. malayi* are a closely related species, a lymphatic filarial worm of mammals. Particularly of cats, dogs and wild carnivores (Denham and McGreevy, 1977). So far, *B. pahangi* has not been known to cause human disease in natural environment, although there is a report of *B. pahangi* by morphology of adult worm in human blood via acid phosphatase activity method in South Kalimantan (Borneo), Indonesia (Palmieri et al., 1985). In addition, according to the experimental inoculation of *B. pahangi* lavae, there was only one human volunteer was found microfilaria infection in blood (Edeson et al., 1960). In Malaysia, five patients were infected with *B. pahangi* based on clinical findings, serology results and PCR confirmation (Tan et al., 2011) and shortly after that infected in human eye (Muslim et al., 2013b). Lately, *B. pahangi* was reported again by mosquitos confirmation with species of *Armigeres subalbatus* as the vector of the zoonotic infectious diseases (Muslim et al., 2013a). Although, *B. pahangi* have been report in human, it has not widely known about this parasite as well as in Thailand that has report only *B. malayi* in a 2-year-old boy living in Surat Thani province (Yokmek et al., 2013) and for *B. pahangi* has demonstrated in cat and dog by using giemsa's staining and the acid phosphatase technique (Chungpivat and Taweethavonsawat, 2008). Later, since microfilariae of *B. pahangi* was presented, the ivermectin injection was used to eliminated (Taweethavonsawat and Chungpivat, 2013). From this first description and investigation study, there was only one of 444 samples infected by natural infection with *B. pahangi* in dog in the plain regions of

Cambodia. However, with the limited number of positive cases in dog of the age of 6-year-old, it was considered that the transmission rate of parasites was probably low because of the absent mosquito vector or low parasitemia in study regions. Furthermore, this low infection may be caused by laboratory diagnosis technique, therefore, should not depend on microscopic examination for microfilaria in the blood sample alone. As a result in this study, the only microscope examination was used to find parasites through conventional methods. These methods are not expensive and easy processing, although they require more experience and skill to distinguish the species of parasites. It is believed that the prevalence of parasite may change if the higher sensitivity has been used for detection in other purposes. Just to give an example, in Thailand, *B. pahangi* and *B. malayi* DNA were differently detected by a real-time fluorescence resonance energy transfer PCR combined with melting curve analysis (Thanchomnang et al., 2010). With the dogs from this study there was not showed any clinical signs of infection but PCV of blood checking was found in abnormal sign (23%). It is not surprised that the symptom of the dog was not related to parasitic infection since very low of parasites (0.22%) was found in blood by giemsa staining and modify knott's test in this survey.

Vector is the most urgent and potentially involved of pathogenic transmission in dogs; particularly tick, *R. sanguineus,* which is the main vector of intra-cellular and extra-cellular parasitic transmission. Unfortunately, tick vectors from this study were found with lower infestation than fleas but higher than lice, therefore, the pathogens were also found with low prevalence. Fleas, *C. canis,* are known to transmit tapeworm such as *Dipylidium caninum*, thus it is believed that this tapeworm may present in the area of this study because fleas are mainly found with high infestation in dog. The other is lice, *H. spiniger*, infestations which are also known as a vector of *Dipetalonema reconditum*. The other is mosquito which the most important vector for extracellular

parasites like microfilaria but in this study did not identify the species or mention with this vector. In addition, some of these are the zoonotic pathogens which need to define the role of *R. sanguineus* in the transmission. Little is understood of the connection between the ecology of *R. sanguineus* and the dynamics of CVBBPs in Cambodia. From this study there are three vectors, *C. canis, R. sanguineus* and *H. spiniger*, affecting dogs were recorded in Cambodia. According to a study at Sakaerat Environmental Research Station, Thailand, vectors were included a mite (*Lealaps echidinus*) 217 (62.00%), tick (Ixodes *sp*.) 40 (11.43%), flea (*Xenopsylla cheopsis*) 90 (25.71%) and pseudoscorpion (*Chelifer cancroides*) 47 (13.43%) (Thanee et al., 2009) were different from my recently study. Although, ticks have obtained in dogs, pathogens may not present or low parasitemia or low infection. During of study, the absent of tick in our areas may be caused by the season of sample collection as to go along with Beugnet has demonstrated that the tick occurred worldwide in climates with mild winters among countries (Beugnet et al., 2011). Generally, the epidemiology of CVBBPs is still unclear and different from area to area and even within each area. The way of transmission of pathogens is closely connected to the behavior of hosts, environment and vectors involved. In addition, According to Irwin has demonstrated that the importance of the disease has to consider on two levels of regional prevalence: (1) regions where the specific parasite is well increased and clinically recognized and (2) regions where indigenous infections or related to the movement of traveling dogs have been recorded. It is also important to control the dog migration from other countries which is the main impact of carrying the pathogens particularly zoonotic potential of CVBBPs from other countries to Cambodia.

Conclusions

The overall prevalence was found that at least five pathogens such as *E. canis, A. platys, B. canis, D. immitis,* and *B. pahangi* by using conventional techniques and confirmed by PRC for intracellular parasites and APA for extracellular parasites. These results were given the positive feedback of CVBBPs presence according to the objective of this study, although the prevalence of parasites has indicated low infection among dogs in the plain regions of Cambodia. Moreover, three species of ectoparaites including *C. canis, R. sanguineus* and *H. spiniger* were also presented. To detect pathogens by morphological observation with conventional methods alone might reveal misinterpretation or misdiagnosis for its sensitivity or specificity particularly zoonosis parasitic species. Anyways, the epidemiology of CVBBPs is complex and unclear for the pattern of transmission from vectors to hosts and an environment involved. Thus, further research of the prevalence of these diseases needed to clarify not only in dogs but also in other animals. Moreover, being based on some researchers have been published that *E. canis* and *B. pahangi* caused zoonosis to human, so it is crucial to provide information concerning for the establishment of pathogens control programs in our countries.

Additionally, according to each local study, Phnom Penh city might be the main sign of a disease existence because of CVBBPs might become established in new geographical locations arises from the increased international mobility of pet dogs and increased contact of these animals with non-urban environments and wildlife disease reservoirs. These factors, coupled with the trend for global climatic change, create real risks for animal and human health.

The advantage of study

- In this primary study can give very handy to all related fields of researchers in order to apply this information for particular purpose in further investigation as well as diagnosis, control, prevention, treatment etc.
- To expect for developing of a better understanding in scientific, diagnostic techniques and improving knowledge the pet owners, veterinarians, public health authorities to beware of accretion regarding CVBBPs infection in Cambodia.
- The veterinarians and students in Cambodia can take this information for further study such as diagnosis, controlling, prevention, treatment and etc.

Recommendations

- In next several years, it may have many infected dogs in Cambodia, so to prevent these parasites I suggest to encourage the people how to control ectoparasites or pathogens in dogs, so it may not happen.
- For further study, I suggest to use polymerase chain reaction (PCR) because it is more sensitive in detecting the pathogens than peripheral blood smear examination.
- Another suggestion is to detect the pathogens from tick vectors which also can indicate the prevalence of parasitic infection.

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APPENDIX

APPENDIX I

The information from fields and laboratory

Table A: The number of blood collection from Takeo, Kompong Speu, Kompong Chhnang, Prey Veng, Svay Rieng and a private clinic in the plain regions of Cambodia and the result from laboratory examination

Table A1: Number of dog information and laboratory results recorded from Toulsang village, Angkor Borey commune, Angkor Borey district, Takeo province

Date of data collection: 15-16/Nov/2014

ID	Age		Sex	Ecto-p	Ecto-p species			Lab examination				
					C. canis	*R. sanguineus*	*H. spiniger*	PCV	BCTBS	APA	MKT	PCR
1	Ad	2y	F	F,T	1	1	0	28	NF	NF	NF	NF
2	Juv	1y	F	F	1	0	0	28	NF	NF	NF	NF
3	Ger	14y	M	F	1	0	0	23	B*	NF	NF	NF
4	Ad	2y	F	F	1	0	0	23	NF	NF	NF	NF
5	Pup	6m	F	F	1	0	0	25	NF	NF	NF	NF
6	Pup	3m	F	F	1	0	0	25	NF	NF	NF	NF
7	Pup	2.5m	M	F,T	1	1	0	32	NF	NF	NF	NF
8	Ad	6y	F	T	0	1	0	25	NF	NF	NF	NF
9	Ad	2y	F	F,T	1	1	0	38	NF	NF	NF	NF
10	Ad	3y	M	T	0	1	0	25	NF	NF	NF	NF
11	Ad	2y	F	F,T	1	1	0	23	NF	NF	NF	NF
12	Pup	3m	M	F	1	0	0	28	E*	NF	NF	YES
13	Pup	4m	M	F	1	0	0	25	NF	NF	NF	NF
14	Pup	3m	F	F	1	0	0	20	NF	NF	NF	NF
15	Ger	14y	M	F,T	1	0	0	15	NF	NF	NF	NF
16	Ad	1.5y	F	F	1	0	0	15	NF	NF	NF	NF
17	Ad	3y	F	F	1	0	0	12	NF	NF	NF	NF
18	Ger	10y	M	F,T	1	1	0	12	NF	NF	NF	NF
19	Ger	10y	M	F,T	1	1	0	20	NF	NF	NF	NF
20	Ad	3y	F	F	1	0	0	24	NF	NF	NF	NF

21	Ad	5y	F	F	1	0	0	21	NF	NF	NF	NF
22	Juv	1y	F	F,T	1	1	0	30	NF	NF	NF	NF
23	Ger	8y	M	F	1	0	0	20	E*	NF	NF	NF
24	Ad	1.5y	M	F	1	0	0	20	E*	NF	NF	NF
25	Ad	3y	F	F,T	1	1	0	23	NF	NF	NF	NF
26	Pup	5m	M	F	1	0	0	30	NF	NF	NF	NF
27	Pup	5m	F	F,T	1	1	0	32	NF	NF	NF	NF
28	Juv	1y	F	F,T	1	1	0	24	NF	NF	NF	NF
29	Juv	9m	M	F	1	0	0	27	NF	NF	NF	NF
30	Ad	4y	F	F,T	1	0	0	18	NF	NF	NF	NF
31	Ad	4y	F	F,T	1	1	0	25	NF	NF	NF	NF
32	Ad	3y	M	F	1	0	0	28	NF	NF	NF	NF
33	Pup	5m	F	F	1	0	0	16	NF	NF	NF	NF
34	Ad	3y	F	F	1	0	0	20	NF	NF	NF	NF
35	Ad	2y	F	F	1	0	0	28	NF	NF	NF	NF
36	Ad	3y	M	F	1	0	0	22	NF	NF	NF	NF
37	Ad	5y	M	F,T	1	1	0	27	NF	NF	NF	NF
38	Ad	5y	M	F	1	0	0	23	NF	NF	NF	NF
39	Ad	5y	F	F,T	1	1	0	37	NF	NF	NF	NF
40	Ad	3y	M	F	1	0	0	21	NF	NF	NF	NF
41	Ad	1y	M	F,T	1	1	0	25	NF	NF	NF	NF
42	Ger	8y	M	F	1	0	0	30	NF	NF	NF	NF
43	Juv	1y	F	F	1	0	0	26	NF	NF	NF	NF
44	Juv	1y	F	F	1	0	0	22	NF	NF	NF	NF
45	Juv	1y	M	F	1	0	0	25	NF	NF	NF	NF
46	Pup	5m	F	F	1	0	0	25	NF	NF	NF	NF
47	Juv	1y	M	F,T	1	1	0	26	NF	NF	NF	NF
48	Ad	4y	M	F	1	0	0	27	NF	NF	NF	NF
49	Ad	2y	F	F	1	0	0	27	NF	NF	NF	NF
50	Ad	3y	M	F	1	0	0	26	NF	NF	NF	NF
51	Ger	10y	M	F	1	0	0	27	NF	NF	NF	NF
52	Juv	1y	M	F,T	1	1	0	41	NF	NF	NF	NF

53	Ad	4y	F	F	1	0	0	37	B*	NF	NF	NF
54	Juv	5m	M	F	1	0	0	15	NF	NF	NF	NF
55	Ad	5y	M	F,T	1	0	0	15	NF	NF	NF	NF
56	Ger	7y	M	F,T	1	0	0	13	E*	NF	NF	NF
57	Pup	5m	F	F	1	0	0	16	B*	NF	NF	NF
58	Ad	3y	F	F,T	1	1	0	23	NF	NF	NF	NF
59	Pup	5m	F	F	1	0	0	27	NF	NF	NF	NF
60	Ad	1.5y	F	F	1	0	0	26	NF	NF	NF	NF
61	Juv	1y	F	F	1	0	0	18	NF	NF	NF	NF
62	Ad	1.5y	M	F,T	1	1	0	22	NF	NF	NF	NF
63	Juv	4m	M	F	1	0	0	20	NF	NF	NF	NF
64	Juv	1y	F	F	1	0	0	27	NF	NF	NF	NF
65	Juv	1y	F	F	1	0	0	25	NF	NF	NF	NF
66	Ad	3y	M	F	1	0	0	26	NF	NF	NF	NF
67	Pup	5m	F	F	1	0	0	17	NF	NF	NF	NF
68	Ad	2y	M	N	0	0	0	24	NF	NF	NF	NF
69	Pup	3m	M	F,T	1	1	0	30	NF	NF	NF	NF
70	Pup	3m	F	F,T	1	1	0	24	NF	NF	NF	NF
71	Ger	7y	M	T	0	1	0	23	NF	NF	NF	NF
72	Ger	7y	M	F	1	0	0	25	NF	NF	NF	NF
73	Ger	12y	F	F	1	0	0	29	NF	NF	NF	NF
74	Ger	8y	F	F	1	0	0	45	NF	NF	NF	NF
75	Ad	5y	M	F	1	0	0	27	NF	NF	NF	NF
76	Ad	4y	F	F	1	0	0	27	NF	NF	NF	NF
77	Juv	6m	F	F	1	0	0	32	NF	NF	NF	NF
78	Ad	5y	F	F	1	0	0	30	NF	NF	NF	NF
79	Ad	1y	M	F,T	1	0	0	39	NF	NF	NF	NF
80	Pup	4m	M	F,T	1	0	0	25	NF	NF	NF	NF
81	Pup	4m	F	F	1	0	0	43	NF	NF	NF	NF
82	Pup	4m	M	F	1	0	0	33	NF	NF	NF	NF
83	Ad	3y	F	T	0	1	0	25	NF	NF k	NF	NF
84	Ger	10y	M	F	1	0	0	18	NF	NF	NF	NF

85	Ger	9y	F	F	1	0	0	30	NF	NF	NF	NF
86	Ad	6y	F	T	0	1	0	29	NF	NF	NF	NF
87	Pup	1m	F	F,T	1	0	0	25	NF	NF	NF	NF
88	Juv	4m	M	F	1	0	0	25	NF	NF	NF	NF
89	Pup	2m	M	F	1	0	0	38	B*	NF	NF	NF
90	Pup	3m	F	F,T	1	1	0	44	NF	NF	NF	NF
91	Ad	2y	F	T	0	1	0	38	NF	NF	NF	NF
92	Ad	6y	F	T	0	1	0	24	NF	NF	NF	NF
93	Ad	3y	M	T	0	1	0	19	NF	NF	NF	NF
94	Ger	7y	M	T	0	1	0	25	NF	NF	NF	NF
95	Ad	5y	M	F	1	0	0	25	NF	NF	NF	NF
96	Juv	6m	F	F	1	0	0	34	E*	NF	NF	NF
97	Ger	7y	F	F	1	0	0	29	NF	NF	NF	NF

Table A2: List of dog information and laboratory results recorded from Banhkong Kaeb village, Trorpaeng Korng commune, Somraong Tong district, Kompong Speu province

Date of data collection: 6-8/Dec/2014

ID	Age		Sex	Ecto-p	Ecto-p species			Lab examination				
					C. canis	*R. sanguineus*	*H. spiniger*	PCV	BCTBS	APA	MKT	PCR
1	Juv	1Y	M	L	0	0	1	26	NF	NF	NF	NF
2	Pup	3M	M	L	0	0	1	28	NF	NF	NF	NF
3	Ger	10Y	M	N	0	0	0	30	NF	NF	NF	NF
4	Ad	2Y	F	T	0	1	0	23	NF	NF	NF	NF
5	Ad	3Y	M	T	0	1	0	24	NF	NF	NF	NF
6	Ad	5Y	F	T	0	1	0	29	E*	NF	NF	NF
7	Pup	5M	F	L,F,T	1	1	1	24	NF	NF	NF	NF
8	Juv	1Y	M	N	0	0	0	34	NF	NF	NF	NF
9	Ad	2Y	M	L,F,T	1	0	0	8	NF	NF	NF	NF
10	Ad	5Y	M	N	0	0	0	32	NF	NF	NF	NF
11	Juv	9M	F	N	0	0	0	35	NF	NF	NF	NF
12	Ad	1.5Y	F	F,T	1	1	0	24	NF	NF	NF	NF
13	Ad	3Y	F	N	0	0	0	30	NF	NF	NF	NF
14	Juv	7M	F	F,T	1	1	0	31	NF	NF	NF	NF
15	Juv	7M	M	F,T	1	1	0	25	NF	NF	NF	NF
16	Ad	3Y	M	F	1	0	0	35	NF	NF	NF	NF
17	Ad	3Y	M	F	1	0	0	29	NF	NF	NF	NF
18	Ad	3Y	M	N	0	0	0	25	NF	NF	NF	NF
19	Ad	5Y	F	F,T	1	1	0	29	NF	NF	NF	NF
20	Juv	8M	M	T	0	1	0	24	NF	NF	NF	NF
21	Ad	1.5Y	F	F	1	0	0	22	NF	NF	NF	NF
22	Ad	4Y	M	T	0	1	0	36	NF	NF	NF	NF
23	Ad	2Y	F	F,L	1	0	1	36	NF	NF	NF	NF
24	Ad	3Y	F	T	0	1	0	25	NF	NF	NF	NF

25	Ad	5Y	F	F	1	0	0	26	NF	NF	NF	NF
26	Ger	10Y	M	F,L	1	0	1	17	NF	NF	NF	NF
27	Ger	10Y	M	T	1	0	0	27	NF	NF	NF	NF
28	Pup	5M	F	F	1	0	0	19	NF	NF	NF	NF
29	Ger	10Y	M	F,L	1	0	1	31	NF	NF	NF	NF
30	Juv	7M	M	L	0	0	1	29	NF	NF	NF	NF
31	Ad	1.5Y	M	F	1	0	0	30	NF	NF	NF	NF
32	Juv	8M	M	F	1	0	0	30	NF	NF	NF	NF
33	Ad	1.5Y	F	L	0	0	1	22	NF	NF	NF	NF
34	Ad	2Y	F	L	0	0	1	35	NF	NF	NF	NF
35	Ad	2Y	M	F,L	1	0	1	31	NF	NF	NF	NF
36	Pup	4M	F	L	0	0	1	37	NF	NF	NF	NF
37	Ger	15Y	M	F,L	1	0	1	35	NF	NF	NF	NF
38	Ger	8Y	F	F,L	1	0	1	45	NF	NF	NF	NF
39	Pup	3M	M	N	0	0	0	40	NF	NF	NF	NF
40	Ad	3Y	M	F	1	0	0	40	NF	NF	NF	NF
41	Ad	1.5Y	F	N	0	0	0	26	NF	NF	NF	NF
42	Ad	2Y	M	F	1	0	0	26	NF	NF	NF	NF
43	Ger	10Y	M	F	1	0	0	13	B*, E*	NF	NF	NF
44	Ad	5Y	M	N	0	0	0	29	NF	NF	NF	NF
45	Ad	1.5Y	M	F,L	1	0	1	29	B*	NF	NF	NF
46	Ad	1.5Y	M	F	1	0	0	25	NF	NF	NF	NF
47	Pup	2M	M	L,T	0	1	1	26	NF	NF	NF	NF
48	Ad	3Y	M	F,L	1	0	1	31	NF	NF	NF	NF
49	Ad	3Y	M	N	0	0	0	17	B	NF	NF	NF
50	Ad	5M	M	F	1	0	0	11	NF	NF	NF	NF
51	Ad	1.5Y	M	T	0	1	0	9	NF	NF	NF	NF
52	Ad	4Y	M	T	0	1	0	10	NF	NF	NF	NF
53	Juv	9M	M	T	0	1	0	10	E*	NF	NF	NF
54	Pup	6M	F	T,F	1	1	0	15	NF	NF	NF	NF
55	Ad	2Y	F	T	0	1	0	23	NF	NF	NF	NF
56	Ad	1.5Y	M	T	0	1	0	18	NF	NF	NF	NF

57	Ad	5Y	M	T,F	1	1	0	19	NF	NF	NF	NF
58	Ad	1Y	M	T,L	0	1	1	10	NF	NF	NF	NF
59	Ad	2Y	F	T,F	1	1	0	25	E*	NF	NF	NF
60	Ad	5Y	M	N	0	0	0	24	E*	NF	NF	NF
61	Ger	8Y	M	F	1	0	0	9	NF	NF	NF	NF
62	Ad	3Y	F	T	0	1	1	25	NF	NF	NF	NF
63	Juv	8M	M	T	0	1	1	22	NF	NF	NF	NF
64	Juv	8M	M	T,L	0	1	1	9	NF	NF	NF	NF
65	Juv	7M	F	T,L	0	1	1	20	NF	NF	NF	NF
66	Ad	3Y	M	T,L	0	1	1	27	NF	NF	NF	NF
67	Ad	4Y	M	T,F	1	1	1	20	NF	NF	NF	NF
68	Ad	2Y	M	T	0	1	0	26	NF	NF	NF	NF
69	Ad	2Y	F	T,L	0	1	0	14	NF	NF	NF	NF
70	Ad	6Y	F	T	0	1	0	26	NF	NF	NF	NF
71	Ad	5Y	M	T	0	1	0	24	B*	NF	NF	NF
72	Ad	4Y	M	T	0	1	0	35	NF	NF	NF	NF
73	Ad	1Y	M	T	0	1	0	27	NF	NF	NF	NF
74	Ger	10Y	M	T	0	1	0	15	NF	NF	NF	NF
75	Ad	3Y	F	F,L	1	0	1	30	NF	NF	NF	NF
76	Ad	3Y	M	L,F,T	1	0	1	29	NF	NF	NF	NF
77	Ger	10Y	M	N	0	0	0	28	NF	NF	NF	NF
78	Ad	4Y	M	T,L	0	1	1	30	NF	NF	NF	NF
79	Ad	3Y	M	T,L	1	1	1	13	B*	NF	NF	NF
80	Ad	2Y	M	T,L	1	1	1	12	NF	NF	NF	NF
81	Juv	1Y	M	T	1	0	1	25	NF	NF	NF	NF
82	Juv	1Y	M	F,L	0	1	0	23	NF	NF	NF	NF
83	Juv	8M	M	T	0	1	0	18	NF	NF	NF	NF
84	Ad	2Y	M	T	0	1	0	23	NF	NF	NF	NF
85	Ad	2Y	M	F	1	0	0	41	NF	NF	NF	NF
86	Ad	2Y	M	T	0	1	0	18	NF	NF	NF	NF
87	Ad	2Y	F	T,L	0	1	1	24	NF	NF	NF	NF
88	Ad	3Y	F	N	0	0	0	21	NF	NF	NF	NF

89	Ad	2Y	F	L	0	0	1	24	NF	NF	NF	NF
90	Ad	2Y	M	N	0	0	0	33	NF	NF	NF	NF
91	Ad	3Y	F	F,L	1	0	1	26	NF	NF	NF	NF
92	Ad	2Y	F	T	1	0	0	24	NF	NF	NF	NF
93	Ad	3Y	M	T,L	0	1	1	29	NF	NF	NF	NF
94	Ger	10Y	M	T,L	0	1	1	30	NF	NF	NF	NF
95	Ger	7Y	F	L	0	0	1	15	NF	NF	NF	NF
96	Ad	3Y	F	T,L	0	1	1	21	NF	NF	NF	NF
97	Ad	2Y	F	F,L	1	0	1	24	B*	NF	NF	NF
98	Ad	2.5Y	M	T,F	1	1	0	23	NF	NF	NF	NF
99	Ad	3Y	M	T,F	1	1	0	25	B*	NF	NF	NF
100	Ad	2Y	F	T,F	1	1	0	18	NF	NF	NF	NF

Table A3: Number of dog information and laboratory result record from Ondong Snay village, Ondong Snay commune, Roleaphea district, Kompong Chhnang province

Date of data collection: 20-21/Dec/14

ID	Age		Sex	Ecto-p	Ecto-p species			Lab examination				
					C. canis	*R. sanguinues*	*H. spiniger*	PCV	BCTBS	APA	MKT	PCR
1	Ger	15Y	F	F	1	0	0	23	NF	NF	NF	NF
2	Ad	3Y	M	F	1	0	0	34	NF	NF	NF	NF
3	Ad	2Y	F	T	0	1	0	26	NF	NF	NF	NF
4	Pup	4M	M	F	1	0	0	25	NF	NF	NF	NF
5	Pup	4M	M	F	1	0	0	20	NF	NF	NF	NF
6	Ger	8Y	M	F	1	0	0	21	NF	NF	NF	NF
7	Ad	3Y	M	N	0	0	0	23	NF	NF	NF	NF
8	Ad	4Y	F	F	1	0	0	34	NF	NF	NF	NF
9	Juv	1Y	F	N	0	0	0	18	NF	NF	NF	NF
10	Ad	3Y	F	F	1	0	0	23	NF	NF	NF	NF
11	Ad	2Y	M	N	0	0	0	35	NF	NF	NF	NF
12	Ger	9Y	F	F	1	0	0	27	NF	NF	NF	NF
13	Pup	3M	M	F	1	0	0	26	NF	NF	NF	NF
14	Juv	1Y	F	N	0	0	0	29	NF	NF	NF	NF
15	Ad	6Y	M	F	1	0	0	27	NF	NF	NF	NF
16	Ad	3Y	M	T	0	1	0	22	NF	NF	NF	NF
17	Ad	2Y	M	F	1	0	0	17	NF	NF	NF	NF
18	Juv	1Y	M	N	0	0	0	29	NF	NF	NF	NF
19	Ger	9Y	F	F	1	0	0	23	NF	NF	NF	NF
20	Juv	1Y	M	F	1	0	0	28	NF	NF	NF	NF
21	Ad	5Y	F	N	0	0	0	34	NF	NF	NF	NF
22	Ger	8Y	F	N	0	0	0	19	NF	NF	NF	NF
23	Ad	6Y	F	N	0	0	0	35	NF	NF	NF	NF
24	Pup	3M	F	F	1	0	0	28	NF	NF	NF	NF

25	Ad	3Y	F	F	1	0	0	30	NF	NF	NF	NF
26	Ad	2Y	F	N	0	0	0	23	NF	NF	NF	NF
27	Ger	10Y	F	F	1	0	0	26	NF	NF	NF	NF
28	Ad	2Y	M	N	0	0	0	23	NF	NF	NF	NF
29	Ad	1.5Y	F	F	1	0	0	19	NF	NF	NF	NF
30	Pup	5M	F	N	0	0	0	18	NF	NF	NF	NF
31	Ad	2Y	M	N	0	0	0	36	NF	NF	NF	NF
32	Juv	1Y	M	F,L	1	0	1	22	NF	NF	NF	NF
33	Ad	2Y	M	F,L	1	0	1	34	NF	NF	NF	NF
34	Ad	2Y	M	F	1	0	0	22	NF	NF	NF	NF
35	Ad	2Y	F	N	0	0	0	37	NF	NF	NF	NF
36	Ad	2Y	M	N	0	0	0	23	NF	NF	NF	NF
37	Pup	3M	M	F	1	0	0	30	NF	NF	NF	NF
38	Juv	1Y	M	N	0	0	0	26	NF	NF	NF	NF
39	Ad	2Y	F	T	0	1	0	37	NF	NF	NF	NF
40	Ad	6Y	M	N	0	0	0	23	filaria	NF	NF	NF
41	Ad	2.5Y	F	N	0	0	0	27	NF	NF	NF	NF
42	Juv	7M	M	F	1	0	0	27	NF	NF	NF	NF
43	Ad	1.5Y	F	T	0	1	0	34	NF	NF	NF	NF
44	Juv	1Y	M	T	0	1	0	21	NF	NF	NF	NF
45	Pup	3M	M	N	0	0	0	21	NF	NF	NF	NF
46	Pup	3M	F	N	0	0	0	22	NF	NF	NF	NF
47	Juv	1Y	M	F	1	0	0	18	NF	NF	NF	NF
48	Ger	9Y	M	F	1	0	0	16	NF	NF	NF	NF
49	Ad	7Y	M	F	1	0	0	18	NF	NF	NF	NF
50	Ad	7Y	M	N	0	0	0	29	NF	NF	NF	NF
51	Ad	1.5Y	M	L	0	0	1	24	NF	NF	NF	NF
52	Juv	1Y	M	F	1	0	0	16	NF	NF	NF	NF
53	Ger	12Y	M	N	0	0	0	22	NF	NF	NF	NF
54	Ad	4Y	F	L	0	0	1	22	NF	NF	NF	NF
55	Ger	10Y	M	F	1	0	0	9	NF	NF	NF	NF
56	Ad	1.5Y	M	N	0	0	0	17	NF	NF	NF	NF

57	Juv	1Y	M	N	0	0	0	15	NF	NF	NF	NF
58	Ad	3Y	M	N	0	0	0	19	NF	NF	NF	NF
59	Ger	10Y	F	N	0	0	0	18	NF	NF	NF	NF
60	Ad	1.3Y	F	N	0	0	0	21	NF	NF	NF	NF
61	Ad	2Y	F	F	1	0	0	22	NF	NF	NF	NF
62	Ad	7Y	F	F	1	0	0	24	NF	NF	NF	NF
63	Juv	1Y	F	F	1	0	0	23	NF	NF	NF	NF
64	Ad	3Y	M	N	0	0	0	23	NF	NF	NF	NF
65	Ger	8Y	M	N	0	0	0	21	NF	NF	NF	NF
66	Ad	2Y	F	N	0	0	0	18	NF	NF	NF	NF
67	Juv	1Y	F	N	0	0	0	21	NF	NF	NF	NF
68	Ad	3Y	M	N	0	0	0	19	NF	NF	NF	NF
69	Ad	2Y	F	N	0	0	0	21	NF	NF	NF	NF
70	Juv	8M	M	F	1	0	0	30	NF	NF	NF	NF
71	Ad	3Y	M	N	0	0	0	28	NF	NF	NF	NF
72	Juv	1Y	F	N	0	0	0	24	NF	NF	NF	NF
73	Juv	11M	M	F,T	1	1	0	17	NF	NF	NF	NF
74	Pup	3M	M	N	0	0	0	24	NF	NF	NF	NF
75	Ger	8Y	M	N	0	0	0	23	NF	NF	NF	NF
76	Ger	10Y	F	N	0	0	0	23	NF	NF	NF	NF
77	Ad	7Y	M	N	0	0	0	31	NF	NF	NF	NF

Table A4: Number of dog information and laboratory results recorded from Tror Keat village, Roka commune, Pea Raeng district, Prey Veng province.

Date of data collection: 29-31/Jan/15

ID	Age		Sex	Ecto-p	Ectoparasitic species			Lab examination				
					C. canis	*R. sanguin*	*H. spinige*	PCV	BCTBS	APA	MKT	PCR
1	Ad	2Y	F	F	1	0	0	35	NF	NF	NF	NF
2	Ad	2.3Y	M	F	1	0	0	18	NF	NF	NF	NF
3	Ad	4Y	M	F	1	0	0	31	NF	NF	NF	NF
4	Ger	15Y	F	F	1	0	0	30	NF	NF	NF	NF
5	Pup	4M	M	F	1	0	0	23	NF	NF	NF	NF
6	Juv	1Y	M	F	1	0	0	19	NF	NF	NF	NF
7	Ger	8Y	F	F	1	0	0	27	NF	NF	NF	NF
8	Juv	1Y	F	F	1	0	0	21	NF	NF	NF	NF
9	Ad	2Y	M	F	1	0	0	25	NF	NF	NF	NF
10	Pup	4M	F	F	1	0	0	25	NF	NF	NF	NF
11	Juv	8M	F	F	1	0	0	29	NF	NF	NF	NF
12	Juv	1Y	F	F	1	0	0	24	NF	NF	NF	NF
13	Pup	4M	F	F	1	0	0	26	NF	NF	NF	NF
14	Ad	4Y	F	N	0	0	0	35	NF	NF	NF	NF
15	Ad	4Y	F	N	0	0	0	28	filaria	NF	NF	NF
16	Ad	2Y	F	F	1	0	0	25	NF	NF	NF	NF
17	Ad	6Y	F	N	0	0	0	34	NF	NF	NF	NF
18	Pup	2M	F	F	1	0	0	16	NF	NF	NF	NF
19	Ad	6Y	F	L	0	0	1	24	NF	NF	NF	NF
20	Ad	5Y	M	L	0	0	1	40	NF	NF	NF	NF
21	Ad	4Y	F	N	0	0	0	30	NF	NF	NF	NF
22	Ad	2Y	F	F	1	0	0	16	NF	NF	NF	NF
23	Ad	3Y	F	N	0	0	0	18	NF	NF	NF	NF
24	Ad	4Y	M	F	1	0	0	36	NF	NF	NF	NF
25	Ad	3Y	F	F	1	0	0	15	NF	NF	NF	NF

26	Pup	5M	M	F	1	0	0	21	NF	NF	NF	NF
27	Juv	1Y	M	F	1	0	0	25	NF	NF	NF	NF
28	Pup	3M	F	F	1	0	0	34	NF	NF	NF	NF
29	Pup	5M	M	F	1	0	0	26	NF	NF	NF	NF
30	Pup	3M	M	F	1	0	0	25	NF	NF	NF	NF
31	Pup	3M	F	F	1	0	0	10	NF	NF	NF	NF
32	Ad	3Y	F	N	0	0	0	25	NF	NF	NF	NF
33	Ger	10Y	M	F	1	0	0	36	NF	NF	NF	NF
34	Ad	5Y	M	F	1	0	0	37	filaria	NF	NF	NF
35	Ad	2Y	F	F	1	0	0	26	NF	NF	NF	NF
36	Ad	4Y	M	F	1	0	0	21	NF	NF	NF	NF
37	Juv	1Y	M	N	0	0	0	40	NF	NF	NF	NF
38	Juv	1Y	M	N	0	0	0	24	NF	NF	NF	NF
39	Ad	3Y	F	L	0	0	1	28	NF	NF	NF	NF
40	Ad	4Y	F	F	1	0	0	27	NF	NF	NF	NF
41	Ad	7Y	F	F	1	0	0	30	NF	NF	NF	NF
42	Juv	1Y	F	L	0	0	1	35	NF	NF	NF	NF
43	Ad	5Y	M	L	0	0	1	21	NF	NF	NF	NF
44	Pup	5M	M	N	0	0	0	35	NF	NF	NF	NF
45	Ad	3Y	M	F	1	0	0	40	NF	NF	NF	NF
46	Pup	2M	M	N	0	0	0	31	NF	NF	NF	NF
47	Juv	1Y	M	F	1	0	0	20	NF	NF	NF	NF
48	Ad	5Y	M	F	1	0	0	26	NF	NF	NF	NF
49	Ad	3Y	M	F	1	0	0	34	NF	NF	NF	NF
50	Ad	4Y	M	N	0	0	0	24	NF	NF	NF	NF
51	Ger	15Y	M	L	0	0	1	15	NF	NF	NF	NF
52	Pup	4M	M	F	1	0	0	10	NF	NF	NF	NF
53	Ad	3Y	F	N	0	0	0	20	NF	NF	NF	NF
54	Juv	8M	M	N	0	0	0	24	NF	NF	NF	NF
55	Juv	8M	F	N	0	0	0	26	NF	NF	NF	NF
56	Juv	8M	M	N	0	0	0	25	NF	NF	NF	NF
57	Pup	2M	F	F	1	0	0	29	NF	NF	NF	NF

58	Pup	2M	M	F	1	0	0	28	NF	NF	NF	NF
59	Juv	1Y	M	F	1	0	0	25	NF	NF	NF	NF
60	Ad	4Y	F	F	1	0	0	28	NF	NF	NF	NF
61	Ad	2Y	M	F	0	0	0	35	NF	NF	NF	NF
62	Ad	8Y	M	F	1	0	0	14	NF	NF	NF	NF
63	Juv	1Y	F	F	1	0	0	24	NF	NF	NF	NF
64	Ad	5Y	M	F	1	0	0	28	NF	NF	NF	NF
65	Ad	4Y	M	N	0	0	0	21	NF	NF	NF	NF
66	Ad	6Y	M	F	1	0	0	36	NF	NF	NF	NF
67	Ad	4Y	M	F	1	0	0	23	NF	NF	NF	NF
68	Ad	5Y	F	L	0	0	1	6	NF	NF	NF	NF
69	Ad	5Y	M	L	0	0	1	24	NF	NF	NF	NF
70	Pup	4M	M	F	1	0	0	26	NF	NF	NF	NF
71	Ad	2Y	M	N	0	0	0	23	NF	NF	NF	NF
72	Ad	3Y	M	F	1	0	0	21	NF	NF	NF	NF
73	Ad	2Y	M	F	1	0	0	26	NF	NF	NF	NF
74	Pup	2M	F	F	1	0	0	22	NF	NF	NF	NF
75	Pup	3M	M	F	1	0	0	17	NF	NF	NF	NF
76	Ad	2Y	F	F	1	0	0	42	NF	NF	NF	NF
77	Ad	2Y	M	F	1	0	0	15	NF	NF	NF	NF
78	Juv	1Y	M	F	1	0	0	34	NF	NF	NF	NF
79	Ad	3Y	F	F	1	0	0	25	NF	NF	NF	NF
80	Ad	2Y	F	N	0	0	0	34	NF	NF	NF	NF
81	Ad	3Y	F	F	1	0	0	26	NF	NF	NF	NF
82	Ad	5Y	M	F	1	0	0	26	NF	NF	NF	NF
83	Ger	10Y	M	F	1	0	0	33	NF	NF	NF	NF
84	Juv	1Y	F	F	1	0	0	20	NF	NF	NF	NF
85	Ad	7Y	F	N	0	0	0	21	NF	NF	NF	NF
86	Ad	2Y	F	N	0	0	0	21	NF	NF	NF	NF
87	Ad	2Y	M	N	0	0	0	30	NF	NF	NF	NF

Table A5: Number of dog information and laboratory results recorded from Phuthi Vong village, Trorsut commune, Svay Theab district, Svay Rieng province

Date of data collection: 01-02/Feb/15

ID	Age		Sex	Ecto-p	Ecto-p species			Lab examination				
					C. canis	*R. sanguineus*	*H. spiniger*	PCV	BCTBS	APA	MKT	PCR
1	Juv	1Y	F	F, T	1	1	0	21	NF	NF	NF	NF
2	Pup	3M	F	F, T	1	1	0	34	NF	NF	NF	NF
3	Ger	10Y	M	F, T	1	1	0	16	NF	NF	NF	NF
4	Ad	2Y	M	F, T	1	1	0	16	NF	NF	NF	NF
5	Ad	3Y	M	F, T	1	1	0	20	NF	NF	NF	NF
6	Ad	5Y	F	F	1	0	0	30	NF	NF	NF	NF
7	Pup	5M	F	F	1	0	0	32	NF	NF	NF	NF
8	Juv	1Y	F	F	1	0	0	29	NF	NF	NF	NF
9	Ad	2Y	M	F, T	1	1	0	25	NF	NF	NF	NF
10	Ad	5Y	F	F	1	0	0	32	NF	NF	NF	NF
11	Juv	9M	F	L, T	0	1	1	30	NF	NF	NF	NF
12	Ad	1.5Y	F	F	1	0	0	38	NF	NF	NF	NF
13	Ad	3Y	M	L	0	0	1	21	NF	NF	NF	NF
14	Juv	7M	M	F, T, L	1	1	1	22	NF	NF	NF	NF
15	Juv	7M	F	F, T, L	1	1	1	24	NF	NF	NF	NF
16	Ad	3Y	M	F	1	0	0	23	NF	NF	NF	NF
17	Ad	3Y	M	F	1	0	0	23	NF	NF	NF	NF
18	Ad	3Y	F	F	1	0	0	24	NF	NF	NF	NF
19	Ad	5Y	F	F	1	0	0	24	NF	NF	NF	NF
20	Juv	8M	M	F	1	0	0	37	NF	NF	NF	NF
21	Ad	1.5Y	M	F	1	0	0	25	NF	NF	NF	NF
22	Ad	4Y	F	F	1	0	0	21	NF	NF	NF	NF
23	Ad	2Y	F	F,	1	0	0	33	NF	NF	NF	NF
24	Ad	3Y	F	F	1	0	0	31	NF	NF	NF	NF

25	Ad	5Y	F	F, T	1	1	0	28	NF	NF	NF	NF
26	Ger	10Y	M	F	1	0	0	27	NF	NF	NF	NF
27	Ger	10Y	F	F	1	0	0	24	NF	NF	NF	NF
28	Pup	5M	F	F	1	0	0	31	NF	NF	NF	NF
29	Ger	10Y	M	F	1	0	0	42	NF	NF	NF	NF
30	Juv	7M	F	F	1	0	0	27	NF	NF	NF	NF
31	Ad	1.5Y	M	F	1	0	0	23	NF	NF	NF	NF
32	Juv	8M	M	F	1	0	0	33	B*	NF	NF	NF
33	Ad	1.5Y	F	F	1	0	0	15	NF	NF	NF	NF
34	Ad	2Y	F	F	1	0	0	23	NF	NF	NF	NF
35	Ad	2Y	F	F	1	0	0	15	NF	NF	NF	NF
36	Pup	4M	F	F, L	1	0	1	24	NF	NF	NF	NF
37	Ger	15Y	F	F	1	0	0	16	NF	NF	NF	NF
38	Ger	8Y	F	F, L	1	0	1	18	NF	NF	NF	NF
39	Pup	3M	M	F, T, L	1	1	0	21	NF	NF	NF	NF
40	Ad	3Y	F	F, T	1	1	0	28	NF	NF	NF	NF
41	Ad	1.5Y	F	F	1	0	0	21	NF	NF	NF	NF
42	Ad	2Y	M	F	1	0	0	20	NF	NF	NF	NF
43	Ger	10Y	F	F, T, L	1	1	1	15	NF	NF	NF	NF
44	Ad	5Y	F	F	1	0	0	21	NF	NF	NF	NF
45	Ad	1.5Y	F	F	1	0	0	25	NF	NF	NF	NF
46	Ad	1.5Y	F	F	1	0	0	29	NF	NF	NF	NF
47	Pup	2M	F	F	1	0	0	15	NF	NF	NF	NF
48	Ad	3Y	F	F	1	0	0	21	NF	NF	NF	NF
49	Ad	3Y	F	F	1	0	0	21	NF	NF	NF	NF
50	Ad	5M	M	F	1	0	0	22	NF	NF	NF	NF
51	Ad	1.5Y	M	F	1	0	0	24	E*	NF	NF	NF
52	Ad	4Y	F	F	1	0	0	19	NF	NF	NF	NF
53	Juv	9M	M	F	1	0	0	19	NF	NF	NF	NF
54	Pup	6M	M	F	1	0	0	19	NF	NF	NF	NF
55	Ad	2Y	M	F	1	0	0	29	NF	NF	NF	NF
56	Ad	1.5Y	F	F, T	1	1	0	29	NF	NF	NF	NF

57	Ad	5Y	F	F	1	0	0	24	NF	NF	NF	NF
58	Ad	1Y	M	F	1	0	0	24	NF	NF	NF	NF
59	Ad	2Y	F	F	1	0	0	26	NF	NF	NF	NF
60	Ad	5Y	F	F	1	0	0	29	NF	NF	NF	NF
61	Ger	8Y	M	F	1	0	0	29	NF	NF	NF	NF
62	Ad	3Y	F	F	1	0	0	24	NF	NF	NF	NF
63	Juv	8M	M	F	1	0	0	25	NF	NF	NF	NF
64	Juv	8M	F	F	1	0	0	29	NF	NF	NF	NF
65	Juv	7M	M	F	1	0	0	20	NF	NF	NF	NF
66	Ad	3Y	M	F	1	0	0	23	NF	NF	NF	NF
67	Ad	4Y	F	F, T	1	1	0	25	NF	NF	NF	NF
68	Ad	2Y	F	F	1	0	0	33	NF	NF	NF	NF
69	Ad	2Y	F	F	1	0	0	24	NF	NF	NF	NF
70	Ad	6Y	M	F	1	0	0	34	NF	NF	NF	NF

Table A6*:* Number of dog information and laboratory result recorded from a private clinic (Veterinary Service Center), #77A, St. 95, Boeung Oeng Kong III commune, Chomkar Morn district in Phnom Penh city.

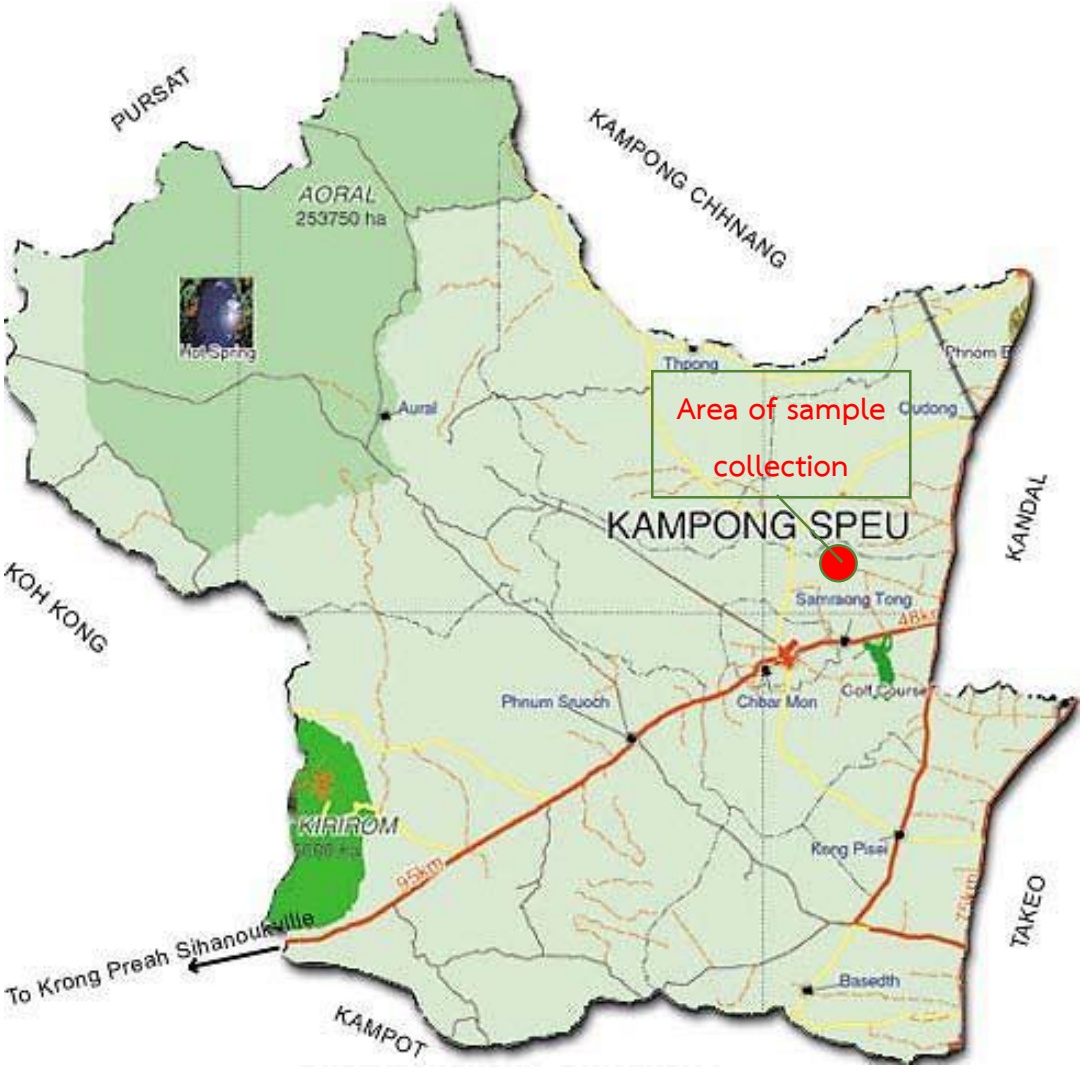
Date of data collection: 04-06/ Feb/15

ID	Age		Sex	Ecto-p	Ecto-p species			Lab examination				
					C. canis	*R. sanguineus*	*H. spiniger*	PCV	BCTBS	APA	MKT	PCR
1	Pup	7M	M	N	0	0	0	12	NF	NF	NF	NF
2	Ad	2Y	M	N	0	0	0	33	NF	NF	NF	NF
3	Pup	3M	M	T	0	1	0	30	NF	NF	NF	NF
4	Pup	3M	F	N	0	0	0	15	NF	NF	NF	NF
5	Ger	10Y	M	N	0	0	0	30	NF	NF	NF	NF
6	Pup	2M	F	N	0	0	0	19	NF	NF	NF	NF
7	Ad	3Y	F	N	0	0	0	10	NF	NF	NF	NF
8	Pup	5M	F	N	0	0	0	26	B*	NF	NF	YES
9	Ad	5Y	F	N	0	0	0	38	E*	NF	NF	NF
10	Ger	12Y	M	N	0	0	0	28	NF	NF	NF	NF
11	Ad	5Y	F	N	0	0	0	35	NF	NF	NF	NF
12	Ad	2Y	F	N	0	0	0	38	NF	NF	NF	NF
13	Ger	10Y	F	N	0	0	0	32	NF	NF	NF	NF

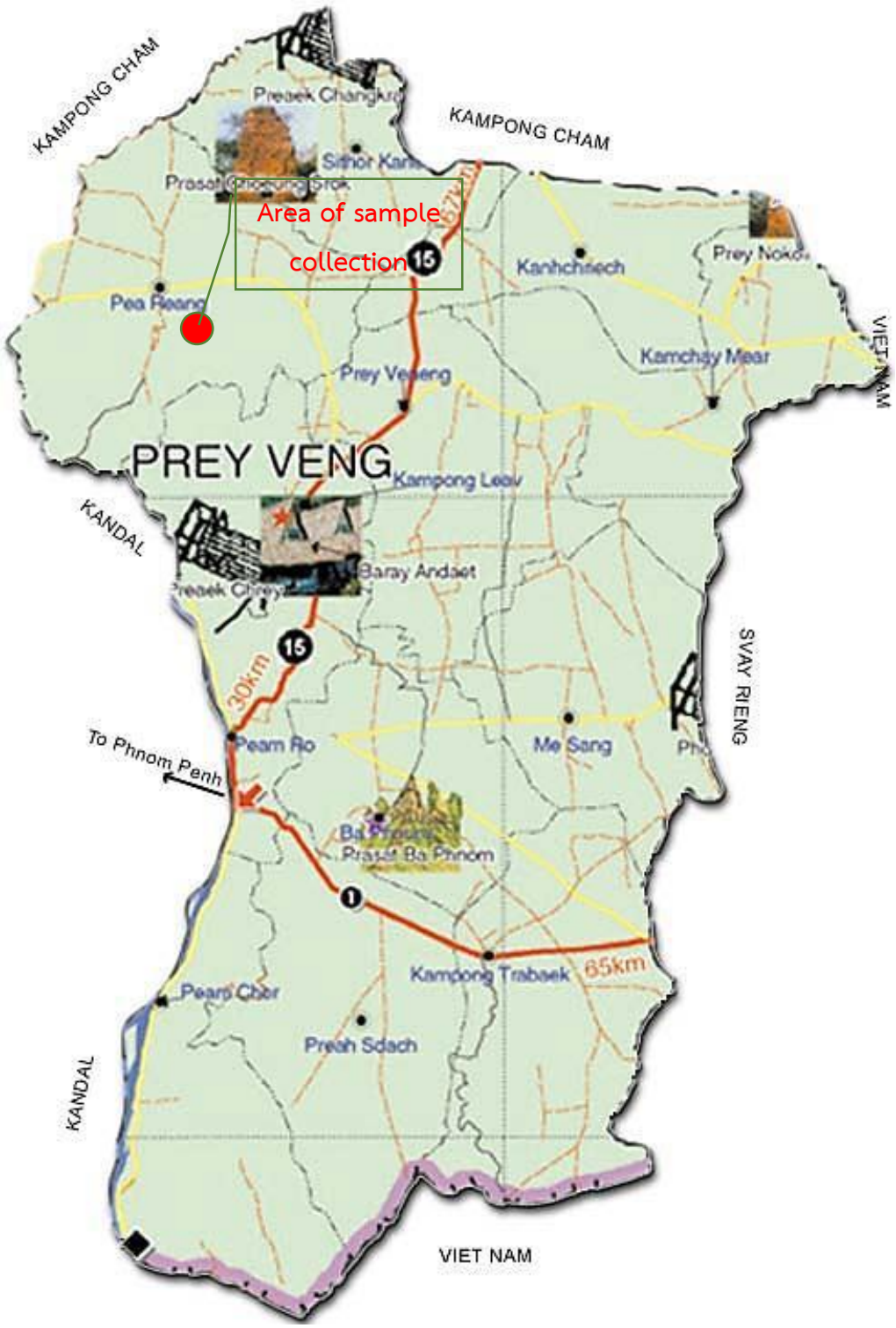
APPENDIX II

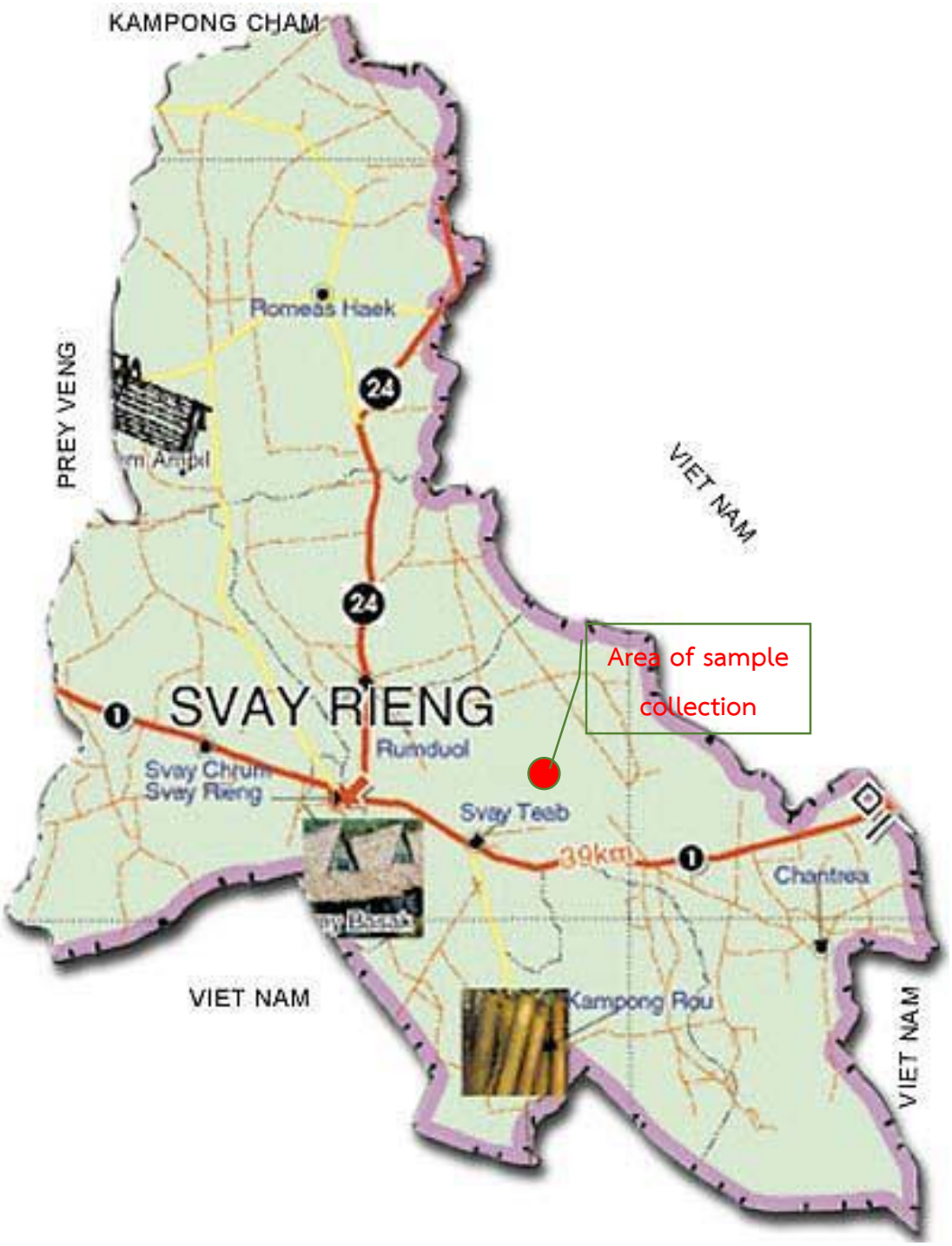
Map of sample collection


KAMPONG SPEU
KANDAL
KAMPOT
KAMPOT
VIET NAM
TAKEO
Area of sample collection
Tram Kak
Doun Kaev
Angkor Borei
Ponhea Lueu
Treang
Bourei Cholsar
Kaoh Andaet
Kiri Vong
Phnom Bayang
Prey Kabbas
Prasat
Neang Khmau
Phnom Chi Sou
Samraong
76km
52km


PURSAT
KAMPONG CHHNANG
AORAL
253750 ha
Hot Spring
Thpong
Aural
Area of sample collection
KAMPONG SPEU
KANDAL
KOH KONG
Samraong Tong
Phnum Sruoch
Chbar Mon
Golf Course
KIRIROM
Kong Pisei
95km
TAKEO
To Krong Preah Sihanoukville
Basedth
KAMPOT


KAMPONG THOM
To Pursat
Banbour
62km
PURSAT
Ponna Reay
Kampong Leaeng
Phnum Kdang
Romeas
Rolea Bier
Phnom Sruouch
Phnom Ta Reach
Phnom Roab Bat
Chol Kiri
KAMPONG CHHNANG
Tuek Phos
Area of sample collection
KAMPONG CHAN
Kampong Tralach
KAMPONG SPEU
Sameakki Mean Chey
KANDAL


KAMPONG CHAM
KAMPONG CHAM
Area of sample collection
Kanhchriech
Prey Nokor
Pea Reang
Kamchay Mear
VIET NAM
Prey Veaeng
PREY VENG
Kampong Leav
KANDAL
Baray Andaet
15
30km
SVAY RIENG
To Phnom Penh
Pearn Ro
Me Sang
Prasat Ba Phnom
1
Kampong Trabaek
65km
Pearn Chor
Preah Sdach
KANDAL
VIET NAM


KAMPONG CHAM
Romeas Haek
PREY VENG
VIET NAM
24
24
SVAY RIENG
Rumduol
Svay Chrum
Svay Rieng
Svay Teab
39km
Chantrea
Area of sample collection
VIET NAM
Kampong Rou
VIET NAM


PHNOM PENH
Monivong Blvd.
Tonle Sap
The Royal
Boeng Kak
Wat Phnom
Pochentong Blvd.
Market
Kampuchea Krom Blvd.
The Juliana Hotel
Norodom Blvd.
Mao Tse Tung Blvd.
Charles de Gaulle Blvd.
Area of sample collection
Sofitel Cambodiana
Olympic Stadium
Monireth Blvd.
Preah Sihanouk Blvd.
Samdech Sothearos Blvd.
Inter-Continental
Monivong Blvd.
Royal Phnom Penh
Mao Tse Tung Blvd.
Bassac

APPENDIX III

The protocol for blood staining

1. Giemsa staining

Giemsa staining was formulated from the manufactory's induction, Merck KGaA, Germany as below:

- ❖ Stock Giemsa:
  - Giemsa's stain 0.6 g
  - Methyl alcohol or absolute or acetone free 50 ml
  - Glycerine 50 ml
- ❖ Buffer water (pH 7.2):
  - Na_2HPO_4 1.0 g or $Na_2HPO_4 12H_2O$ 2,5g
  - KH_2PO_4 0.7 g
  - Distilled water 1,000 ml

2. Wright giemsa staining

Wright giemsa was prepared from the manufactory's induction, Merck KGaA, Germany as below:

- ❖ Stock wright giemsa:
  - Wright giemsa 12g
  - Giemsa 1,6g
  - Methanol or Acetone free 100% 4000 ml
- ❖ Stock buffer wright giemsa (pH 6.8)
  - Na_2HPO_4 9.47 g/l
  - $KH_2 PO_2$ 9.08 g/l

3. Acid phosphatase activity (APA)

The incubating medium was contained of:

❖ **Solution 1**: Michaelis veronal acetate buffer

- Sodium acetate 9.714g
- Sodium barbital 14.714g
- Distilled water with pH 10 500ml

❖ **Solution 2**: Naphthol AS-TR phosphate

- Naphthol AS-TR phosphate salt 0.05g
- N-N dimethyl formamide 5.0g

❖ **Solution 3**: Patarosaniline

- Pararosaniline hydrochloride 1.0g
- HCl 5.0g
- Distilled water 20.0g

❖ **Solution 4**: Sodium nitrite 4 %

- Sodium nitrite 4.0g
- Distilled water 100 ml

VITA

My name is Koemseang Nhuong. I was born on 8th January, 1988 in Takeo province. In 2008, I finished my general academic from Hun Sen Angkor Borey high school, Takeo, Cambodia. After that I pursued Bachelor degree in a field of Veterinary Medicine, Faculty of Animal Science and Veterinary Medicine, Royal University of Agriculture (RUA) and at the same time I got a scholarship in a major of Teaching English as a Foreign Language, Faculty of Education Arts and Humanities, Beltei International Institute, Phnom Penh, Cambodia, from 2008-2012. Moreover, during of my studies I had joined some of activities and worked as a volunteer such as Research Assistant in Parasitology Laboratory, Division of Research and Extension, RUA; Phnom Penh, Cambodia; Join training of CeVa Animal Health Asia Pacific, Thailand; Poster Presentation on " The 4th ICERD-International Conference on Environmental and Rural Development, Siem Reab, 2013; Technical Trainer at Mong Reththy Group M's Pig ACMC for three months in 2012; Liaison Officer (LO) for ASEAN Meeting (The 32nd AMAF, The 10th AMAF+3, The 2nd ASEAN-China Ministerial on SPS and Related Meeting), RUA and Ministry of Agriculture, Forestry and Fisheries (MAFF), Phnom Penh in 2010 and other activities and also got some certificates.

Shortly, I got a scholarship award of master's degree from Chulalongkorn University Graduate Scholarship Program for ASEAN countries for two years. A long with this I had an oral presentation in an international conference "The 14th Chulalongkorn University Veterinary Conference (CUVC2015)", Thailand. Also, I presented some of my work and progress reports during study.