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Detection of Pathogens on the Brown Dog Tick, *Rhipicephalus sanguineus* sensu lato (s.l.) (Arachnida: Acari: Ixodidae) in the Philippines

Kathlyn Bartolome-Cruz

College of Veterinary Medicine, Cagayan State University,
Carig, Tuguegarao City, Cagayan 3500 Philippines

The objective of this study was to determine the negative effects of *Rhipicephalus sanguineus* sensu lato (s.l.) on infested dogs and its vector potential in three sites of the Philippines. To perform this study, blood samples were obtained from 953 dogs naturally infested with *R. sanguineus* (s.l.) ticks from three distinct localities in the Philippines (Los Baños, Laguna; Quezon City, Metro Manila; and Pasay City, Metro Manila). In the molecular detection of hemoparasites in *R. sanguineus* (s.l.) infested dogs, 6.40% were diagnosed positive with hemoparasites: *Hepatozoon* spp. (3.67%), *Babesia* spp. (2.00%), and *Ehrlichia* spp. (0.73%). *R. sanguineus* (s.l.) infested dogs positive with hemoparasites age range were 1–3 years old. Males (52.46%) were more infected than female (47.54%). The crossbreeds (24. 60%) were the most infected with hemoparasite. In the detection of tick-carried pathogens vectored by *R. sanguineus* (s.l.), 29 untreated dogs were collected with ticks. Age range were observed within 1–3 years old, there were more male 20 (68.97%) than female 9 (31.03%), and most were crossbreed (51.72%) dogs. Nested PCR total detection rate was 12.50%: *Babesia* spp. (2.08%), *Hepatozoon* spp. (2.08%), and *Ehrlichia* spp. (8.33%). Engorged adult female and male ticks were detected positive with the tick-carried pathogens. Co-infection of *Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. was also detected. BLASTS analysis confirmed the sequence identities of the positive tick samples as *Ehrlichia canis.*

Key words: molecular detection, *Rhipicephalus sanguineus* sensu lato (s.l.), tick-carried pathogens

INTRODUCTION

Rhipicephalus sanguineus sensu lato (s.l.) or commonly known as the brown dog tick stands out as the most relevant and economically important ectoparasite affecting the small-animal practitioner, specifically the dog breeders (Graf et al. 2004). Endless concerns with its control and prevention demand a vast knowledge of this parasite's life cycle, aiming to perform treatments in seasons that are not favorable to the ticks.

*Corresponding author: kbcruz@up.edu.ph

Due to its veterinary and public health relevance, *R. sanguineus* (s.l.) is one of the most studied ticks in other countries (Walker et al. 2005). It is the most widespread tick in the world, making it an important reservoir and vector of pathogens (Dantas-Torres 2010) affecting domestic animals and humans (Jongejan & Uilenberg 2004; Otranto et al. 2009).

The brown dog tick or kennel tick is a parasite of dogs but can occasionally parasitize other hosts, including humans. It is the vector of canine ehrlichiosis caused by *Ehrlichia canis* (Rickettsiales: Anaplasmataceae), canine babesiosis caused by *Babesia vogeli* or *Babesia gibsoni* (Piroplasmida:

Babesiidae), and canine hepatozoonosis caused by *Hepatozoon canis* (Eucoccidiorida: Hepatozoidae). Moreover, *R. sanguineus* (s.l.) is a vector of many disease agents – some of them being of zoonotic concern as *Coxiella burnetii* (Legionellalea: Coxiellaceae)*, Ehrlichia canis* (Rickettsiales: Anaplasmaticeae)*, Rickettsia conorii* (Rickesialles: Rickettsiacea), and *Rickettsia rickettsii* (Rickesialles: Rickettsiacea) (Dantas-Torres 2008, 2010).

In this study, the negative effects of *R. sanguineus* (s.l.) on infested dogs and its vector potential was determined.

MATERIALS AND METHODS

Description of the Studied Area and Sampling

This study was conducted from Jan 2016 to May 2017. The veterinary importance of *R. sanguineus* (s.l.) were studied in three sampling areas, namely: Veterinary Teaching Hospital of the College of Veterinary Medicine at the University of the Philippines Los Baños (VTH UPLB), Laguna; Veterinary Teaching Hospital of the College of Veterinary Medicine at the University of the Philippines Diliman (VTH UPD), Quezon City, Metro Manila; and Private Veterinary Clinic at Pasay City, Metro Manila.

To perform this study, blood samples were obtained from 953 dogs (VTH UPD, 517; VTH UPLB, 185; and Private Veterinary Clinic, 251) naturally infested with *R. sanguineus* (s.l.) ticks from three previous described distinct localities in the Philippines (Los Baños, Laguna; Quezon City, Metro Manila; and Pasay City, Metro Manila). These animals were inspected for the presence of ticks – the abnormal clinical signs were observed and diagnostic test like packed cell volume (PCV), complete blood count (CBC), and blood parasite examination (BPE) were performed and gathered in the sampling areas.

The identification and description of *R. sanguineus* (s.l.) were performed at the Department of Veterinary Paraclinical Sciences of the College of Veterinary Medicine at the University of the Philippines Los Baños, Laguna. The tick samples were processed for molecular analysis at the Veterinary Molecular Biology Laboratory of the Department of Veterinary Paraclinical Sciences at the College of Veterinary Medicine, University of the Philippines Los Baños, Laguna.

Ethical Considerations

The procedures on animals in this study have been approved by the Institutional Animal Care and Use Committee of UPLB and with the approval of the attending veterinarians, chief veterinarians, and/or proprietor of the veterinary establishment.

Detection and Frequency of Hemoparasite Infection

Blood Collection, Staining, and Examination. All standard procedures for blood collection, staining, and examination of hemoparasite in dog samples can be found in Appendix I. BPE or hemoparasite examination of the stained blood smears were done using immersion microscopy (1000x). The analyzed samples were considered positive by cytological examinations for canine babesiosis when revealed the presence of paired large merozoites pair tear-drop forms in the erythrocyte. The same applies for canine hepatozoonosis (with the presence of intracytoplasmic ellipsoidal-shaped gamonts in the neutrophils) and ehrlichiosis (revealing the presence of *Ehrlichia* morulae, an intracytoplasmic inclusion bodies within a white blood cell). Stained blood smears that were positive for hemoparasite were recorded and documented.

Frequency of Hemoparasite Infection. Records of *R. sanguineus* (s.l.) infested dogs positive for hemoparasite in the three sampling areas from Jan 2016 to Mar 2017 were gathered to obtain the data for the signalment (respiratory rate, heart rate, weight, and temperature); history/chief complaint; clinical signs; diagnostic test results; and diagnosis and status of the patient. The frequency of *R. sanguineus* (s.l.) infested dogs positive for hemoparasite were associated with dog's age range, gender (male or female), and breed.

Detection of Tick-Carried Pathogens in *R. sanguineus* (s.l.)

Host Selection, Tick Collection, and Sorting. Out of 97 dogs, 29 subjects untreated with acaricides admitted in previous described three sampling areas were used in this part of the study. The animals that did not receive the acaricide treatment were inspected for the presence of *R. sanguineus* (s.l.). The collected tick samples were sorted as follows: 1 engorged adult female tick – 1 polypropylene tube, 2 partially engorged adult female – 1 polypropylene tube, and 5 unfed adult female/ unfed adult male/ nymph – 1 polypropylene tube. In total, 52 (18 adult male, 23 adult female, and 11 nymph) samples of ticks were collected, all of which were submitted to deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR).

Ticks' DNA Extraction. Boiling method adapted from Takano et al. (2014) was done to extract DNA from the ticks. Each pooled tick sample was washed first with 500 µL 1x phosphate buffer solution (PBS). After washing, the PBS was removed by aspiration. Two hundred microliters (200 µL) of 25 mM sodium hydroxide (NaOH) was added directly to unfed adult female/ unfed adult male/ nymph tick samples, while 500 µL of the same solution was added to fully and partially engorge adult female ticks. The ticks were crushed thoroughly using sterile tube pestle to expel the contents completely. Afterwards, the tubes containing

the homogenates were placed in boiling water bath for 10 min. The samples were cooled down for approximately 20 min afterwards. Sixteen microliters (16 µL) of 1 M Tris HCl buffer was added to each tube sample containing unfed adult female/ unfed adult male/ nymph ticks and 40 µL of the same solution to each tube sample containing nearly to fully and partially engorged adult female ticks. The samples were centrifuged at 20,000 g for 5 min and the supernatant (DNA) was transferred to a new sterile tube.

Detection of Marker Genes Using Conventional PCR. To confirm the success of DNA extraction from the 52 tick samples, PCR was performed to detect the marker gene mt-rrs in ticks (Ushijima et al. 2003; Andoh et al. 2015). For each sample, 10 µL of PCR mixture was prepared by mixing 5 µL of 2x Gflex buffer; 0.4 µL each of 5 µM forward and reverse primers of mt-rrs (Appendix Table 1); 2.1 µL sterile nuclease-freewater; 0.1 µL Tks Glex® DNA polymerase (Takara, Shiga, Japan); and 2 µL DNA.

PCR was carried out in a thermal cycler (Hercuvan®, Hercuvan Lab Systems, USA and Proflex®, Applied Biosystems, USA). The PCR condition for mt-rrs target gene (Ushijima et al. 2003) were as follows: initial denaturation for 10 min at 94 °C followed by a 30 temperature-step cycle program consisting of denaturation for 30 s at 94 ° C, annealing for 30 s at 56 °C, and an extension for 30 s at 72 °C. Afterwards, a final extension for 5 min at 72 °C ensued.

Nested PCR for Detection of Tick-carried Pathogens. Extracted DNA from the 48 tick samples with positive bands for *mt-rrs* genes were selected during the first round of nested PCR for detecting the presence of *Babesia* spp./ *Hepatozoon* spp. and *Ehrlichia* spp. Except for the primers used, the PCR mixture was prepared with the same composition as described above in PCR for mt-rrs genes. The second round of nested PCR established the use of 1 µL of the product obtained from the first round.

For the first round of PCR, the primers used for DNA amplification of *Babesia* spp./ *Hepatozoon* spp. were $BT1_1$ and $HPF2P_1$. For the second round, all primers used in the study are listed in Appendix Table 2. The conditions were adjusted according to the recommended annealing temperature of the primers. For the conventional amplification of the *18S rRNA* target gene for *Babesia* spp./ *Hepatozoon* spp. (Criado-Fornelio et al. 2006; Tateno et al. 2015), the samples undergone an initial denaturation for 10 min at 94 °C followed by a 30 temperature- step cycle program consisting of denaturation for 40 sec at 95 °C, annealing for 40 sec at 63 °C, and an extension for 1 min and 30 sec at 72 °C. To conclude, a final extension for 5 min at 72 °C followed, thereby producing the first PCR product. The next round of PCR was carried out under similar condition. For *Babesia* spp. (Birkenheuer et al. 2003; Tateno et al. 2015), initial denaturation was done for 5 min at 95 °C followed by a 30 temperature- step cycle program consisting of denaturation for 30 sec at 94 °C, annealing for 30 sec at 58 °C, and an extension for 1 min and 30 sec at 72 °C. Afterwards, a final extension was done for 5 min at 72 °C. For *Hepatozoon* spp. (Criado-Fornelio et al. 2006; Tateno et al. 2015), initial denaturation was done for 10 min at 94 °C followed by a 30 temperature-step cycle program consisting of denaturation for 40 sec at 95 °C, annealing for 40 sec at 60 °C, and an extension for 1 min at 72 °C. Afterwards, a final extension was done for 5 min at 72 °C. Negative controls using sterile nuclease-free water were included in each PCR run. For nested PCR detection of *Babesia* spp., DNA extracted from an *in vitro* culture of *B. gibsoni* acquired from the Laboratory of Infectious Diseases of Kagoshima University, Japan was used as a positive control.

For the DNA amplification of *Ehrlichia* spp., the primers used were $gro607F_1$ and $gro1294R_1$ for the first round of PCR. For the second round, all primers used in the study are listed in Appendix Table 2. Also, the conditions were adjusted according to the recommended annealing temperature of the primers. For the conventional amplification of the *GroEL* target gene for *Ehrlichia* spp. (Andoh et al. 2015), the samples underwent an initial denaturation for 10 min at 94 °C followed by a 30 temperature-step cycle program consisting of denaturation for 30 sec at 94 °C, annealing for 30 sec at 57 °C, and an extension for 1 min at 72 °C. A final extension for 5 min at 72 °C followed, thereby producing the first PCR product. The next round of PCR was carried out under similar condition. Initial denaturation was done for 5 min at 94 °C followed by a 30 temperature-step cycle program consisting of denaturation for 30 sec at 94 °C, annealing for 30 sec at 57 °C, and an extension for 30 sec at 72 °C. Afterwards, a final extension was done for 3 min at 72 °C.

Gel Electrophoresis and Documentation. The products of the nested PCR reactions were mixed with 1 µL loading buffer and were loaded on 2% agarose gel in 1x TAE (Tris-HCl, acetic acid, EDTA) as buffer. A 100 (base pairs) bp ladder (GeneDirex ®, Axil Scientific, USA) was used as the molecular weight standard. After electrophoresis, the gels were viewed using the BioRad gel documentation system.

Purification and Sequence Analysis. In the study, only positive samples for *Ehrlichia* spp. were selected for sequence reading. The first PCR products of those positive samples were subjected to another round of PCR, with total reaction mixture of 50 µL to obtain high concentration of the amplicons. After electrophoresis, extraction from the gel was done by cutting the band corresponding to the expected PCR amplicon size and putting it in a microcentrifuge tube. Purification of amplicons was performed using Nucleospin® Gel and PCR clean up kit

(Macherey-Nagel, Leicesteshire, England). Briefly, buffer NTI three times the volume of the incised gel was added to the gel and placed in a hot water bath at 50 °C for 20 min to ensure complete dissolution of the gel. Dissolved gel was transferred to a spin filter and then centrifuged at 12,000 rpm for 1 min. Washing was done twice followed by final centrifugation to remove excess ethanol and lastly, elution with ultra-pure nuclease-free water. Capillary sequencing was performed for the samples brought to the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan. Homologues of the resulting nucleotide sequence readings were compared from sequences of reported *Ehrlichia* isolates through the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) of the US National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/BLAST.cgi.).

Data Analysis

Data comparison were performed using Chi-squared test for association for the frequency of *R. sanguineus* (s.l.) infested dogs positive for hemoparasite, frequency of hemoparasite infection, and frequency of tick-borne diseases. A two proportion test was done for the frequency of hemoparasite infestation based on dog gender. Differences were considered statistically significant when $P \leq 0.05$ (Walpole 1982). Statistical analyses were performed using built-in functions and add-in data analysis in Microsoft Excel 2013 (v15.0).

RESULTS AND DISCUSSION

Detection and Frequency of Hemoparasite Infection

Collectively observed clinical signs in *R. sanguineus* (s.l.) infested dogs were presence of fever, presence of scabs, pale mucous membrane (pallor), rashes (red streaks), redness of the skin, pruritus/itchiness, inappetence (decreased/loss of appetite), vomiting, regurgitation, retching, unsteadiness, weakness, lethargy, shivering, panting, head shaking, weight loss, runny nose, nose bleeding (epistaxis), watery eyes, respiratory distress, dyspnea, coughing, retching, bloody diarrhea, watery stool, muscle pain, limping/lameness (hind limb), nausea, enlarged and painful lymph nodes, and restlessness.

Observed clinical signs in *R. sanguineus* (s.l.) infested dogs positive with hemoparasites were inappetence (decreased appetite), lethargy, weakness, vomiting, pale mucous membrane (pallor), episodes of limping, muscle pain (reluctance to move), epistaxis, feverish, pruritus, dyspnea, coughing and retching, and soft, watery stool. Indicative of canine hepatozoonosis positive dogs were episodes of limping and muscle pain (Babeth 2002; Shaw et al. 2001). Also, indicative of canine ehrlichiosis was presence of epistaxis (Breitschwerdt et al. 1998; Neer et al. 2002).

Table 1 shows that out of the 953 *R. sanguineus* (s.l.) infested dogs admitted in the three sampling sites, 61 (6.40%) dogs were diagnosed positive with hemoparasite.

Table 1. Frequency and diagnosis of *R. sanguineus* (s.l.) infested dogs positive with hemoparasite.

Month and Year	No. of Tick-infested Dogs Admitted	No. of Dogs Positive With Hemoparasites	Diagnosis		
			Babesiosis	Hepatozoonosis	Ehrlichiosis
Jan 2016	67	6	1	5	0
Feb 2016	61	12	4	8	0
Mar 2016	72	3	0	3	0
Apr 2016	41	5	1	3	1
May 2016	73	3	0	3	0
Jun 2016	63	6	2	1	3
Jul 2016	55	2	2	0	0
Aug 2016	69	2	1	1	0
Sep 2016	74	2	0	2	0
Oct 2016	45	2	0	1	1
Nov 2016	79	4	2	2	0
Dec 2016	63	4	3	1	0
Jan 2017	63	5	1	3	1
Feb 2017	62	2	1	1	0
Mar 2017	66	3	1	1	1
Total (%)	953	61 (6.40)	19 (2.00)	35 (3.67)	7 (0.73)

Result shows that canine hepatozoonosis was mostly diagnosed with a frequency of 3.67% followed by canine babesiosis with 2.00% and canine ehrlichiosis with 0.73%, while Table 2 shows the age range, gender, and breed of *R. sanguineus* (s.l.) infested dogs positive with hemoparasite.

Statistical analysis showed no significant difference on the frequency of hemoparasite positive dogs infested with *R. sanguineus* (s.l.) from Jan 2016 to Mar 2017 by month (Chi-squared test = 22.078, *P*=0.054). Analysis also showed that there was significant difference among the tick-borne diseases, wherein canine hepatozoonosis constitutes the highest proportion of hemoparasite positive dogs (Chi squared test = 18.970, *P*=0.000). Results showing the high frequency of canine hepatozoonosis caused by *Hepatozoon* spp. in *R. sanguineus* (s.l.) infested dogs can be attributed to the nature of transmission of the tick-carried pathogen *Hepatozoon* spp., which is transmitted by new animals via ingestion of an infected tick especially in heavily-infested dogs (Ewing & Panciera 2003). Results of the studies done by Kordick et al. (1999), Shaw et al. (2001), Babeth (2002), and Corales et al. (2014) explains that "the coinfection may occur in a heavily tick infested dog or exposure to arthropods infected with single pathogen species at different points in time or to vector(s) concurrently infected with multiple agents (*Ehrlichia*, *Bartonella*, *Babesia*, *Hepatozoon*, *Leishmania*, and *Rickettsia*)."

Results show that the *R. sanguineus* (s.l.) infested dogs positive with hemoparasite have a high frequency of infection observed in dogs 1–3 years old. Regarding to dog's gender, males showed to be more susceptible to the infection by hemoparasites (52.46%) than females (47.54%). Among dog breeds, the crossbreeds showed to be the most infected with hemoparasite having 24.60% of the animals from this category.

Findings were similar to earlier researches wherein the high frequency of hemoparasite infection for young dogs (1–3 years old), which can be due to immunological status as older dogs are resistant to *R. sanguineus* (s.l.) tick reinfestation (Inokuma et al. 1997; Kordick et al. 1999; Tinoco-Gracia et al. 2009; Dantas-Torres et al. 2009).

Possible reasons for the result obtained on the frequency of *R. sanguineus* (s.l.) tick infestation between dog

Table 2. Frequency of *R. sanguineus* (s.l.) infested dogs positive with hemoparasite based on age range, gender, and breeds.

Age and Gender	Breed																Total (%)
	Beagle	Belgian Mallinois	Chihuahua	Crossbreed	Dachshund	Golden Retriever	Labrador Retriever	Lasa Apso	Mini Pinscher	Pitbull	Pomeranian	Poodle	Portuguese	Shih Tzu	Siberian Huskey	Yorkshire Terrier	
<1 y.o.	1			2										1	1		5 (17.24)
1–3 y.o.			2	2		1	1							4	3		13 (44.83)
3–6 y.o.	1			1		1			1								4 (13.79)
6–9 y.o.					1	1	1							2			5 (17.24)
9–12 y.o.																	0 (0)
12–15 y.o.					1	1											2 (6.9)
Female																	**29 (47.54)**
<1 y.o.	1			2									1	1			4 (12.5)
1–3 yo.		1	1	7		1	1	1						5	2	1	20 (62.5)
3–6 y.o.				1	1				1	1					1		5 (15.63)
6–9 y.o.						1					1	1					3 (9.38)
9–12 y.o.																	0 (0)
12–15 y.o.																	0 (0)
Male																	**32 (52.46)**
Total (%)	2 (3.28)	1 (1.64)	3 (4.92)	15 (24.60)	3 (4.92)	6 (9.84)	3 (4.92)	1 (1.64)	1 (1.64)	1 (1.64)	1 (1.64)	1 (1.64)	1 (1.64)	13 (21.31)	8 (13.11)	1 (1.64)	**61 (100.00)**

gender – wherein male is higher than female – are gender-related susceptibility or a matter of exposition (Silveria et al. 2009), characteristics of the population studied (Dantas-Torres et al. 2009), and host exposure to ticks (Laummaunwai et al. 2014).

Similar possible reason resulting in the high frequency hemoparasite positive dogs infested with *R. sanguineus* (s.l.) in crossbreeds is shown by prior researches of Dantas-Torres (2010) and Louly et al. (2009) explaining that "some dog breeds are more susceptible or resistant to *R. sanguineus* (s.l.) than others."

Statistical analysis showed that there was a significant difference on the level of infection with hemoparasite by age range (Chi-squared test = 671.915, P=0.000) and dog breed (Chi-squared test = 4112.368, P=0.000). On the other hand, statistical analysis showed that there was no significant difference on the level of infection with hemoparasite by dog gender (two proportions test = 0.0001, P=0.969).

Diagnostic tests, PCV, and CBC from the 3 sampling areas revealed that almost all of the hemoparasite positive dogs have anemia. For canine babesiosis, there was also thrombocytopenia (Lobetti 1998; Jacobsen 2006); for canine hepatozoonosis, presence of neutrophilia (Babeth 2002; Shaw et al. 2001) was also pronounced; and for canine ehrlichiosis, thrombocytopenia and leucopenia (Breitschwerdt et al. 1998; Neer et al. 2002) were also seen.

BPE or hemoparasite examination results revealed presence of paired large merozoites pair tear-drop forms in the red blood cell for canine babesiosis patients (Figs. 1a, 1b, and 1c). Presence of intracytoplasmic ellipsoidal-shaped gamonts in the neutrophils is apparent for canine hepatozoon patients (Figs. 1d and 1e). For canine ehrlichiosis, no intracytoplasmic inclusion bodies were revealed within a white blood cell since an immunochromatographic test kit was used.

Among the 61 patients positive with hemoparasite, 54 (88.52%) recovered and 7 (11.48%) died.

Detection of Tick-Carried Pathogens in *R. sanguineus* (s.l.)

From the 97 *R. sanguineus* (s.l.) infested dogs collected with tick samples, 29 dogs were untreated with acaricides. Table 3 shows the age range, gender, and breed of the 29 *R. sanguineus* (s.l.) infested untreated dogs, tick samples from which were collected in the three sampling sites used for the detection of tick-carried pathogens.

Results show that most of the untreated dogs were 1–3 years of age; there were more male (68.97%, n=20) than female (31.03%, n=9); and the crossbreed showed to be the highest in number (51.72%, n=15).

Result shows similarity to previous researches wherein tick burden is heavier on young dogs in comparison to older ones (Tinoco-Gracia et al. 2009; Dantas-Torres

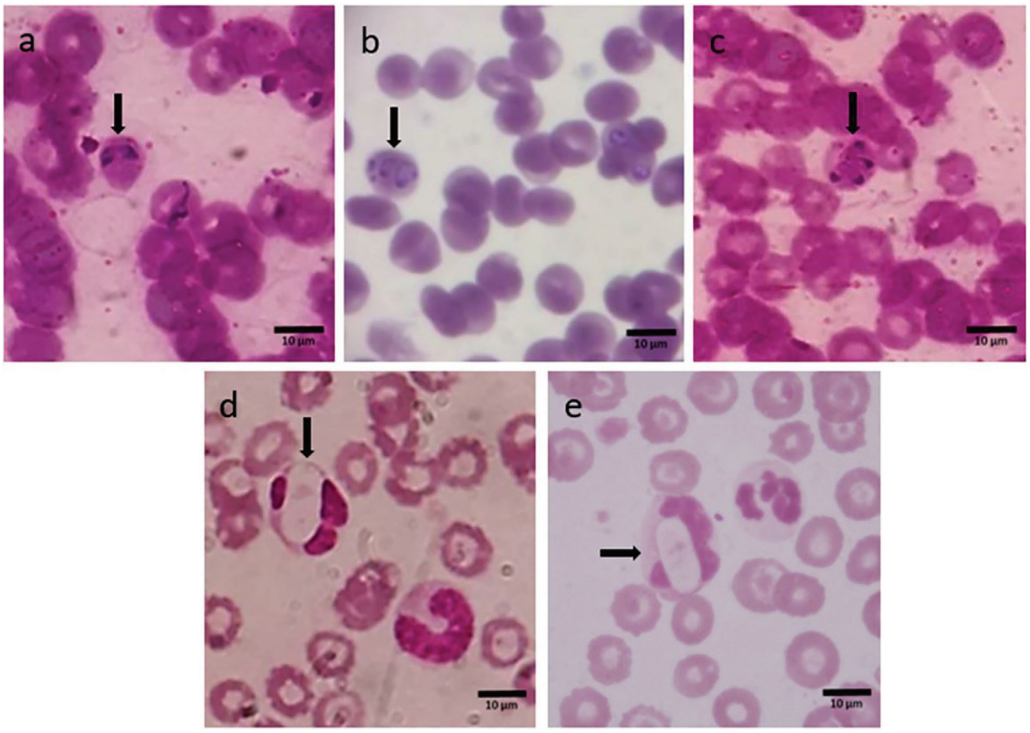

Figure 1. *R. sanguineus* (s.l.) infested dogs' blood parasite examination results [a, b, and c – *Babesia* spp. positive; d and e – *Hepatozoon* spp. positive]. Arrows showing the tick-carried pathogens.

Table 3. Age range, gender, and breed of *R. sanguineus* (s.l.) infested untreated dogs used for the detection of tick-carried pathogen (TCP).

Age and Gender	Breed						Total (%)
	Beagle	Chow chow	Crossbreed	Pomeranian	Shih Tzu	Siberian Husky	
<1 y.o.					4	1	5 (25)
1–3 y.o.			7				7 (35)
3–6 y.o.			2		1		3 (15)
6–9 y.o.	1	1			1		3 (15)
9–12 y.o.				1			1 (5)
12–15 y.o.							0 (0)
>15 y.o.			1				1 (5)
Male							**20 (68.97)**
<1 y.o.	1		1				2 (22.22)
1–3 y.o.			3		2		5 (55.56)
3–6 y.o.			1		1		2 (22.22)
6–9 y.o.							0 (0)
9–12 y.o.							0 (0)
12–15 y.o.							0 (0)
>15 y.o.							0 (0)
Female							**9 (31.03)**
Total (%)	2 (6.9)	1 (3.45)	15 (51.72)	1 (3.45)	9 (31.03)	1 (3.45)	29

Table 4. Detection rate of tick-carried pathogen (TCP) using nested PCR.

Sampling Area	Tick-infested Untreated Dogs	Tick Samples Positive for *mt-rrs* Genes	Tick Samples Positive for Tick-carried Pathogens			Total
			Babesia spp.	*Hepatozoon* spp.	*Ehrlichia* spp.	
VTH UPD	5	11	0	0	0	0
VTH UPLB	11	14	1	1	1	3
Private Vet. Clinic	13	23	0	0	3	3
Total (%)	**29**	**48**	**1 (2.08)**	**1 (2.08)**	**4 (8.33)**	**6 (12.50)**

et al. 2009) and that young dogs heavily infested by ticks might develop anemia, particularly if they are also infected by tick- carried pathogens such as *Ehrlichia* spp. (Kordick et al. 1999). Likewise, past research indicated that young dogs were found to have a higher frequency of tick infestation than older dogs; this situation could be due to resistance of old dogs to reinfestation (Inokuma et al. 1997). Again, the high frequency of tick infestation in male than in female dogs is possibly due to gender-related susceptibility or a matter of exposition (Silveria et al. 2009) and that certain breeds of dog have susceptibility to tick infestation (Dantas-Torres 2010; Louly et al. 2009).

Table 4 shows the detection rate of tick-carried pathogen (TCP) using nested PCR. Result shows a total detection rate of 12.50% (n=6/48) from the ticks collected in 29 untreated *R. sanguineus* (s.l.) infested dogs.

In the present study, 2.08% (n=1/48) of the tick samples showed amplification of a fragment of 339 bp of the target gene of *Babesia* spp. (Fig. 3), which was detected in an engorged adult female tick. Similarly, 2.08% (n=1/48) of the tick samples showed amplification of a fragment of 665 bp of the target gene of *Hepatozoon* spp. (Fig. 3), which was detected in an engorged adult female tick. Finally, 8.33% (n=4/48) of the tick samples showed amplification of the 315 bp of target gene of *Ehrlichia* spp. (Fig. 2), which was detected in three engorged adult females and one adult male.

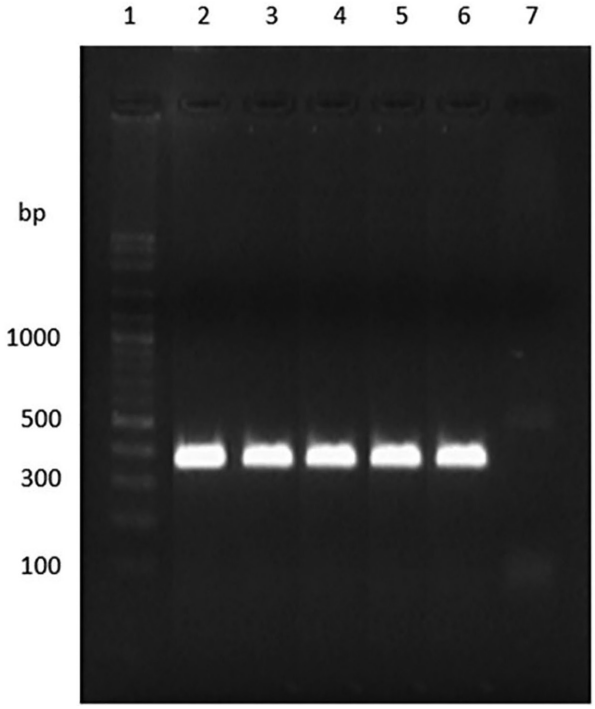

Figure 2. Nested PCR amplification of the groEL genes of *Ehrlichia* spp. Detection of *Ehrlichia* spp. amplicons with expected 315 bp.
Lane 1: 100 bp molecular weight marker
Lane 2: positive control for *Ehrlichia* spp.
Lane 3: tick #13 of dog 11 from UPLB VTH; positive for *Ehrlichia* spp.
Lane 4: tick #14 of dog 11 from UPLB VTH; positive for *Ehrlichia* spp.
Lane 5: tick #33 of dog 19 from Private Vet Clinic; positive for *Ehrlichia* spp.
Lane 6: tick #46 of dog 26 from Private Vet Clinic; positive for *Ehrlichia* spp.
Lane 7: negative control (ultra-pure water) for *Ehrlichia* spp.

Result further shows co-infection of the three tick-carried pathogens – *Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. – detected from an engorged adult female tick collected from Crossbreed11 at VTH UPLB.

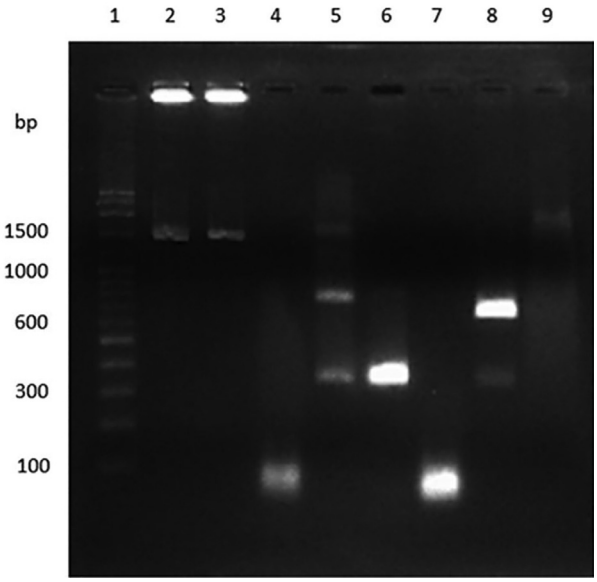

Figure 3. Nested PCR amplification of the 18S rRNA genes of *Babesia* spp./ *Hepatozoon* spp. Detection of *Babesia* spp. amplicons with expected 339 bp and *Hepatozoon* spp. amplicons with expected 665 bp.
Lane 1: 100 bp molecular weight marker
Lane 2: positive control for *Babesia* spp./ *Hepatozoon* spp.
Lane 3: tick #13 of dog 11 from UPLB VTH; positive for *Babesia* spp./ *Hepatozoon* spp.
Lane 4: negative control (ultra-pure water) for *Babesia* spp./ *Hepatozoon* spp.
Lane 5: positive control for *Babesia* spp.
Lane 6: tick #13 of dog 11 from UPLB VTH; positive for *Babesia* spp.
Lane 7: negative control (ultra-pure water) for *Babesia* spp.
Lane 8: tick #13 of dog 11 from UPLB VTH; positive for *Hepatozoon* spp.
Lane 9: negative control (ultra-pure water) for *Hepatozoon* spp.

After the nested PCR, the sequence of the positive amplicons corresponding to groEL gene (*Ehrlichia* spp.) was determined. Confirmed was the sequence identities of the positive tick samples as *Ehrlichia canis* with identity match of 95% (tick sample 13, Crossbreed11); 94% (tick sample 14, Crossbreed11); 95% (tick sample 33, Crossbreed15); and 95% (tick sample 46, Beagle2).

Among the developmental stages of *R. sanguineus* (s.l.) collected, the engorged adult female and adult male were detected positive for the tick-carried pathogens *Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. (Table 5). Findings show that the adult stages of *R. sanguineus* (s.l.) can possibly transmit tick-carried pathogens and act as vector of *Babesia* spp., *Hepatozoon* spp., and *Ehrlichia* spp. (Petney 1993; Jongejan & Uilenberg 2004; Dantas-Torres 2008; Nicholson et al. 2010).

Table 5. PCR and Nested PCR results of the collected developmental stages of the *R. sanguineus* (s.l.).

Dog #	BPE* Result	Tick #	Developmental Stage	PCR Marker Gene	Nested PCR	
					***Babesia* spp./ *Hepatozoon* spp.**	***Ehrlichia* spp.**
D1	BP Suspect**	1	Engorged Nymph	positive	negative	negative
D2	BP Suspect**	2	Engorged Nymph	positive	negative	negative
D3	BP Suspect**	3	Adult Male	positive	negative	negative
D4	BP Suspect**	4	Adult Female	positive	negative	negative
D5	BP Suspect**	5	Engorged Nymph	positive	negative	negative
		6	Adult Male	positive	negative	negative
D6	BP Suspect**	7	Engorged Nymph	positive	negative	negative
D7	BP Suspect**	8	Adult Female	positive	negative	negative
D8	BP Suspect**	9	Adult Male	positive	negative	negative
		10	Adult Female	positive	negative	negative
D9	BP Suspect**	11	Engorged Female	positive	negative	negative
D10	BP Suspect**	12	Adult Male	positive	negative	negative
D11	BP Suspect**	13	Engorged Female	positive	positive	positive
		14	Adult Male	positive	negative	positive
		15	Adult Female	positive	negative	negative
D12	BP Suspect**	16	Engorged Female	positive	negative	negative
		17	Adult Male	positive	negative	negative
		18	Adult Female	positive	negative	negative
D13	BP Suspect**	19	Engorged Female	positive	negative	negative
		20	Adult Male	positive	negative	negative
		21	Adult Female	negative	na	na
D14	BP Suspect**	22	Engorged Female	positive	negative	negative
		23	Adult Male	positive	negative	negative
		24	Adult Female	positive	negative	negative
D15	BP Suspect**	25	Engorged Female	positive	negative	negative
		26	Adult Male	positive	negative	negative
D16	BP Suspect*	27	Engorged Nymph	positive	negative	negative
		28	Adult Male	positive	negative	negative
D17	BP Suspect**	29	Engorged Nymph	positive	negative	negative
		30	Adult Male	positive	negative	negative
		31	Adult Female	positive	negative	negative
D18	BP Suspect**	32	Engorged Nymph	negative	n/a	n/a
D19	BP Suspect**	33	Engorged Female	positive	negative	positive
		34	Adult Male	positive	negative	negative
D20	BP Suspect**	35	Engorged Female	positive	negative	negative
		36	Adult Male	positive	negative	negative
D21	BP Suspect**	37	Adult Female	positive	negative	negative
D22	BP Suspect**	38	Engorged Nymph	negative	n/a	n/a
		39	Adult Male	positive	negative	negative
		40	Adult Female	positive	negative	negative
D23	BP Suspect**	41	Engorged Female	positive	negative	negative
		42	Adult Male	positive	negative	negative
D24	(+) *Ehrlichia* spp. ***	43	Engorged Female	negative	n/a	n/a
		44	Adult Female	positive	negative	negative

Table 5 continuation

D25	BP Suspect**	45	Engorged Female	positive	negative	negative
D26	(+) *Ehrlichia* spp.***	46	Engorged Female	positive	negative	positive
		47	Adult Male	positive	negative	negative
D27	BP Suspect**	48	Engorged Nymph	positive	negative	negative
		49	Adult Male	positive	negative	negative
D28	BP Suspect**	50	Engorged Nymph	positive	negative	negative
		51	Adult Male	positive	negative	negative
D29	BP Suspect**	52	Engorged Nymph	positive	negative	negative

CONCLUSION

These results will provide additional information on the occurrence of these tick-carried pathogens in the Philippines. These evidences will also serve as guide and source of knowledge regarding the negative effects of *R. sanguineus* (s.l.) for effective management skill and technique geared towards the prevention and control of both ticks and hemoparasite.

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APPENDICES

Appendix I. Blood collection, staining and examination for blood parasites.

Methods for Cephalic Venipuncture for Dogs

***Proper Restraint**:*

To collect blood from the cephalic vein, which is located on the cranial aspect of the foreleg, the animal is restrained in sternal recumbency or in standing position. Small dogs may be picked up and held in a standing position. Small dogs may be picked up and held in the restrainer's arms with a foreleg extended. Some large dog breeds prefer to remain standing or seated with a foreleg held in extension.

***Procedure for a Venipuncture**:*

1. Attach a 20–25 gauge needle to a 1–3 ml syringe.
2. Occlude the vein with digital pressure.
3. Wipe the skin and hair on top of the vein with an alcohol-soaked cotton ball to help identify the vein.
4. Insert the needle with the bevel facing up through the skin and into the vein at a 25-degree angle.
5. Slowly retract the syringe plunger and collect a blood sample.
6. Release the pressure on the vein and release the syringe plunger when a sufficient volume of blood has been collected.
7. Remove the needle from the vein.
8. Apply digital pressure to the venipuncture site as soon as the needle is removed until hemostasis occurs.
9. Remove the needle from the syringe.
10. Transfer the blood from the syringe to the vial with anticoagulant.
11. Mix the blood and anticoagulant thoroughly.

Methods for Blood Smear Preparation

Blood smear should be prepared within a couple of hours of blood sample collection to avoid artifactual changes that will distort the structure of blood cells. Blood films are prepared in various ways, including the slide (wedge) and coverslip methods. It is essential that a monolayer of intact cells be present on the slide so that accurate examination and differential leukocyte counts can be performed.

Slide Blood Smear Method:

1. A clean glass slide is placed on a flat surface, and a small drop of well-mixed blood is placed on one end of the slide by using an applicator stick.
2. This slide is held in place with one hand, and a second glass slide (spreader slide) is placed on the first slide and held between the thumb and the forefinger with the other hand at an angle of about 30 degrees in front of the drop of blood.
3. The spreader slide is then backed into the drop of blood and as soon the blood flows along back side of the spreader slide, the spreader slide is rapidly pushed forward.
4. Once prepared, the slide is immediately dried by waving it in the air or by holding it in front of a hair dryer set on a warm air setting. Holding the slide close to a dryer set on a hot air setting can result in fragmentation of cells.

Blood Smear Staining Method

Romanowsky-type Stains

Blood smear are routinely stained with a Romanowsky-type stain (e.g., Wright or Wright-Giemsa stain). Romanowsky-type stains are composed of a mixture of eosin and oxidized methylene blue (azure) dyes. The azure dyes stain acids resulting in blue to purple colors and eosin stains bases resulting in red coloration. These staining characteristics depend on the pH of the stains and rinse water, as well as the nature of the cells present.

Various rapid stains are available; however, the quality of stained blood films is generally somewhat lower than that obtained with longer staining procedures. The commercial Hema-Quick stain is a commonly used Romanowsky-type rapid blood stain.

Examination of Stained Smear Films:

1. Blood films should first be scanned by using a low-power objective to estimate the total leukocyte count and to check for the presence of erythrocyte agglutination, leukocyte aggregates, platelet aggregates, microfilaria, and abnormal cells that might be missed during the differential leukocyte count.
2. On glass slides, examine the feathered end of blood films made. Leukocytes and platelet aggregates may be concentrated in this area.
3. On glass slides, the film will be too thick to evaluate blood cell structure at the back of the slide where the drop of blood was applied and too thin at the feathered edge where cells are distorted. The optimal for evaluation is generally in the front half of the smear behind the feathered edge. This area should appear as a well-stained monolayer of cells. A monolayer is defined as a field in which erythrocytes are close together, with approximately one-half of the erythrocytes touching each other.
4. On a coverslip blood films, examine the center of rather than at the feathered edge. This is the area where aggregates of cells are found.

Source: Marte 2013

APPENDIX TABLES

Appendix Table 1. Primers for amplification of marker genes used in the study.

Target Gene	Primer Name	Sequence (5'→3')	Expected Product Length	References
mt-rrs	mt-rrsF	CTGCTCAATGATTTTTTAAATTGCTGTGG	460 bp	Ushijima et al. (2003); Andoh et al. (2015)
	mt-rrsR	CCGGTCTGAACTCAGATCAAGTA		Ushijima et al. (2003); Andoh et al. (2015)

Appendix Table 2. Primers used for amplification of TCPs of interest in the study.

Pathogen	Target Gene	Primer Name	Sequence (5'→3')	Expected Product Length	References
Babesia spp./ *Hepatozoon* spp.	18S rRNA	BT1	GGTTGATCCTGCCAGTAGT	1669 bp	Criado-Fornelio et al. (2007); Tateno et al. (2015)
		HPF2P	GACTTCTCCTTCTTTAAGTGATAAG		
Babesia spp.		455-479F	GTCTTGTAATGGAATGATGGTGAC	339 bp	Birkenheuer et al. (2003); Tateno et al. (2015)
		793-772R	ATGCCCCCAACCGTTCCTATTA		
Hepatozoon spp.		HEP1	CGCGAAATTACCCAATT	665 bp	Criado-Fornelio et al. (2006); Tateno et al. (2015)
		HEP4	TAAGGTGCTGAAGGAGTCGTTTAT		
Ehrlichia spp.	groEL	gro607F[1]	GAAGATGCWGTWGGWTGTACKGC	614 bp	Andoh et al. (2015)
		gro1294R[1]	AGMGCTTCWCCTTCWACRTCYTC		
		gro677F[2]	ATTACTCAGAGTGCTTCTCARTG	315 bp	Andoh et al. (2015)
		gro1121R[2]	TGCATACCRTCAGTYTTTTCAAC		