J. Trop. Resour. Sustain. Sci. 12 (2024): 12-18
https://doi.org/10.47253/jtrss.v12i1.1360

Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Asymptomatic canine vector-borne diseases and diagnostic performance: Comparison between blood smears vs. conventional PCR

Tan Jun Jie[1], Ruhil Hayati Hamdan[1], Ibrahim Abdul-Azeez Okene[2], Nadiah Syuhada Roslan[3], Nur Atikah Hashim[3], Faqihah Ibrahim[3], C.W. Salma. C.W. Zalati[1], Tan Li Peng[1]*

[1]Faculty of Veterinary Medicine, Universiti Malaysia Kelantan Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia.
[2]Veterinary Medicine and Food Security Research Group, College of Health Sciences, Higher Colleges of Technology, Sharjah Men Campus, Sharjah, United Arab Emirates.
[3]Veterinary Teaching Hospital, Universiti Malaysia Kelantan, 16310 Bachok, Kelantan, Malaysia.

Received 09 August 2023
Accepted 22 January 2024
Online 30 June 2024

Keywords:
asymptomatic, dogs, diagnostic methods, haemoparasites, malaysia.

⊠*Corresponding author:
Dr. Tan Li Peng
Faculty of Veterinary Medicine, Universiti Malaysia Kelantan Pengkalan Chepa, 16100 Kota Bharu, Kelantan, Malaysia.
Email: li.peng@umk.edu.my

Abstract

Across the globe, vector-borne diseases (VBD) are causes of health and economic concern, particularly for companion animals such as dogs and cats. The common clinical presentation ranges from subclinical to acute infection even a proportion with symptomatic manifestation. The diagnosis of asymptomatic animals with VBDs is quite challenging since veterinary practitioners do rely on presenting clinical signs to inform the choice of diagnostic plan. In Malaysia, blood smears (thin and thick) are employed in the diagnosis of VBDs such as babesiosis, anaplasmosis, theileriosis and trypanosomiasis. This method is readily available and inexpensive; however, the sensitivity and specificity are low as the diagnosis is strongly dependent on the experience of the examiner. Conventional polymerase chain reaction (PCR) on the other hand is used in larger veterinary clinics and hospitals in Malaysia and is only utilised when there is a suspicion of VBDs. This study attempts to compare the thin blood smear method and conventional PCR in the diagnosis of of *Anaplasma phagocytophilum*, *A. platys*, *Babesia canis*, *B. gibsoni*, *Ehrlichia canis* in dogs in Malaysia. Thirty clinically healthy dogs (17 males and 13 females) averaging 2 years of age were randomly selected from clinic walk-ins to screen for VBDs with both blood smear and conventional PCR. In general, the conventional PCR was 2.5 times more sensitive and specific than the blood smear in the detection of VBDs including *Babesia canis*, *Babesia gibsoni* and *Ehrlichia* canis in the asymptomatic sampled dogs. The detection rate for blood smears was 20% (6/30) in comparison with 50% (15/30). Therefore, it is imperative to conduct screenings for VBDs in dogs, even when they present as asymptomatic, in order to avert the oversight of potential health emergencies. Conventional PCR emerges as the recommended methodology for screening asymptomatic dogs for VBDs.

1. INTRODUCTION

Dogs are competent hosts for a number of zoonotic agents, and new public health concerns are emerging in developing countries (Otranto *et al*., 2009). Tick-borne diseases around the world are a major cause of morbidity and mortality among the canine species (Dantas-Torres, 2008). In Malaysia, tick-borne pathogens such as *Ehrlichia sp*., *Anaplasma sp*., *Babesia sp*. and *Rickettsia sp*. infections in both humans and animals have been reported in the past (Jing *et al*, 2017) thus suggesting the zoonotic potential of some of them. Statistics from the Malaysian Veterinary Research Institute (VRI) Annual report, (2009-2011) revealed that vector-borne diseases (VBDs) are circulating within the Malaysian dog population (domestic and stray). Through blood meal, ticks are capable of transmitting haemoparasites (VBDs) to dogs with a single pathogen infection or multi-pathogens infection. The occurrence of multi-pathogens vector-borne haemoparasitic infection is reported to be 39% in Malaysian dogs (Abd Rani *et al*., 2011).

The most common vector-borne haemoparasitic infections of dogs in Malaysia are babesiosis, ehrlichiosis and anaplasmosis. Babesiosis is a haemoparasitic disease with a potential for zoonosis causing intra- and extravascular haemolysis, jaundice and pyrexia in animals and humans (Rotondano *et al*., 2015). The common causative agents of canine babesiosis have been divided into two distinct groups, namely Babesia canis and B. gibsoni (Nalubamba *et al*., 2011; Ghasemzade *et al*., 2022). On the other hand, important species of Anaplasma include Anaplasma phagocytophilum and A. platys which cause canine and human anaplasmosis (Parola and Raoult, 2001). Anaplasma species like Babesia are intraerythrocytic parasites transmitted by tick vectors to a wide hot range of animals and humans.

eISSN Number: 2462-2389

Both the *Anaplasma* and *Ehrlichia* species are recently classified as bacteria based on the 16sRNA profiling. While *Anaplasma* is intraerythrocytic, *Ehrlichia* – depending on the species – are intramonocytic or intragranulocytic in predilection. Infected dogs would often display mild to medium clinical signs from any of these pathogens, severe clinical presentations are observed in concurrent infections with multiple vector-borne haemoparasitic pathogens. Disease severity is particularly worsened by the severe thrombocytopaenia caused by *Ehrlichia canis* when it co-infects with other haemoparasites (Gaunt *et al*., 2010). *Ehrlichia canis* is the most prevalent and medically virulent type of canine ehrlichiosis and can also induce human ehrlichiosis (Perez *et al*. 1996). In Malaysia, available literature loosely reflects the true distribution of VBDs in the canine population, especially in areas where dog companionship is uncommon such as the east coast region.

Canine vector-borne haemoparasitic diseases are potentially zoonotic. Human infected with these pathogens are generally presents with nonspecific symptoms such as fever, chills, malaise, headache, and myalgias (Guzman *et al*., 2023). Severe manifestations usually occur in immunocompromised individuals. Rashes and nonspecific gastrointestinal (GI) or respiratory symptoms may be observed occasionally in anaplasmosis and ehrlichiosis cases (Snowden *et al*., 2022). Babesiosis on the other hand can cause hemolytic anemia due to the nature of *Babesia* infect and destroy red blood cells. hemolytic anemia can lead to jaundice and dark urine (Islam *et al*., 2023).

In Kelantan for instance, with a 96.6% ethnic Malay-Muslim population, dog ownership is unpopular, hence data on canine-related diseases among owned and stray dogs are rather scarce. Limited information could be obtained regarding the prevalence of canine vector-borne diseases (CVBDs) in dogs in this state. More importantly, owned dogs often carry haemoparasitism in clinically unrecognisable form – asymptomatic. Hence, this study was designed to determine the detection and prevalence of some common tick-borne haemoparasites of dogs in the study area using microscopic examination and conventional PCR while comparing the efficacy of both diagnostic methods. Most of these haemoparasites are potentially zoonotic, thus may be transmitted from the pets to owners. Therefore, monitoring the prevalence and examining the risk factors of these pathogens are important to safeguard the human population against outbreaks of zoonotic diseases in the study area.

2. MATERIALS AND METHODS

Thirty owned dogs that were presented to the Veterinary Teaching Hospital University Malaysia Kelantan (HPVUMK) and one private veterinary clinic located in Kota Bharu for routine examinations, vaccinations or treatments were selected randomly into the study. Information on the identified risk factors such as the presence of ticks upon presentation and management of the dog were obtained during history taking from the owners.

2.1. Ethical Clearance

All procedures were reviewed and approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Malaysia (UMK/FPV/ACUE/FYP/4/2020).

2.2. Sample Collection

Blood samples were collected from dogs after obtaining the consent of the owners. All procedures on animals were reviewed and approved by the Animal Care and Use Committee of Universiti Malaysia Kelantan. Blood samples (approx. 1.5 ml) were collected from the cephalic vein using 23G needle and a 3 ml syringe and then transferred into 2 ml ethylenediamine-tetra-acetic acid (EDTA) tube. Two thin blood smears were performed immediately for each blood sample using Diff-Quick stain followed by microscopic examination for the detection of the presence of blood parasites. Blood samples were then stored in chiller for not more than 24 h before performing the DNA extraction and also subjected to conventional PCR for molecular detection of *Anaplasma phagocytophilum*, A. *platys*, *Babesia canis*, *B. gibsoni* and *Ehrlichia canis*. Dogs were also thoroughly examined for the presence of ticks.

2.3. Molecular Detection

Genomic DNA was extracted from 200 µl of blood using the Genomic DNA Mini Kit (Blood/Cultured Cell (Geneaid, Taiwan) according to the manufacturer's protocol. The extracted DNA was subjected to PCR using the primers and the conditions as stated in Table 1. The PCR was performed in C1000 Touch Thermal Cycler (Bio-Rad, USA), and each PCR reaction mixture (25 µl) included 12.5 µl of GoTaq ® Green Master Mix, 1.0 µl of each forward and reverse primers, 5 µl of template DNA, and 5.5 µl nuclease-free water. The amplified PCR products were separated using Midori Green Advance DNA-stained agarose gel (2%) electrophoresis and visualized using Geldoc™ EZ Imager (Bio-Rad, USA). Positive controls of *Babesia* and *Ehrlichia* from previous clinical cases were included.

2.4. Statistical Analysis

The association between the prevalence of canine vector-borne pathogens and risk factors such as age, sex, breed, management, and presence of ticks were determined using univariate analysis of odd ratios at 95% confidence intervals using Fisher's exact test. The difference between the two diagnostic tools - microscopy and conventional PCR was compared determined using chi-square test.

eISSN Number: 2462-2389

Statistical analyses were performed using Statistical Package for Social Science (SPSS®) version 20.

3. RESULTS AND DISCUSSION

A total of 30 dogs that were brought to the Veterinary Teaching Hospital University Malaysia Kelantan (HPVUMK) and one private veterinary clinic were sampled with puppies (n = 6) and adults (n = 24). There were 17 males and 13 females of different breeds. There were 7 dogs kept indoors while 23 dogs were kept outdoors. Upon the physical examination, 13 dogs were found to be infested with ticks. All the dogs in this study were asymptomatic for haemoparasitism thus expressing no apparent clinical signs of fever, lymphadenomegaly, jaundice, pale mucus membrane, splenomegaly or haematuria.

As the canine species are at high risk of contracting haemoparasitism from vectors, such as ticks, timely detection of the disease is essential to achieve a better prognosis (Bajer *et al*., 2022). In many cases, subclinical to asymptomatic infections are not noticed in routine clinical examination and are often missed until the disease is full-blown. In Malaysia, a tropical country conducive to the sustained presence of haemoparasite vectors, the dog population is at even higher risk of continuous exposure and morbidity from vector-borne diseases such as babesiosis, anaplasmosis and ehrlichiosis. This study randomly selected 30 apparently healthy dogs visiting veterinary hospitals and clinics in the Kelantan state of Malaysia for routine health services. In the state of Kelantan with a relatively low domestication rate for dogs, there is a higher tendency of having asymptomatic owned dogs for VBDs which would strongly reflects a higher prevalence of VBDs in the stray population. From this study, the 50% prevalence of the various haemoparasites in the owned dogs is of concern when reflected on the stray population (Nazari *et al*., 2013; Azzag *et al*., 2015). Three out of five common vector-borne pathogens (*B. canis*, *B. gibsoni* and *E. canis*) were amplified using PCR while only two pathogens (B. canis and B. gibsoni) were detected through blood smear microscopic evaluation.

Microscopic examinations and 150 PCR tests were conducted on 30 blood samples to screen for five common haemoparasitic pathogens (*Anaplasma phagocytophilum*, *A. platys*, *Babesia canis*, *B. gibsoni* and *Ehrlichia canis*) using both microscopy and conventional PCR. Hence each sample was replicated for each of the five pathogens screened. Vector-borne haemoparasitic infections were identified in 20.0% (6/30) of by microscopic examination of blood smears as compared to 50.0% (15/30) using Polymerase Chain Reaction (PCR). Among all the positive cases, 16.7% (5/30) of them were and classified as a co-infection of haemoparasitism with the detection of more than one haemoparasite in that sample dog (Table 2). Overall, 4.0% (6/150) of the screened samples were found positive for canine vector-borne pathogens through blood smear, while 13.3% (20/150) tested positive by using conventional PCR.

Comparing the diagnostic efficiency of microscopy and conventional PCR revealed a higher detection rate and sensitivity when the PCR is used. Polymerase chain reaction expectedly perform better than microscopy since it detects fragments of the genetic materials in both actively infected and recovering asymptomatic dogs (Zulkifli *et al*., 2011; Kalaivanan, 2017; Rucksaken *et al*., 2019). Detection rate and specificity by microscopy increases as observer experience increases and when there is a high parasitic load (Valkiunnas *et al*., 2008).

There was a significant difference in sensitivity between the diagnostic methods (microscopy and conventional PCR) used ($\chi 2 = 8.811$, $p = 0.003$), in which PCR was more sensitive in this study. *Anaplasma* spp. was not detected in any of the samples; *Babesia canis* and *B. gibsoni* were detected in both blood smear and PCR techniques. There were 3.3% and 16.7% of the sample detected with *B. canis* and *B. gibsoni* respectively using blood smear, while 20% and 33.3% of the sample were detected with *B. canis* and *B. gibsoni* through PCR. *Ehrlichia canis* were detected in 13.3% of the samples via PCR but none from the blood smear.

The prevalence presented in this study depicts the trend reported by Rajamanickam *et al*. (1985). Babesia gibsoni (33.3%) was the most common canine vector-borne pathogen detected in this study, followed by *B. canis* (20.0%) and *E. canis* (13.3%). Few studies have documented low to moderate prevalence of *B. gibsoni* (0.0-17.7%) in Peninsular Malaysia (Rajamanickam *et al*., 1985; Zulkifli *et al*., 2011; Mokhtar *et al*., 2013; Prakash *et al*., 2018). A study conducted in 2017 – 2018, covering the Klang Valley region and Kota Bharu reported no detection of *B. gibsoni* from the samples collected (Colella *et al*., 2020). Most of these studies were conducted in Selangor, the state in the central region of Peninsular Malaysia, higher prevalence of *B. gibsoni* in this study could be due to geographical factors in which *B. gibsoni* infection remains high in the East Coast region such as Kelantan as compared to *Anaplasma* spp. which was not detected or previously reported in the East Coast regions but were common in the West Coast regions of Peninsular Malaysia (Mokhtar *et al*., 2013; Koh *et al*., 2016; Sipin *et al*., 2020). The variation in haemoparasite species detected suggests a geographical preponderance even within a country with closely similar terrain. However, in the case of Kelantan, it remains unclear if the proximity to beaches and higher humidity plays any role in the detection of *Babesia* spp. and *Ehrlichia* spp. as well as the non-detection of *Anaplasma* spp. Similar reports have shown epigenetic traits for other pathogens of animals (Paddock and Goddard, 2015; Wang *et al*., 2019).

Published investigations on *E. canis* detection via blood smear method revealed a prevalence of only 0.2% in Selangor state (Rajamanickam *et al*., 1985), and recently the prevalence of *E. canis* infection was determined to be 15.0% in Perak state using indirect immunofluorescence assay (IFA) (Rahman *et al*., 2010) and molecular prevalence of *E. canis* in the tested samples was only 2.0% from several states of Malaysia (Nazari *et al*., 2013). Our results on the prevalence of *E. canis* (13.3%) using PCR were similar to the one detected with IFA which is considered the "gold standard" for *E. canis* detection. Therefore, the sensitivity and ability of molecular detection of *E. canis* in the early or asymptomatic stages of the disease before the development of antibodies provide a better prognosis. Targeted prompt treatment can then be instituted even before the onset of clinical signs.

eISSN Number: 2462-2389

Comparison between PCR and blood smear for for *A. phagocytophilum* and *A. platys* infections could not be done due to the non-detection in all examined samples. This finding deviates from those of Koh *et al*. (2016) and Mokhtar *et al*. (2013) who reported 13.3% (4/30) and 4.3% (2/47) *A. platys* and *A. phagocytophilum* infection through PCR among the dogs in Peninsular Malaysia. Negative detection of *A. phagocytophilum* and *A. platys* might be due to various PCR sensitivity between laboratories or the absence of *Anaplasma* spp. in this region. As mentioned by Mokhtar *et al*. (2013) and Koh *et al*. (2016), their study was the first study which identified *A. platys* and *A. phagocytophilum* through PCR detection among dogs in Malaysia. Therefore, the true prevalence of *A. phagocytophilum* and *A. platys* infections in Peninsular Malaysia might be considered low to be isolated and identified in the current study.

Statistical analysis revealed no association of canine vector-borne pathogens infections with any of the selected risk factors including age, sex, breed, management and presence of ticks (Table 3). Even so, the commonly identified risk factors investigated in this were insignificantly associated with the occurrence of the disease. This may be explained by the routine care provided by the owners as the dogs would receive routine deticking and possibly antiprotozoal protection. Most importantly the highlight of this study is the detection of VBDs despite the absence of clinical signs which renders the risk factors trivial in the occurrence. It may thus indicate the need for reclassification of the clinical presentation of VBDs in dogs in East coast region of Malaysia. Currently, the VBDs presents as either subclinical, acute or peracute, all of which shows some clinical signs with differing intensities (Megat Abdul Rani *et al*., 2010; Solano-Gallego *et al*., 2016).

This study reveals the occurrence of mixed infections or coinfections with haemoparasites in asymptomatic owned dogs. While further investigation is required, coinfections with VBDs have been anecdotally reported to be presented as subclinical to chronic in most owned dog populations in Kelantan (Koh *et al*., 2016). It thus portends a rather essential enzootic relevance because the dogs presented with clinical VBD coinfections are at higher risk of cardiovascular and haematological complications (Köster *et al*., 2015). Such coinfections in stray dogs may serve to maintain the cycle of transmission within the eco-space.

4. CONCLUSION

The occurrence of asymptomatic VBDs in owned dogs portrays the necessity for frequent screening of susceptible animals in routine health check-ups. A 20% and 50% detection rate by microscopy and conventional PCR respectively strongly indicates that a larger population of dogs are morbid with VBDs that would be undiagnosed and untreated. The results of such asymptomatic VBDs could indicate health adversities for dogs including the risk of haematological and cardiovascular challenges as has been observed in dogs with clinical infections. Even as the studies on risk factors did not correlate with the occurrence of VBDs it does indicate that there are other determinant factors at play in the maintenance of the disease in an enzootic state among owned dog population. It remains unclear from this study if the occurrence of coinfections with VBDs in asymptomatic dogs would metamorphose into clinical VBD cases or would show more severe menifestation than cases with singular VBD morbidity.

eISSN Number: 2462-2389

Table 1: Primers for canine vector-borne pathogens and their PCR amplification conditions

Pathogens	Primer Sequences (5'-3')	Expected Size (bp)	References
Anaplasma phagocytophilum	MSP465f TGATGTTGTTACTGGACAGA MSP980r CACCTAACCTTCATAAGAA	550	Caspersen *et al*., 2002
Anasplasma platys	Platys-F AAGTCGAACGGATTTTGTC Platys-R CTTTAACTTACCGAACC	500	Beall *et al*., 2008
Babesia canis	PIRO-A1 AGGGAGCCTGAGAGACGGCTACC PIRO-B TTAAATACGAATGCCCCCAAC	450	Laha *et al*., 2014
Babesia gibsoni	Gib599 CTCGGCTACTTGCCTTGTC Gib1270 GCCGAAACTGAAATAACGGC	690	Otranto *et al*., 2009
Ehrlichia canis	ECA AACACATGCAAGTCGAACGGA HE3 TATAGGTACCGTCATTATCTTCCCTAT	400	Wen *et al*., 1997

Table 2: Prevalence and Diagnostic Performance of Canine Vector-borne Pathogens through Blood Smear and PCR (n = 30)

Pathogens	Methods	Positive (n)	Prevalence (%)
Anaplasma phagocytophilum	Smear	0	0.0
	PCR	0	0.0
Anasplasma platys	Smear	0	0.0
	PCR	0	0.0
Babesia canis	Smear	1	3.3
	PCR	6	20.0
Babesia gibsoni	Smear	5	16.7
	PCR	10	33.3
Ehrlichia canis	Smear	0	0.0
	PCR	4	13.3
Co-infection	Methods	Positive (n)	Prevalence (%)
Babesia gibsoni + *Babesia canis*	PCR	3	10.0
Babesia gibsoni + *Ehrlichia canis*	PCR	2	6.7

eISSN Number: 2462-2389

Table 3: Risk Factors associated with Canine Vector-Borne Pathogens

Risk factors	Positive	Negative	P-value
Life Stage			.088
Puppy	2	4	
Adult	17	7	
Sex			.346
Male	12	5	
Female	7	6	
Breed			.361
Mongrel	4	4	
Pedigree	15	7	
Management			.612
Indoor	5	2	
Outdoor	14	9	
Tick Infestation			.880
Yes	8	5	
No	10	7	
Diagnostic Methods			.003*
Blood Smear	6	84	
PCR	20	70	

(* significant at 95% confidence intervals)

REFERENCES

Abd Rani, P.A.M., Irwin, P.J., Coleman, G.T., Gatne, M., Traub, R.J. (2011). A survey of canine tick-borne diseases in India. Parasites Vectors, 4, 141.

Azzag, N., Petit, E., Gandoin, C., Bouillin, C., Ghalmi, F., Haddad, N., Boulouis, H.J. (2015). Prevalence of select vector-borne pathogens in stray and client-owned dogs from Algiers. Comparative Immunology, Microbiology and Infectious Diseases, 8, 1-7.

Bajer, A., Beck, A., Beck, R., Behnke, J.M., Dwużnik-Szarek, D., Eichenberger, R.M., Farkas, R., Fuehrer, H.P., Heddergott, M., Jokelainen, P., et al. (2022). Babesiosis in Southeastern, Central and Northeastern Europe: An emerging and re-emerging tick-borne disease of humans and animals. Microorganisms, 10, 945.

Beall, M.J,, Chandrashekar, R., Eberts, M.D., Cyr, K.E,, Diniz, P.P., Mainville, C., Hegarty, B.C., Crawford, J.M., Breitschwerdt, E.B. (2008). Serological and molecular prevalence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia* species in dogs from Minnesota. Vector-borne and Zoonotic Diseases, 8, 455-464.

Bergmann, M., and Hartmann, K. (2017). Vector-borne diseases in cats in Germany. Tierarztl Prax Ausg K Kleintiere Heimtiere, 45, 329-335.

Caspersen, K., Park, J.H., Patil, S., Dumler, J.S. (2002). Genetic variability and stability of *Anaplasma phagocytophila* msp2 (p44). Infection and Immunity, 70, 1230-1234.

Colella, V., Nguyen, V.L., Tan, D.Y., Lu, N., Fang, F., Zhijuan, Y., Wang, J., Liu, X., Chen, X., Dong, J., *et al*. (2020). Zoonotic vectorborne pathogens and ectoparasites of dogs and cats in Eastern and Southeast Asia. Emerging Infectious Diseases**,** 26,1221-1233.

DOS - Department of Statistic Malaysia (2022). Key Findings Population and Housing Census of Malaysia 2020. https://cloud.stats.gov.my/index.php/s/BG11nZfaBh09RaX#pdfviewer Accessed 12 January 2023.

Dantas-Torres, F. (2008). The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. Veterinary Parasitology, 152,173-85.

Gaunt, S., Beall, M., Stillman, B.A., Lorentzen, L., Diniz, P.P.V.P., Chandrashekar, R., Breitschwerdt, E.B., (2010). Experimental infection and co-infection of dogs with *Anaplasma platys* and *Ehrlichia canis*: hematologic, serologic and molecular findings. Parasites Vectors, 3, 33

Ghasemzade, M., Esmaeilnejad, B., Asri-Rezaei, S., Hadian, M. (2022). Molecular identification of *Babesia canis* canis genotype A in a dog from Iran. Veterinary Medicine and Science, 8**,** 21–25.

Jing, K.J., Shiang, L.F., Sieng, K.C., Bolongon, G.G., Noor Azleen, M.K., Bakar, S.A. (2017). Mini review: zoonotic agents from ticks in Malaysia. Journal of Wildlife and Parks, 32, 67–77.

Kalaivanan, M.K., and Palanivel, K.M. (2017). Detection of Co-Infection by *Babesia canis* and *Ehrlichia canis* in a subclinically infected dog. Indian Journal Animal Health, 56, 37-42.

Koh, F.X., Panchadcharam, C., Tay, S.T. (2016). Vector-borne diseases in stray dogs in peninsular Malaysia and molecular detection of *Anaplasma* and *Ehrlichia* spp. from *Rhipicephalus sanguineus* (Acari:Ixodidae) ticks. Journal of Medical Entomology, 53, 183-187.

Köster, L.S., Lobetti, R.G., Kelly, P. (2015). Canine babesiosis: a perspective on clinical complications, biomarkers, and treatment. Veterinary medicine (Auckland, NZ), 6,119-128.

Laha, R., Bhattacharjee, K., Sarmah, P.C., Das, M., Goswami, A., Sarma, D., Sen, A. (2014). Babesia infection in naturally exposed pet dogs from a north-eastern state (Assam) of India: detection by microscopy and polymerase chain reaction. Journal of Parasitic Diseases, 38, 389–393.

Megat Abd Rani, P.A., Irwin, P.J., Gatne, M., Coleman, G.T., Traub, R.J. (2010). Canine vector-borne diseases in India: a review of the literature and identification of existing knowledge gaps. Parasites Vectors, 3, 28

Mokhtar, A.S., Lim, S.F., Tay, S.T. (2013). Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. Tropical Biomedicine**,** 30, 345–348.

Nalubamba, K.S., Hankanga, C., Mudenda, N.B., Masuku, M. (2011). The epidemiology of canine *Babesia* infections in Zambia. Preventive Veterinary Medicine, 99, 240-244.

Nazari, M., Lim, S.Y., Watanabe, M., Sharma, R.S., Cheng, N.A., Watanabe, M. (2013). Molecular detection of *Ehrlichia canis* in dogs in Malaysia. PLoS Neglected Tropical Diseases, 7, e1982.

Otranto, D., Dantas-Torres, F., Breitschwerdt, E.B. (2009). Managing canine vector-borne diseases of zoonotic concern: part one. Trends in Parasitology, 25, 157-63.

Paddock, C.D., Goddard, J. (2015). The evolving medical and veterinary importance of the Gulf Coast tick (Acari: Ixodidae). Journal of Medical Entomology, 52, 230–252.

Parola, P. and Raoult, D. (2001). Ticks and tickborne bacterial diseases in humans: An emerging infectious threat. Clinical Infectious Diseases, 32, 897–928.

Perez, M., Rikihisa, Y., Wen, B. (1996). *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. Journal of Clinical Microbiology, 34, 2133–2139.

Prakash, B.K., Low, V.L., Vinnie-Siow, W.Y., Tan, T.K., Lim, Y.A., Morvarid, A.R., Abu Bakar, S., Sofian-Azirun, M. (2018). Detection of *Babesia* spp. in dogs and their ticks from Peninsular Malaysia: Emphasis on *Babesia gibsoni* and *Babesia vogeli* infections in *Rhipicephalus sanguineus* sensu lato (Acari:Ixodidae). Journal of Medical Entomology, 55, 1337-1340.

Rahman, W.A., Ning, C.H., Chandrawathani, P. (2010). Prevalence of canine ehrlichiosis in Perak State, Penisular Malaysia. Tropical Biomedicine, 27, 13–18.

Rajamanickam, C., Wiesenhutter, E., Zin, F. M., Hamid, J. (1985). The incidence of canine haematozoa in Peninsular Malaysia. Veterinary Parasitology, 17, 151-157.

Rotondano, T.E.F., Almeida, H.K.A., Krawczak, F.S., Santana, V.L., Vidal, I.F., Labruna, M.B., Azevedo, S.S., Almeida, A.M.P., Melo, M.A. (2015). Survey of *Ehrlichia canis, Babesia* spp. and *Hepatozoon* spp. in dogs from a semiarid region of Brazil. Revista Brasileira de Parasitologia Veterinária, 24, 52-58.

Rucksaken, R., Maneeruttanarungroj, C., Maswanna, T., Sussadee, M., Kanbutra, P. (2019). Comparison of conventional polymerase chain reaction and routine blood smear for the detection of *Babesia canis*,

eISSN Number: 2462-2389

Hepatozoon canis, *Ehrlichia canis*, and *Anaplasma platys* in Buriram Province, Thailand. Veterinary World, 12, 700-705.

Sipin, Q., Mustaffa Kamal, F., Watanabe, M., Megat Abdul Rani, P.A., Low, V.L., Abdul Aziz, N.A. (2020). Molecular detection of tick-borne haemopathogens in shelter dogs and *Rhipicephalus sanguineus* (sensu lato) ticks from Peninsular Malaysia. Comparative Immunology Microbiology Infectious Diseases, 73, 101563.

Solano-Gallego, L., Sainz, Á., Roura, X., Estrada-Peña, A., Miró, G. (2016). A review of canine babesiosis: The European perspective. Parasites Vectors, 9, 336.

Valkiunas, G., Iezhova, T.A., Krizanauskiene, A., Palinauskas, V., Sehgal, R.N., Bensch, S. (2008). A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology, 94, 1395-401.

Wang, J., Liu, J., Yang, J., Liu, Z., Wang, X., Li, Y., Luo, J., Guan, G., Yin, H. (2019). Molecular detection and genetic diversity of *Babesia canis canis* in pet dogs in Henan Province, China. Parasitology International 71, 37– 40.

Wen, B., Rikihisa, Y., Mott, J.M., Greene, R., Kim, H.Y., Zhi, N., Couto, G.C., Unver, A., Bartsch, R. (1997). Comparison of nested PCR with immunofluorescent antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. Journal of Clinical Microbiology, 35, 1852-1855.

Zulkifli, A.R., Hassan, L., Watanabe, M. (2011). Molecular study of Babesia in canine blood and comparison between conventional and molecular diagnostic methods. 6th Proceedings of the Seminar on Veterinary Sciences, Selangor, Malaysia.

eISSN Number: 2462-2389