Molecular detection of tick-borne haemopathogens in shelter dogs and *Rhipicephalus sanguineus* (*sensu lato*) ticks from Peninsular Malaysia

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Abstract

Background

Canine tick-borne haemopathogens (TBH) constitute a significant concern worldwide. The detection of these TBH is mainly achieved by microscopic evaluation, seroprevalence, and molecular detection. The present study was designed to investigate the molecular detection of Anaplasma platys , *Babesia gibsoni, Babesia vogeli* , and *Ehrlichia canis* in shelter dogs and *Rhipicephalus sanguineus (sensu lato)* ticks infesting them in Malaysia.

Results

A total of 220 blood samples and 140 ticks were collected from 10 animal shelters in Peninsular Malaysia. The presence of haemopathogens was detected using conventional PCR, sequenced, and identified at the species level. Of the 220 blood samples, 77 (35%) were positive with at least one of the four haemopathogens, of which *E. canis* predominated (20%) followed by B. gibsoni and *B. vogeli* both with detection of 7%, and *A. platys* (12%). In tick samples, 5 (3.57%) of the collected samples were positive with at least one of the three haemopathogens. Low detection of *E. canis* and A. platys were present (n = 2; 1.43%) as well as *B. vogeli* shows only 1 sample positive (0.71%) and no detection of B. gibsoni . For co-infection in dogs, single infection is common (24%), while co-infection with two haemopathogens (10%) was also observed. The occurrence of infection with three TBH was also observed in the sampled dogs (1%).

Conclusion

E. canis is the most common TBH affecting shelter dogs in Peninsular Malaysia. Co-infection is quite common and the most common co-infection present was *E. canis* and *A. platys*. The study highlighted the first extensive molecular detection of TBH in dogs and R. sanguineus (sensu lato) ticks in Malaysia

Background

Tick-borne diseases are a common problem worldwide and has increase in their importance due to the capability of causing critical illness as well as possibility of death in infected human and animals [1-3]. Tick-borne haemopathogens (TBH) were closely related to the companion animals which act as

resevoir of the various pathogen but also a potential victim that could harm this cats and dogs [4]. Recently, there is a trend of increase in the reported cases world wide which bring an alarming issues in human and animal well-being. As a result of TBH disease complex epidemiology, knowledge of this TBH in various host and different region of the world is critical for control [1, 5].

Tropical and subtropical countries are susceptible to tick vector infestation due to the warm, humid climate that is beneficial for their growth and proliferation [6,7]. *R. sanguineus* (*sensu lato*) also known as the brown dog tick, is the most common tick-vector in Malaysia and acts as a vector to most of the tick-borne haemopathogens in dogs [8,9]. Their life-cycle that makes it possible for them to infest multiple hosts makes them feasible to carry several pathogens at once [10]. Besides, *R. sanguineus* (*sensu lato*) ticks can retain the pathogens to its next progeny (transovarial), or life stages (transtadial) [11,12]. These capabilities make them be of vital importance in not only veterinary aspects but also public health, due to the close living environment between their primary host, dogs, with humans [13-15]. TBH that are most common and important in dogs are *B. vogeli*, *B. gibsoni*, *E. canis*, and *A. platys* [16].

Canine monocytic ehrlichiosis (CME) and infectious cyclic thrombocytopenia are among tick-borne bacterial diseases that are common in dogs, in Southeast Asia, with *E. canis* and *A. platys* as the causative agents, respectively [17]. Both diseases cause thrombocytopenia in infected dogs. *E. chaffeensis* and *E. ewingii* are zoonotic and infect monocytes and granulocytes of both dogs and humans, respectively [19-20]. Unlike the other two common species in dogs, *E. canis* are rarely found infecting humans. Worldwide, *E. canis* is considered as the most pathogenic, and have the potential to cause fatality to the infected dogs [21]. In general, two dominant species were reported to cause canine anaplasmosis, namely *A. phagocytophilum* and *A. platys* [21]. These *Anaplasma* species infecting dogs were known to cause granulocytic anaplasmosis, and thrombocytic anaplasmosis, respectively [21-23]. The clinical signs of *Anaplasma platys* infection are ranging from benign clinical signs [24-27], to a more severe clinical signs, identical to those shown by *E. canis*-infected dog [28-30]. Meanwhile, Babesiosis is the most common tick-borne protozoan disease in dogs with *Babesia* spp. as the aetiological agents. *B. vogeli* and *B. gibsoni* were mainly found in tropical and subtropical

areas and *B. gibsoni*, particularly, contributes to a more significant and severe clinical sign to the infected dog, compared to the other *Babesia* spp. [31].

In Malaysia, the abundance presence of stray animals and the increase in dog ownership especially in urban areas provide favourable conditions for tick survival and dispersion thus increase the chances of TBh transmission. There is also increase in complaint or cases of TBH among pet owners in the veterinary clinic and most of veterinarian are seeing a number of 3-4 cases per week which is quite alarming (author personal observation and personal communication).There are several reports on detection of TBH for these four important TBH in dogs in some region of the country [16,21, 32- 39]. Most of the previous work is based on conventional identification of the parasites microscopically from stained blood film [32-34] or seroprevalence [21,33, 35-36] while few on molecular detection [16,21, 35, 37 - 39] in recent works. However, these studies investigated certain small region within the country and most of the region are within or around the capital city of Malaysia.

Here we investigated the occurrence of selected TBH in shelter dogs where most of them are the rescued stray or neglected companion dogs, and ticks collected from those dogs, and further to examine the presence of the haemopathogen in dogs and the *R. sanguineus* (*sensu lato*) tick collected from same dogs,from six region in Peninsular, Malaysia.

Methods

Geographical area, study population and sample collection

This study included cites in west part of Peninsular Malaysia which includes three states in north region (Kedah, Perak, Pulau Pinang), three states and cities in central region (Putrajaya,Selangor, Kuala Lumpur), one state in south (Johor) and one state in east (Pahang) (Fig. 1). Blood were sampled and if present, ticks were collected from shelter dogs with no regards on age, sex and breed. The shelter dogs were selected randomly based on the manageable handling. The veterinarian involved in blood sampling and the postgraduate student collect the ticks with helps from workers from the shelter in restraining the animal. Blood samples were stored in EDTA tubes and frozen at -20°C prior to DNA extraction. The management of the shelter pertaining control of ectoparasites were asked to shelter owner or manager and were recorded.

Tick collection and morphological identification

Ticks collection from shelter dogs was conducted by inspecting each dog thoroughly for ticks; head part to toes, especially those parts of the dog's body that unreachable by the dog (eg., ears, neck, chest, legs, armpit, interdigital spaces). After inspection of the dog's body part, a slight pressure was applied to detect any presence of lumps from the dog's head to the tail. Ticks found were collected by forceps and placed in a tube. The tube was then labelled with the dog's identification and stored at -20°C before DNA extraction. Ticks were classified into either engorged or non-engorged ticks. The morphological inspection of ticks was done under a stereomicroscope, where ticks were identified to species, life stages, and gender using pictorial guides [40- 41].

DNA extraction, PCR assays, and sequencing

DNA extraction of ticks and blood was conducted by using the commercially available extraction kits, DNEasy Blood, and Tissue Kit (Qiagen, Germany). The ticks were extracted following the manufacturer protocol on the tissue sample while blood samples according to the blood extraction protocol. All DNA was then eluted in 100µl of elution buffer and stored at -20°C before PCR screening.

PCR amplification was conducted for the confirmation of tick species targeting the 450-500bp fragment of the 28S rRNA gene using primers 28SF (5'-GACTCTAGTCTGACTCTGTG-3') and 28SR (5'-GCCACAAGCCAGTTATCCC-3') [42]. The PCR detection for *A. platys*, *B. gibsoni*, *B. vogeli*, and *E. canis* for both blood and tick samples were performed using species-specific primers in a thermal cycler (Bio-Rad Laboratories, USA) with the cycling conditions indicated in Table 1.

Amplified PCR products were sequenced for further phylogenetic analysis. PCR products were sequenced using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were subjected to BLAST identity search (NCBI) to compare with known sequences of *B. vogeli*, *A. platys*, and *E. canis* in the GenBank.

Statistical analysis

The detection of pathogens in blood samples and ticks were analysed using global prevalence to calculate expected frequencies and the presence of TBH was compared across regions, sex, and age

using Chi-squared tests (SPSS Statistics version 21, IBM, USA). 95% confidence interval was used, where a p-value of 0.05 or less ($p \leq 0.05$) was considered to indicate a statistically significant difference.

Sequence analysis and phylogenetic tree

Sequence alignment for all the genes was conducted using DAMBE7 [46]. Phylogenetic relationships based on nuclear genes were determined using a maximum likelihood (ML) algorithm using MEGA X [47]. At least 1000 bootstrap replicates were used to infer statistical support at branch nodes. Outgroup sequences for *A. platys, B. vogeli*, and *E. canis* following their targeted gene were as tabulated in Table 2. Sequences of dog DNA, *Canis familiaris* (DQ983934 or AY623831), and *R. sanguineus* (*sensu lato*) DNA (MH481870 or KU198407) were used as outgroup for each haemopathogen phylogenetic tree.

Results

Data collection

A total of 220 blood sample were obtained from 10 shelter in eight different areas in Peninsular Malaysia. The majority of the sampled were adults (72.72%) and female (68.18%) dogs. All the dogs sampled were appeared healthy and did not show any clinical signs related to TBH infections at the time of sampling. There were 66 dogs were having ticks and a total of 140 ticks sample were subjected for TBH detection by PCR assay. All the ticks were identified as *R. sanguineus* (*sensu lato*) with all of them were adult and 44 (31.43%) were male ticks while 96 (68.57%) were female ticks. Twelve ticks were engorged (8.57%) while the rest were unengorged ticks (n=128, 91.42%). Representative samples of 30 ticks (21%) (GenBank accession numbers MN160243-49) were sequenced, and they showed a similarity between 94-100% with *R. sanguines* (*sensu lato*) sequences from NCBI GenBank (AF120312).

Detection of tick-borne haemopathogen in dogs

In general, 76 dogs (34.55%) were infected with at least one of the four TBH. *E. canis* was the most commonly detected TBH where 43 dogs (19.55%; 95% CI: 14.65-25.54) were positive, while *A. platys,* was detected in 26 dogs (11.82%, CI: 8.01-17.01) and *B. gibsoni* and *B. vogeli* were the least detected

in 16 dogs (7.27%, CI: 4.35-11.75). Single infection was common, being detected in 53 dogs (24.09%) with either one of the TBH. Co-infection with any two of the TBH were detected (*E. canis* + *A. platys*, *E. canis* + *B. gibsoni*, *E. canis* + *B. vogeli,* and *A. platys* + *B. gibsoni*) was also observed in 21 dogs (9.55%). Triple infection where three pathogen were detected in the same dogs (*E. canis* + *A. platys* + *B. vogeli*, *A. platys* + *B. vogeli* + *B. gibsoni)* were observed in two dogs (0.9%). The most common co-infection observed was *E. canis* + *A. platys*, with 12 dogs tested positive (Table 3). Co-infection with all four haemopathogens was not observed in the study.

The detection of TBH in dogs were compared to age, sexes and region by using Chi-square analysis (Table 4). Male dogs showed a significantly higher *A. platys* infection rate compared to their female counterparts (= 6.33, *df* = 1, P < 0.05). There was a significant difference between young and adult dogs for *B. vogeli* infection (= 6.33, *df* = 1, P < 0.001) where more young dogs being infected (n=11). With regards to region, dogs from the eastern region exhibited the highest detection rate of *B. gibsoni* infection (n=8), while infection with *E. canis* (n=33) and *A. platys* (n=20) were both dominant in dogs from the northern region. *B. vogeli* infection was only detected in dogs from the northern (n=9) and central (n=7) regions. Dogs from the southern region were observed to have the lowest infection rate, with only *B. gibsoni* being detected. There were significant differences in infection rates of *A. platys* (= 9.20, *df* = 3, P < 0.05)*, E. canis* (= 15.30, *df* = 3, P < 0.001)*,* and *B. gibsoni* (= 51.80, *df* = 3, P < 0.001) between the four regions.

Detection of tick-borne haemopathogen in ticks

Out of 140 tick samples, 2 (1.43%) and 1 (0.71%) were positive for *A. platys, E. canis,* and *B. vogeli* respectively. No detection of *B. gibsoni* obtained in this study. All positive samples were from non-engorged ticks and all the ticks that were positive for TBH were removed from infected dogs.

Molecular characterization of tick-borne haemopathogen

A total of four DNA samples from *B. gibsoni* positive blood samples (25%) of 18S rRNA gene with the size of ~690bp were sequenced (GenBank accession numbers MN068981-84). The sequence analyses revealed the similarity between 99-100% with *B. gibsoni* sequences from India, Malaysia, South Korea, and Taiwan obtained from NCBI GenBank (Fig. 2).

Four (25%) DNA positive samples from dogs and only one from ticks of *B. vogeli* 18S rRNA gene with the size of ~450bp were sequenced (GenBank accession numbers MN075251-54; MN194598). The 18S rRNA gene showed 99% similarity with other *B. vogeli* sequences from Myanmar, Brazil, Taiwan, and Malaysia obtained from GenBank (Fig. 3).

E. canis 16S rRNA gene with the size of ~ 400bp was sequenced from 10 dog samples (26%) (GenBank accession numbers MN075258-68) and two positive samples from ticks (GenBank accession numbers MN159066-67). All 16S rRNA gene sequences formed a monophyletic clade with other *canis* obtained from GenBank with 99% similarity except for one sequence from the central region that was different from other sequences where it forms in another clade from the rest of the sequences (Fig. 4).

The 16S rRNA gene of *A. platys* with the size of ~500bp was sequenced nine dog samples (35%) (GenBank accession numbers MN075275-83) and from two tick samples (GenBank accession numbers MN159064-65). All sequences were 95-100% identical to each other and clustered together with *A. platys* obtained from GenBank except two sequences from dogs that were different from the rest of the sequences and formed a different clade that was from the northern region. The sequence of *A. platys* from the dog's blood (GenBank accession numbers MN075276) was similar to the sequence of *A. platys* from tick (GenBank accession numbers MN159064) collected from the same dog (Fig. 5).

Discussion

The present study is the first comprehensive study on the detection of TBH in ticks and dogs in Malaysia. The present work investigated the relationship of the tick-borne pathogens in dogs and ticks, the co-infection presence, and phylogenetic analysis of both sequences from dogs and ticks. In general, the present study showed *E. canis* as predominated TBH in dogs, followed by *A. platys, B. gibsoni* and *B. vogeli.* In ticks, there is detection of *A. platys, E. canis, B. vogeli* and no detection of *B. gibsoni*.

Previous work on TBH in dogs in Malaysia utilizing microscopical examination of the blood smear, serology, however, recent work have changed the method of detection to molecular method. The used of microscopical examination of the blood smear and serology methods are both lacking in

terms of sensitivity. The use of serology method, notably, lacking in differentiating past from on-going infection after antibiotic treatment due to the continued existence of antibodies, thus resulting in false-positive results [1, 48-50]. The availability of these works on TBH provide little epidemiological data in Malaysia as most of the work were limited to small areas in the country. Our study included eight states in Peninsular Malaysia as well as the capital region of Malaysia where the cities in this states have high density of human as well as pets and stray dogs. Stray dogs has an important role of maintaining the TBH disease as a result of being neglected [1, 6]. Animal shelters is a place where strays dogs being rescued and able to represent the bigger picture of the condition of stray dogs and what diseases they carry. Therefore, the current work use molecular methods and a large scale of sampling area in the present study were expected to give a better picture of TBH detection in dogs in Peninsular Malaysia.

E. canis infection was reported to be generally higher than the other TBH observed in current work and Malaysian dogs, which also agree with those reported from other Southeast Asian countries [1, 51 - 52]. For other TBH, previous work have reported varying detection of *E. canis* (0-55.6%), *A. platys* (3.3-13.3%), *B. gibsoni* (0-17.7%) and *B. vogeli* (0-10%) in dogs in Peninsular Malaysia [16, 32, 34 - 36,39, 40, 54]. Meanwhile, a higher detection of TBH, *E. canis* (33-56.7%), *A. platys* (27-38.5%), and *Babesia* (65.4%) was reported in dogs from East Malaysia, [21,37]. The differences in the prevalence of TBH obtained were most likely due to the number of dogs, geographical area of sampling, selection criteria and target gene [1].

Male dogs more likely to encounter *A. platys* infection based on our findings and young dogs have higher infection rate of *B. vogeli*. Some previous studies reported significantly higher haemopathogen infection in young dogs compared to adult dogs [1, 54-55]. The best explanation are young dogs normally prone to have tick infestation with higher burden compared to adult dogs [56]. Other works showed higher infection rates of TBH in female as a result of they are less active than male, which allows high chances of getting infested by ticks and being infected with TBH [57]. However the higher detection in male in current study warrant further investigation or could be due to the population of study were different from other study. Higher detection of TBH were observed from northern region

while previous work reported higher detection in the central and eastern regions [35-36, 38]. The previous work were mainly focusing on the central region in capital city of Malaysia where other region were understudied. In relation to management practices, most of the infected samples were from animal shelters that do not practice giving preventive medicine to the dogs residing in their shelter. Treatment was only given to dogs with clinical signs, which as far as tick-borne haemopathogens is concerned; the infection can occur subclinically [16]. Inadequate funding and lack of staff are the reasons that prevent these animal shelters from giving the dogs the medication they need. The southern region animal shelter recorded low detection of TBH as they provides good preventive measures and treatments for their dogs. The animal shelter practices application of dipping bath and doxycycline treatment (10mg/kg) once a day for a month, orally, to new dogs. In addition, dipping bath was conducted once every three weeks to all dogs residing in the shelter. Therefore in our work, management does play a significant role which variation in tick-borne haemopathogen prevalence is related to differences in management practices of different shelters [36].

The low detection of TBH in ticks in present works were alligned with other reports where low detection of the pathogens in the tick vector were found compared to the detection in dogs [1, 58-63]. Climatic conditions play roles in the detection, activity, and survival of the tick vector [38, 64-66]. The climate was considered as one of the leading causes of these tick-borne haemopathogens prevalence changes between regions due to its capability to alter the disease transmission dynamics and their geographical distribution, although other factors such as ecological changes and importation of animals in an area can also contribute to the changes[67-69]. Furthermore, the chance of the pathogen transmission is also relatively high through co-feeding, where the previously uninfected ticks can be infected by blood-feeding in close proximity to an infected tick [70]. The other possible reasons for the low prevalence of TBH in ticks due to the other ticks infected with TBH might already fall off the dog host prior to sample collection. The attachment period of ticks was different in terms of their life stages. Female *R. sanguineus* (*sensu lato*) in its adult form, for example, usually blood-feeding ranging from five to 21 days, while nymph blood-feeding for three to 11 days before they

drop off to the ground [71]. Approximately 5% of the tick vectors could be found on the dog host, while the rest can be found in the environment [10]. Future studies are recommended to work on the detection of TBH in ticks, which were not only attached to the dog host but also off-host ticks to understand further the true prevalence of the pathogens they could carried.

The co-infection of TBH has been reported in Malaysian dogs [35,37,38]. To the best of our knowledge, there has been no report of *A. platys* and *B. gibsoni* co-infection in the literature in Malaysia, thus indicates that this is the first report of the co-infection in the country. The most common co-infection seen in dogs in the present work was *E. canis* and *A. platys* co-infection, which agree with the report from other previous studies elsewhere [52, 72-74]. The co-infection of TBH in dogs is common and has been reported in countries worldwide. Co-infection of pathogens in the host warrant proper surveillance due to the possibility of pathophysiological alteration by multiple pathogens, and the difficulty in giving proper treatment to the co-infected dogs [75-77]. Although co-infection of these TBH is possible in ticks [38-39,78], no co-infection of these haemopathogens were observed in ticks in the present study. From the current work, ticks positive for TBH were all the ticks removed from infected dogs which showed the high possibility of the ticks obtaining the infection from the infected dogs, although the ticks could obtained the infection from their previous blood meal [38].

In general, the phylogenetic analysis of the 16S rRNA and 18S rRNA gene of the tick-borne haemopathogens showed that the Malaysian isolates shared high similarity and clustered together with isolates from other Asian countries (Philippines, China, Thailand, Myanmar, Taiwan, Japan, India, and Vietnam.) as well as countries outside Asia (Brazil, Cape Verde, Panama, Italy, Portugal, Venezuela, and Nigeria). These results suggested that the 16S rRNA and 18S rRNA gene of the tick-borne haemopathogens are conserved regions, which have high similarity within species. Although it is only for one sample, the similarity of sequence in *A. platys* in dogs and ticks most likely suggesting the tick could be a source of infection for *A. platys* in the dog, and this could be a good indicator in tick-borne disease diagnosis and tracking the source of infection. However, there is no specific pattern or separation between isolates of tick-borne haemopathogens in dogs and ticks might be due

to the conserved region of the gene of interest used in present work. A more variable region can be used for detailed phylogenetic analysis, for example, mitochondrial gene.

Conclusions

The study highlighted the first extensive molecular detection of tick-borne haemopathogens in dogs and ticks in Malaysia. *E. canis* was the main tick-borne haemopathogen in dogs, and the most common co-infection present was *E. canis* and *A. platys.* However, the detection of tick-borne haemopathogen in ticks was low compared to dogs and the phylogenetic analysis show similarity of isolates in present work with published sequences from various countries. It is crucial to investigate the presence of these tick-borne haemopathogens in tick populations that act as a vector for these tick-borne haemopathogens as there is still limited information available in the literature. Future studies on the relationship between tick-borne haemopathogens in dogs and their ticks would be beneficial in understanding the nature of transmission of these tick-borne haemopathogens.

Declarations

Ethics approval and consent to participate

The application for animal ethical clearance has been approved by the Institutional Animal Care and Use Committee Universiti Putra Malaysia (IACUC) (AUP no: UPM/IACUC/AUP-R028/2018). Consent was asked from the shelters before sampling. The shelter's owner has been explained on the nature of the research, and the animals in shelters were handled concerning the animal ethics laws.

Consent for publication

Not applicable.

Availability of data and materials

Sequences have been submitted in the GenBank database under the following Accession Numbers:GenBank accession numbers MN068981-84 for *B. gibsoni*, GenBank accession numbers MN075251-54; MN194598 for *B. vogeli*, GenBank accession numbers MN075258-68; MN159066-67 for *E. canis*,GenBank accession numbers MN075275-83; MN159064-65 for *A. platys* and GenBank accession numbers MN160243-49 for *Rhipicephalus sanguineus* (*sensu lato*).

Competing interests

The authors declare that they have no competing interest

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Authors' contributions

QS and NA conceived the study and all authors participated in its design. QS, NA, FM, MK, PA planned and undertook blood collections, QS performed the PCR assay, and QS and NA conducted the statistical analysis and drafted the manuscript. NM and RS give their valuable opinion and help in data analysis. All authors read and approved the final version of the manuscript.

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References

1. Galay RL, Manalo AAL, Dolores SLD, Aguilar IPM, Sandalo KAC, Cruz KB, et al. Molecular detection of tick-borne pathogens in canine population and *Rhipicephalus sanguineus* (*sensu lato*) ticks from southern Metro Manila and Laguna Philippines. Parasites & Vectors. 2018;11:643-650.
2. Wei L, Kelly P, Ackerson K, Zhang J, El-Mahallawy HS, Kaltenboeck B, et al. First report of Babesia gibsoni in Central America and survey for vector-borne infections in dogs from Nicaragua. Parasit Vectors. 2014;7:126-131.
3. Dantas-Torres F, Otranto D. Dogs, cats, parasites, and humans in Brazil: opening the black box, Parasit Vectors. 2014;7:22-46.

4. Claerebout E, Losson B, Cochez C, Casaert S, Dalemans AC, De DA, et al. Ticks and associated pathogens collected from dogs and cats in Belgium. Parasit Vectors. 2013;6:183-191.
5. Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 2012;28:437–446.
6. Irwin PJ, Jefferies R. Arthropod-transmitted diseases of companion animals in Southeast Asia. Trends Parasitol. 2004;20:27–34.
7. Mariana A, Mohd KB, Halimaton I, Suhaili ZA, Shahrul-Anuar MS, Nor ZM. Acarine ectoparasites of Panti Forest Reserve in Johore, Malaysia. Asian Pac J Trop Biomed. 2011;1:1-5.
8. Harrus S, Waner T. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. Vet J. 2011;187:292-296.
9. Solano-Gallego L, Baneth G. Babesiosis in dogs and cats-expanding parasitological and clinical spectra. Vet Parasitol. 2011;181:48–60.
10. Dantas-Torres F. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): From taxonomy to control. Veterinary Parasitology. 2008;152:173–185.
11. Bremer WG, Schaefer JJ, Wagner ER, Ewing SA, Rikihisa Y, Needham GR, et al. Transstadial and intrastadial experimental transmission of Ehrlichia canis by male *Rhipicephalus sanguineus*. Vet. Parasitol. 2005;131:95-105.
12. Dantas-Torres F. Rocky Mountain spotted fever. Lancet Infect Dis. 2007;7:724–732.
13. Felz MW, Durden LA, Oliver Jr JH. Ticks parasitizing humans in Georgia and South Carolina. J Parasitol. 1996;82:505–508.
14. Uspensky I, Ioffe-Uspensky I. The dog factor in brown dog tick *Rhipicephalus sanguineus* (Acari: Ixodidae) infestations in and near human dwellings. Int J Med

Microbiol. 2002;291:156–163.

15. Dantas-Torres F, Figueredo LA, Brandaˆo-Filho SP. *Rhipicephalus sanguineus* (Acari: Ixodidae), the brown dog tick, parasitizing humans in Brazil. Rev Soc Bras Med Trop. 2006;39:64–67.

16. Nazari M, Lim SY, Watanabe M, Sharma RS, Cheng NA, Watanabe M. Molecular detection of *Ehrlichia canis* in dogs in Malaysia. PLoS Negl Trop Dis. 2013;7:e1982.

17. Sainz Á, Roura X, Miró G, Estrada-Peña A, Kohn B, Harrus S, Solano-Gallego L. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. Parasit Vectors. 2015;8:75-94.

18. Breitschwerdt EB, Hegarty BC, Hancock SI. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. Journal of Clinical Microbiology. 1998;36:2645-2651.

19. Goldman EE, Breitschwerdt EB, Grindem CB, Hegarty BC, Walls JJ, Dumler JS. Granulocytic Ehrlichiosis in dogs from North Carolina and Virginia. J Vet Intern Med. 1998;12:61-70.

20. McBride JW, Walker DH. Molecular and cellular pathobiology of *Ehrlichia* infection: Targets for new therapeutics and immunomodulation strategies. Expert Rev Mol Med. 2013;13:e3.

21. Konto M, Tukur SM, Watanabe M, Abd-Rani PAM, Sharma RSK, Fong LS, Watanabe M. Molecular and serological prevalence of *Anaplasma* and *Ehrlichia* among stray dogs in East Malaysia. Trop Biomed. 2017;34:570-575.

22. Ndip LM, Ndip RN, Esemu SN, Walker DH, McBride JW. Predominance of *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks from kennel-confined dogs in Limbe, Cameroon. Exp Appl Acarol. 2010;50:163–168.

23. Skotarczak B. The role of companion animals in the environmental circulation of tick-

borne bacterial pathogens. Ann Agric Environ Med. 2018;25:473–480.

24. Baker DC, Gaunt SD, Babin SS. Anemia of inflammation in dogs infected with *Ehrlichia platys*. Am J Vet Res. 1988;49:1014-1016.

25. Bradfield JF, Vore SJ, Pyron WH. *Ehrlichia platys* infection in dogs. Lab Anim Sci. 1996;46:565–568.

26. Simpson RM, Gaunt SD, Hair JA, Kocan KM, Henk WG, Casey HW. Evaluation of *Rhipicephalus sanguineus* as a potential biologic vector of *Ehrlichia platys*. Am J Vet Res. 1991;52:1537–1541.

27. Walker DH, Dumler JS. Emergence of the ehrlichioses as human health problems. Emerg Infect Dis. 1996;2:18–29.

28. Arraga-Alvarado C. Ehrlichiosis canina en Maracaibo, Estado ZuliaVenezuela: reporte de 55 casos. Revista Cientifica FCV-LUZ. 1992;2:41-52.

29. Glaze MB, Gaunt SD, Babin SS. Uveitis associated with Ehrlichia platys infection in a dog. J Am Vet Med Assoc. 1986;189: 916-917.

30. Wilson BJ. *Ehrlichia platys* in a Michigan dog. J Am Anim Hosp Assoc. 1992;28:381-383.

31. Miyama T, Sakata Y, Shimada Y, Ogino S, Watanabe M, Itamoto K, et al. Epidemiological survey of Babesia gibsoni infection in dogs in eastern Japan. Vet Med Sci. 2005;67:467–471.

32. Rajamanickam C, Wiesenhutter E, Zin FM, Hamid J. The incidence of canine haematozoa in Peninsular Malaysia. Vet Parasitol. 1985;17:151-157.

33. Rahman WA, Ning CH, Chandrawathani P. Prevalence of canine ehrlichiosis in Perak State, Peninsular Malaysia. Trop Biomed. 2010;27:13–18.

34. Erwanas AI, Chandrawathani P, Premaalatha B, Zaini CM, Lilt-Rozita MH, Jamnah O, et al. Parasitic infections found in pet and stray dogs in Ipoh, Malaysia. Malaysian J Vet

Res. 2014;5:27-34.

35. Koh FX, Panchadcharam C, Tay ST. Vector-borne diseases in stray dogs in Peninsular Malaysia and molecular detection of *Anaplasma* and *Ehrlichia* from *Rhipicephalus sanguineus* (Acari: Ixodidae) ticks. J Med Entomol. 2016;53:183-187.

36. Lau SF, Dolah RN, Mohammed K, Watanabe M, Abdul Rani PAM. Canine vector-borne diseases of zoonotic concern in three dog shelters in Peninsular Malaysia: The importance of preventive measures. Trop Biomed. 2017;34:72–79.

37. Mohammed KSM, Tukur M, Watanabe M, Megat-Abdul-Rani PA, Sharma RSK, Fong LS, et al. Molecular and serological detection of tick-borne hemopathogens among stray dogs in East Malaysia. J Vet Med. 2017;4:1074-1078.

38. Low VL, Prakash, Lim YAL, Tan TK, Vinnie-Siow WY, Sofian-Azirun M, et al. Detection of Anaplasmataceae agents and co-infection with other tick-borne protozoa in dogs and *Rhipicephalus Sanguineus sensu lato* Experimental and Applied Acarology. 2018;75:429-435.

39. Prakash BK, Low VL, Vinnie-Siow WY, Tan TK, Lim YAL, Movarid AR, et al. Detection of *Babesia* in dogs and their ticks from Peninsular Malaysia: emphasis on *Babesia gibsoni* and *Babesia vogeli* infections in *Rhipicephalus sanguineus sensu lato* (Acari: Ixodidae). J. Med. Entomol. 2018;55:1337-1340.

40. Walker A, Bouattour A, Camicas JL, Estrada-Peña A, Horak IG, Latif AA. Ticks of domestic animals in Africa: a guide to identification of species. UK: Bioscience Reports Edinburgh. 2014.

41. Barker SC, Walker AR. Ticks of Australia: The species that infest domestic animals and humans. Zootaxa Magnolia Press. 2014;3816:001-144.

42. Inokuma H, Yoshizaki Y, Shimada Y, Sakata Y, Masaru O, Takafumi O. Epidemiological Survey of *Babesia* Species in Japan Performed with Specimens from Ticks Collected

from Dogs and Detection of New *Babesia* DNA Closely Related to *Babesia odocoilei* and *Babesia divergens* Journal of Clinical Microbiology. 2003;41:3494–3498.

43. Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part one. Trends Parasitol. 2009;25:157-163.

44. Beall MJ, Chandrashekar R, Eberts MD, Cyr KE, Diniz PPVP, Mainville C, et al. Serological and Molecular Prevalence of *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Ehrlichia* Species in Dogs from Minnesota. Vector-borne and Zoonotic Diseases. 2008;8:455-464.

45. Wen B, Rikihisa Y, Mott JM, Greene R, Kim HY, Zhi N, et al. Comparison of nested PCR with immunofluorescent antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J Clin Microbiol. 1997;35:1852-1855.

46. Xia X. DAMBE7: New and improved tools for data analysis in molecular biology and evolution. Mol Biol Evol. 2018;35:1550–1552.

47. Kumar S, Stecher G, Li M, Knyaz C, Tamura K.MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol. 2018;35:1547–1549.

48. Suksawat J, Xuejie Y, Hancock SI, Hegarty BC, Nilkumhang P, Breitschwerdt EB. Serologic and molecular evidence of coinfection with multiple vector-borne pathogens in dogs from Thailand. J Vet Intern Med. 2001;15:453-462.

49. Wong SSY, Teng JLL, Poon RWS, Choi GKY, Chan KH, Yeung ML, et al. Comparative evaluation of a point-of-care immunochromatographic test SNAP 4Dx with molecular detection tests for vector-borne canine pathogens in Hong Kong. Vector Borne Zoonotic Dis. 2011;11:1269-1277.

50. Maggi RG, Birkenheuer AJ, Hegarty BC, Bradley JM, Levy MG, Breitschwerdt EB. Comparison of serological and molecular panels for diagnosis of vector-borne diseases in dogs. Parasit Vectors. 2014;7:127-135.

51. Piratae S, Pimpjong K, Vaisusuk K, Chatan W. Molecular detection of *Ehrlichia canis*, *Hepatozoon canis* and *Babesia canis vogeli* in stray dogs in Mahasarakham province, Thailand. Ann Parasitol. 2015; 61:183-187.

52. Nasution AYA, Hadi UK, Retnani EB. Prevalence of ectoparasites and endoparasites on companion dogs which visit animal clinic at north Jakarta. Journal of Entomology and Zoology Studies. 2018;6:1099-1104.

53. Mokhtar AS, Lim SF, Tay ST. Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. Trop Biomed. 2013;30:345-348.

54. Lakshmanan B, John L, Gomathinayagam S, Dhinakarraj G. Prevalence of *Ehrlichia canis* in Chennai. Indian Vet. J. 2006;7:307–312.

55. Milanjeet HS, Singh NK, Singh ND, Singh C, Rath SS. Molecular prevalence and risk factors for the occurrence of canine monocytic ehrlichiosis. Vet Med. 2014;59:129–136.

56. Gray J, Dantas-Torres F, Estrada-Peña A, Levin M. Systematics and ecology of the brown dog tick, Rhipicephalus sanguineus. Ticks Tick Borne Dis. 2013;4:171–180.

57. Konto M, Biu AA, Ahmed MI, Charles S. Prevalence and seasonal abundance of ticks on dogs and the role of Rhipicephalus sanguineus in transmitting Babesia species in Maidugiri, north-eastern Nigeria. Vet World. 2014;7:119–124.

58. Inokuma H, Raoult D, Brouqui P. Detection of *Ehrlichia platys* DNA in Brown Dog Ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. Journal of Clinical Microbiology. 2000;38:4219-4221.

59. Foongladda S, Inthawong D, Kositanont U, Gaywee J. Rickettsia, Ehrlichia, Anaplasma, and Bartonella in ticks and fleas from dogs and cats in Bangkok. Vector-Borne Zoonotic Dis. 2011;11: 1335–1341.

60. Ybanez AP, Perez ZO, Gabotero SR, Yandug RT, Kotaro M, Inokuma H. First molecular

detection of *Ehrlichia canis* and *Anaplasma platys* in ticks from dogs in Cebu, Philippines. Ticks and Tick-borne diseases. 2012;3:287-292.

61. Chao LL, Yeh ST, Hsieh CK, Shih CM. First detection and molecular identification of *Babesia vogeli* from Rhipicephalus sanguineus (Acari: Ixodidae) in Taiwan. Exp Appl Acarol. 2016;68:539–551.

62. Livanova NN, Fomenko NV, Akimov IA, Ivanov MJ, Tikunova NV, Armstrong R, et al. Dog survey in Russian veterinary hospitals: tick identification and molecular detection of tick-borne pathogens. Parasites & Vectors. 2018;11:591-600.

63. Nguyen VL, Colella V, Latta R, Bui KL, Dantas-Torres F, Otranto D. Ticks and associated pathogens from dogs in northern Vietnam. Arthropods and Medical Enttomology. 2019;118:139-142.

64. Nijhof AM, Bodaan C, Postigo M, Nieuwenhuijs H, Opsteegh M, Franssen L, et al. Ticks and associated pathogens collected from domestic animals in The Netherlands. Vector Borne Zoonotic Dis. 2007;7:1–11.

65. Cardoso L, Yisaschar-Mekuzas Y, Rodrigues FT, Costa Á, Machado J, Diz-Lopes D, et al. Canine babesiosis in northern Portugal and molecular characterization of vector-borne co-infections. Parasit Vectors. 2010;3:27-35.

66. Madder M, Pascucci I. Ticks and tick-borne diseases: geographical distribution and control strategies in the Euro-Asia region. CABI. 2012;3:27-32.

67. Gray JS, Dautel H, Estrada-Peña A, Kahl O, Lindgren E. Effects of climate change on ticks and tick-borne diseases in Europe. Interdisciplinary perspectives on infectious diseases. 2009. Article ID 593232, 12 pages.

68. Estrada-Peña A, Ortega C, Sánchez N, DeSimone L, Sudre B, Suk JE, et al. Correlation of *Borrelia burgdorferi sensu lato* prevalence in questing *Ixodes ricinus* ticks with specific abiotic traits in the western Palearctic. Applied and environmental

microbiology. 2011;77:3838-3845.

69. Begum RA, Siwar C, Abidin RDZRZ, Pereira JJ. Vulnerability of climate change and hardcore poverty in Malaysia. Journal of Environmental Science and Technology. 2011;4:112-117.

70. Gondard M, Cabezas-Cruz A, Charles RA, Vayssier-Taussat M, Albina E, Moutailler S. Ticks and Tick-Borne Pathogens of the Caribbean: Current Understanding and Future Directions for More Comprehensive Surveillance. Front. Cell. Infect. Microbiol. 2017;7:1-16.

71. Pegram RG, Keirans JE, Clifford CM, Walker JB. Clarification of the Rhipicephalus sanguineus group (Acari, Ixodoidea, Ixodidae). II. *sanguineus* (Latreille, 1806) and related species. Syst Parasitol. 1987;10:27–44.

72. Dagnone AS, Souza AI, Andre MR, Machado RZ. Molecular diagnosis of Anaplasmataceae organisms in dogs with clinical and microscopical signs of ehrlichiosis. Rev Bras Parasitol Vet. 2009;18:20-25.

73. Ramos R, Ramos C, Araujo F, Oliveira R, Souza I, Pimentel D. Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil). Parasitol Res. 2010;107:1115-1120.

74. Huggins LG, Koehler AV, Ng-Nguyen D, Wilcox S, Schunack B, Inpankaew T, Traub RJ. Assessment of a metabarcoding approach for the characterisation of vector-borne bacteria in canines from Bangkok. Thailand Parasites Vectors. 2019;12:394-404.

75. Shaw SE, Day MJ, Birtles RJ, Breitschwerdt EB. Tick-borne infectious diseases of dogs. Trends Parasitol. 2001;17:74-80.

76. Gaunt SD, Beall MJ, Stillman BA, Lorentzen L, Diniz PPVP, Chandrashekar R, et al. Experimental infection and co-infection of dogs with *Anaplasma platys* and *Ehrlichia canis*: hematologic, serologic and molecular findings. Parasit Vectors. 2010;3:33-42

77. Mylonakis ME, Koutinas AF, Baneth G, Polizopoulou Z, Fytianou A. Mixed *Ehrlichia canis*, *Hepatozoon canis*, and presumptive *Anaplasma phagocytophilum* infection in a dog. Vet Clin Path. 2004;33:249-251.

78. Kamani J, Baneth G, Mumcuoglu KY, Waziri NE, Eyal O, Guthmann Y, et al. Molecular Detection and Characterization of Tick-borne Pathogens in Dogs and Ticks from Nigeria. PLoS Negl Trop Dis. 2013;7:e2108.

Tables

Table 1 Primers used in this study and PCR conditions (annealing temperature).

Haemopathogens	Primer sequence (5'-3')	Gene target (
Babesia gibsoni	Gib599F (5'-CTCGGCTACTTGCCTTGTC-3') Gib1270R (5'-GCCGAAACTGAAATAACGGC-3')	18S rRNA (69
Babesia vogeli	C172F (5'-GTTTATTAGTTTGAAACCCGC-3') C626R (5'-GAACTCGAAAAAGCCAAACGA-3')	18S rRNA (45
Anaplasma platys	PlatysF (5'-AAGTCGAACGGATTTTTGTC-3') PlatysR (5'-CTTTAACTTACCGAACC-3')	16S rRNA (50
Ehrlichia canis	ECA (5'-AACACATGCAAGTCGAACGGA-3') HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3')	16S rRNA (40

Table 2 Outgroups used in phylogenetic tree construction

Pathogen (gene)	Outgroups		Previous sequenc
Anaplasma platys (16S rRNA)	GQ395385 [Cape Verde]		MN075275 [Ea7]
	KJ659045 [China]		MN075276 [Ea8]
	KF360842 [Panama]		MN075277 [Ea11
	EU439943 [Italy]		MN075278 [Nb2]
	JQ894779 [Philippines]		MN075279 [Nb15
	AF536828 [Japan]		MN075280 [Nc13
	EF139459 [Thailand		MN075281 [Nd4]
	KU500912 [Selangor]		MN075282 [Wa19
	MG793446 [Brazil]		MN075283 [Wd25
	KU500913 [Sarawak]		
	MG050136 [India]		
	KR232559 [*Ehrlichia canis*]		
Babesia vogeli (18S rRNA)	KU361219 [Malaysia]		MN075251 [Nb1]
	LC168631 [Myanmar]		MN075252 [Wd2]
	KJ494656 [Brazil]		MN075253 [Wa1]
	JF682473 [Taiwan]		MN075254 [Wb18
	EF601930 [*Babesia bovis*]		
	MG585382 [*Theileria orientalis*]		
Babesia gibsoni (18S rRNA)	EU430494 [South Korea]		MN068981 [Nd7]
	KU500915; KU500917 [Malaysia]		MN068982 [Wb25
	KF112075 [India]		MN068983 [Wd25
	EF587268 [Taiwan]		MN068984 [Sa2]
	LC094958 [*Theileria orientalis*]		
Ehrlichia canis (16S rRNA)	JF429693 [Malaysia]	DQ003032 [Venezuela]	MN075258 [Ea6]
	EF051166 [Portugal]	DQ228511 [Taiwan]	MN075259 [Ea14
	KJ995844 [Brazil]	KY434111 [Nigeria]	MN075260 [Na12
	JX893523 [Philippines]	HQ290362 [India]	MN075261 [Nb9]
	MH686052 [Vietnam]	HM466921 [Theileria]	MN075262 [Nc6]
	AB287435 [Japan]		MN075263 [Nc7]

Table 3 Co-infections of tick-borne haemopathogens in dogs

	Dogs (N=220)
Double infection	21 (9.55%)
Ehrlichia canis + *Anaplasma platys*	12 (5.45%)
Ehrlichia canis + *Babesia vogeli*	2 (0.91%)
Ehrlichia canis + *Babesia gibsoni*	3 (1.36%)
Anaplasma platys + *Babesia gibsoni*.	4 (1.82%)
Triple infection	2 (0.9%)
Ehrlichia canis + *Anaplasma platys* + *Babesia vogeli*.	1 (0.45%)
Anaplasma platys + *Babesia vogeli* + *Babesia gibsoni*	1 (0.45%)

Table 4 Occurence of TBH according to age, sex and region. Chi-square analysis showed the association () and *P*-values are shown.

Factor Category	No. of dogs	No of (%)infected with TBH			
		Anaplasma platys	*Ehrlichia canis*	*Babesia gibsoni*	*Babesia v*
Sex:					
Male	70	14 (20%)	12 (17.14%)	4 (5.71%)	4 (5.71%)
Female	150	12 (8%)	31 (20.67%)	12 (8%)	12 (8%)
		P = 0.0119	*P* = 0.4977	*P* = 0.4572	*P* = 0.457
		= 6.33	= 0.46	= 0.55	= 0.55
Age:					
Young	60	10 (16.67%)	8 (13.33%)	2 (3.33%)	11 (18.33
Adult	160	16(10%)	35 (21.88%)	14 (8.75%)	5 (3.13%
		P = 0.1931	*P* = 0.1370	*P* = 0.1298	*P* = 0.000
		= 1.69	= 2.21	= 2.30	= 14.18
Region:					
North	113	20(17.7%)	33(29.2%)	4(3.54%)	9(7.96%)
Central	71	4(5.63%)	8(11.27%)	2(2.82%)	7(9.86%)
East	15	2(13.33%)	2(13.33%)	8(53.33%)	-
South	21	-	-	2(9.52%)	-
		P=0.0267	*P* = 0.0016	*P* = 0.0002	*P* = 0.307
		=9.20	= 15.30	=51.80	= 3.61

Figures

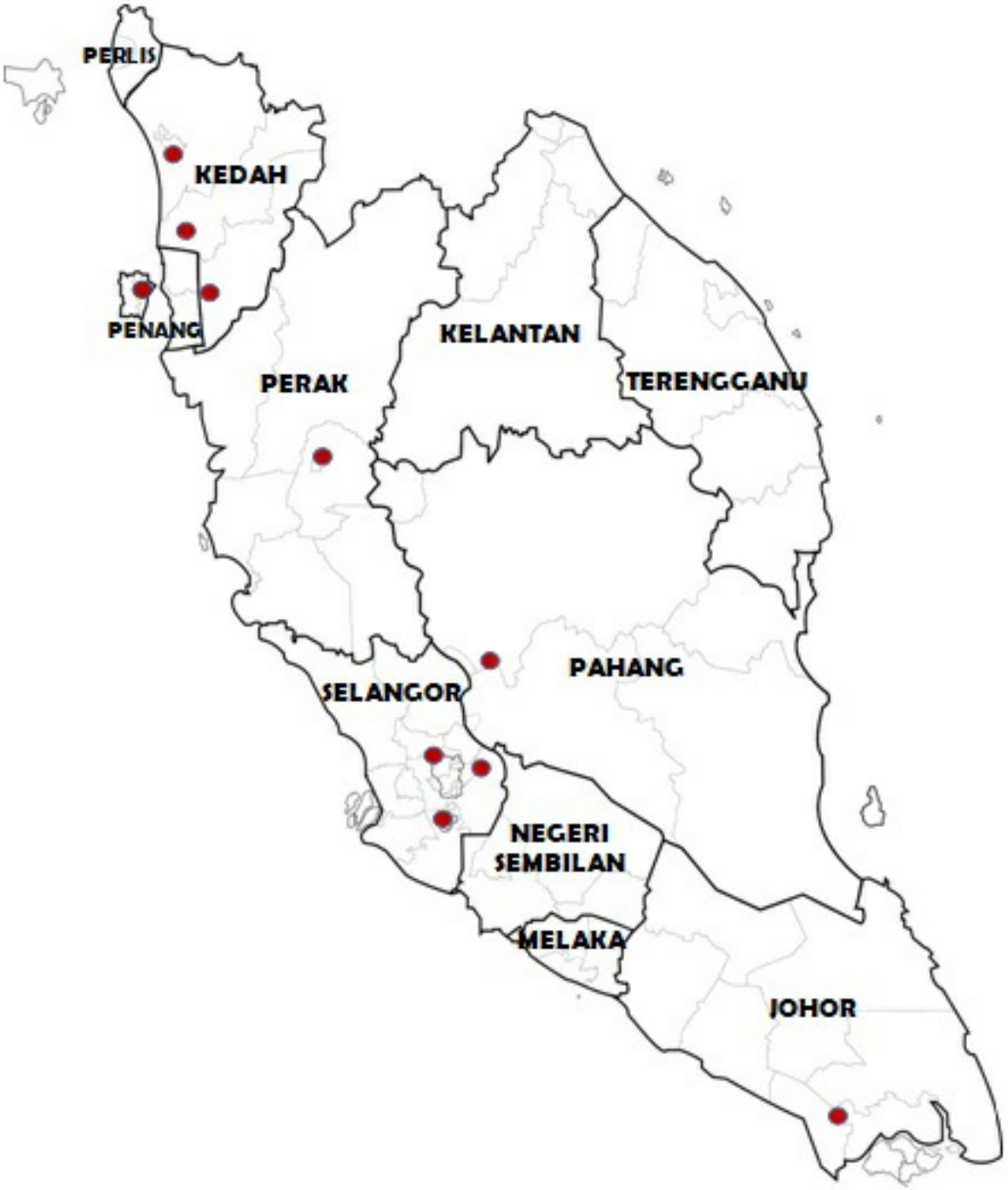

Figure 1

Map of sampling locations in Peninsular Malaysia. Sampling sites are marked red.

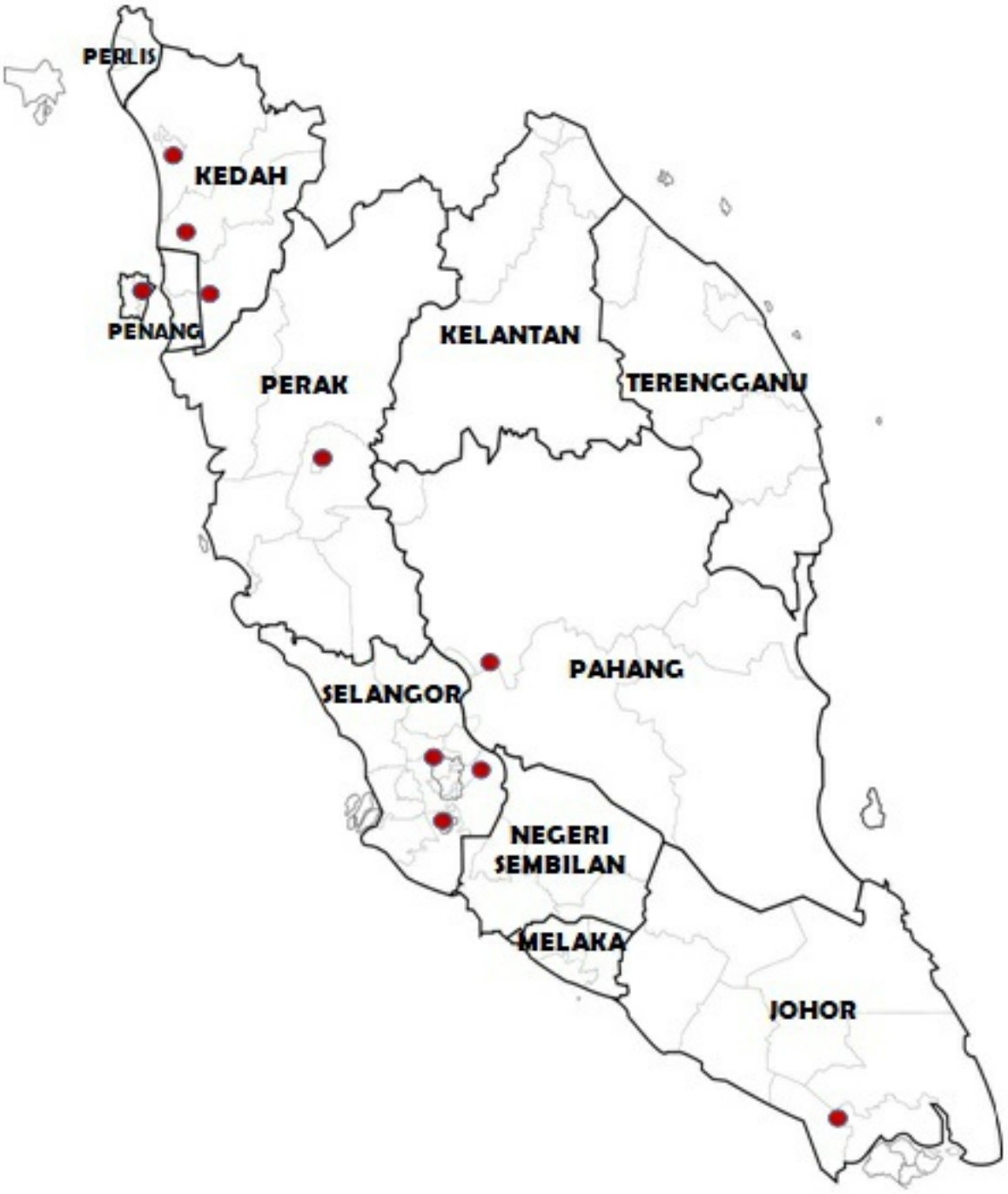

Figure 1

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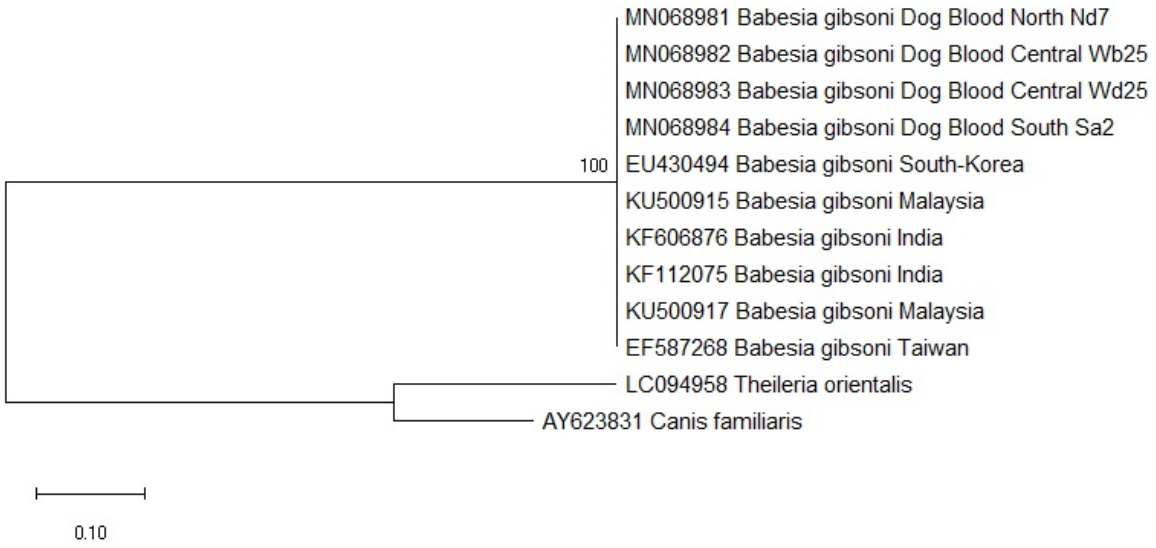
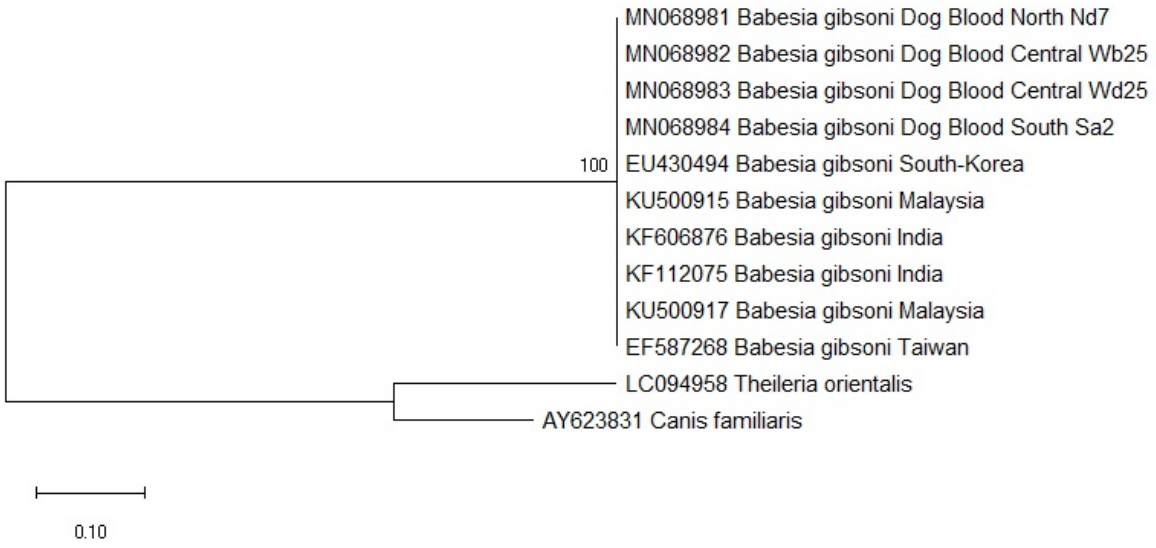


Figure 2

Phylogenetic tree of B. gibsoni constructed using 18S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.



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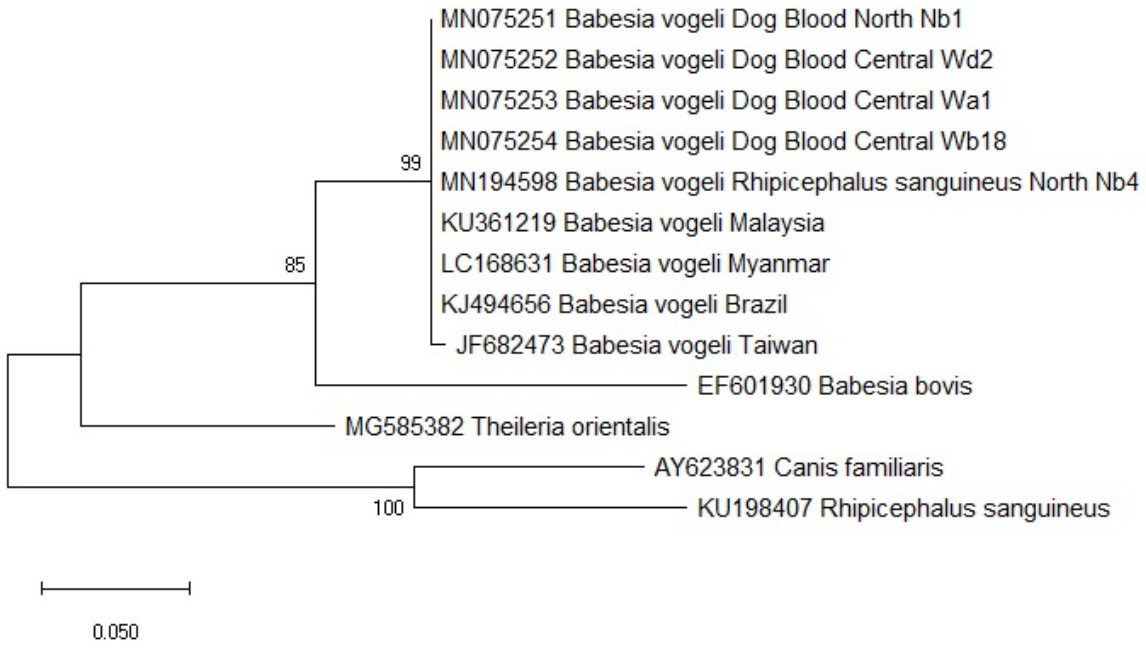

Figure 3

Phylogenetic tree of B. vogeli constructed using 18S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.

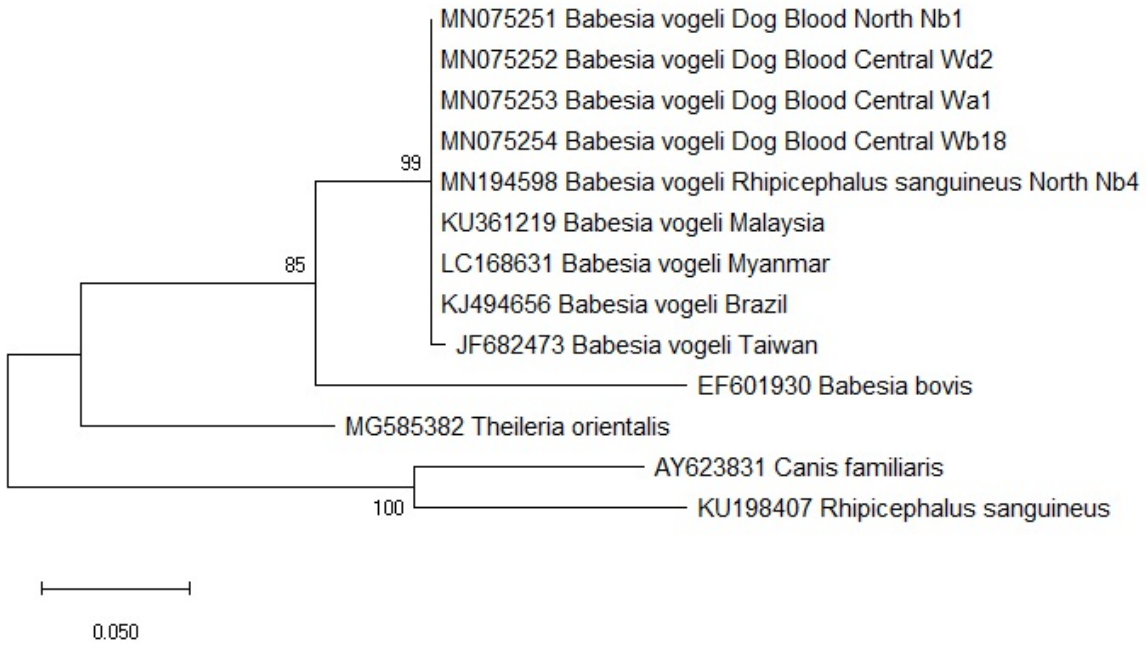

Figure 3

Phylogenetic tree of B. vogeli constructed using 18S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.

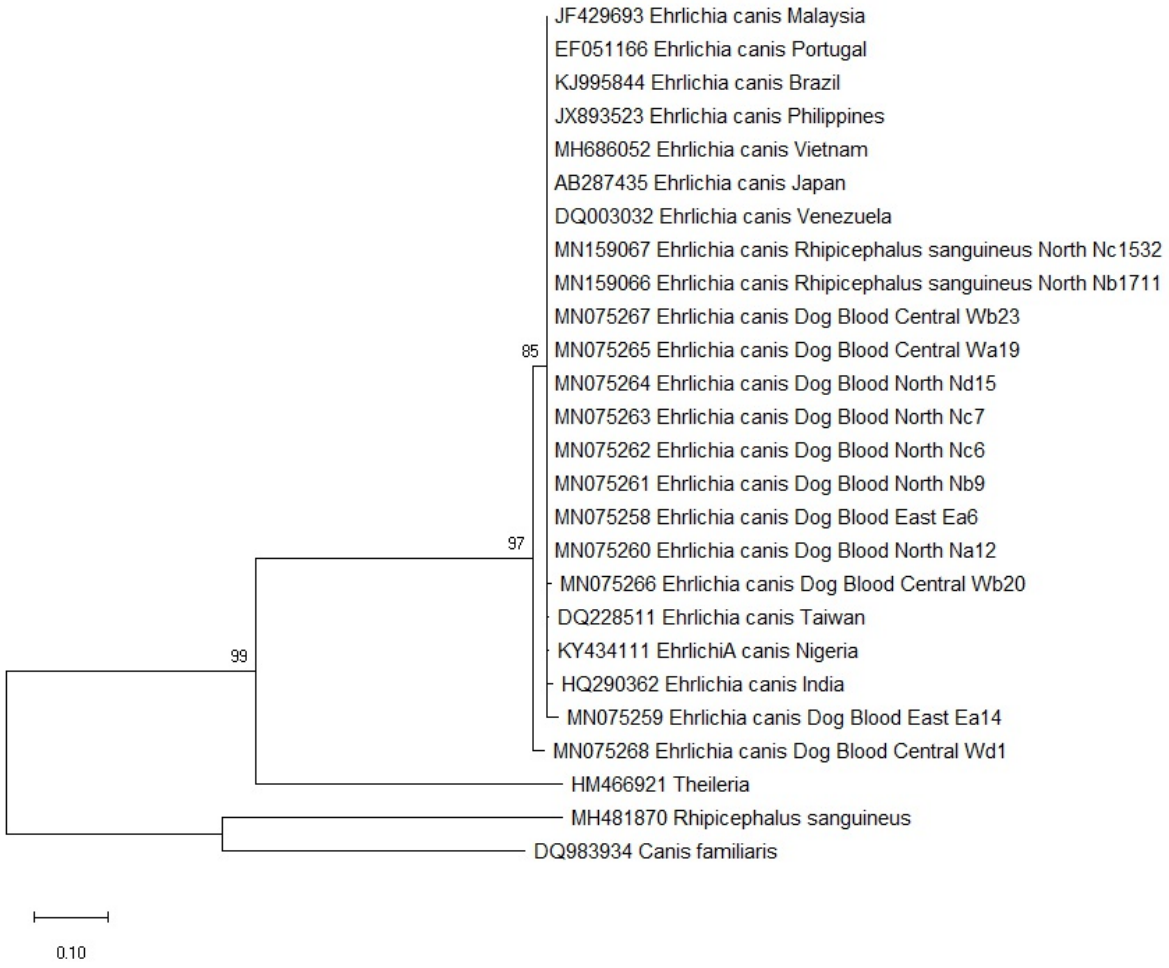

Figure 4

Phylogenetic tree of E. canis constructed using 16S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.

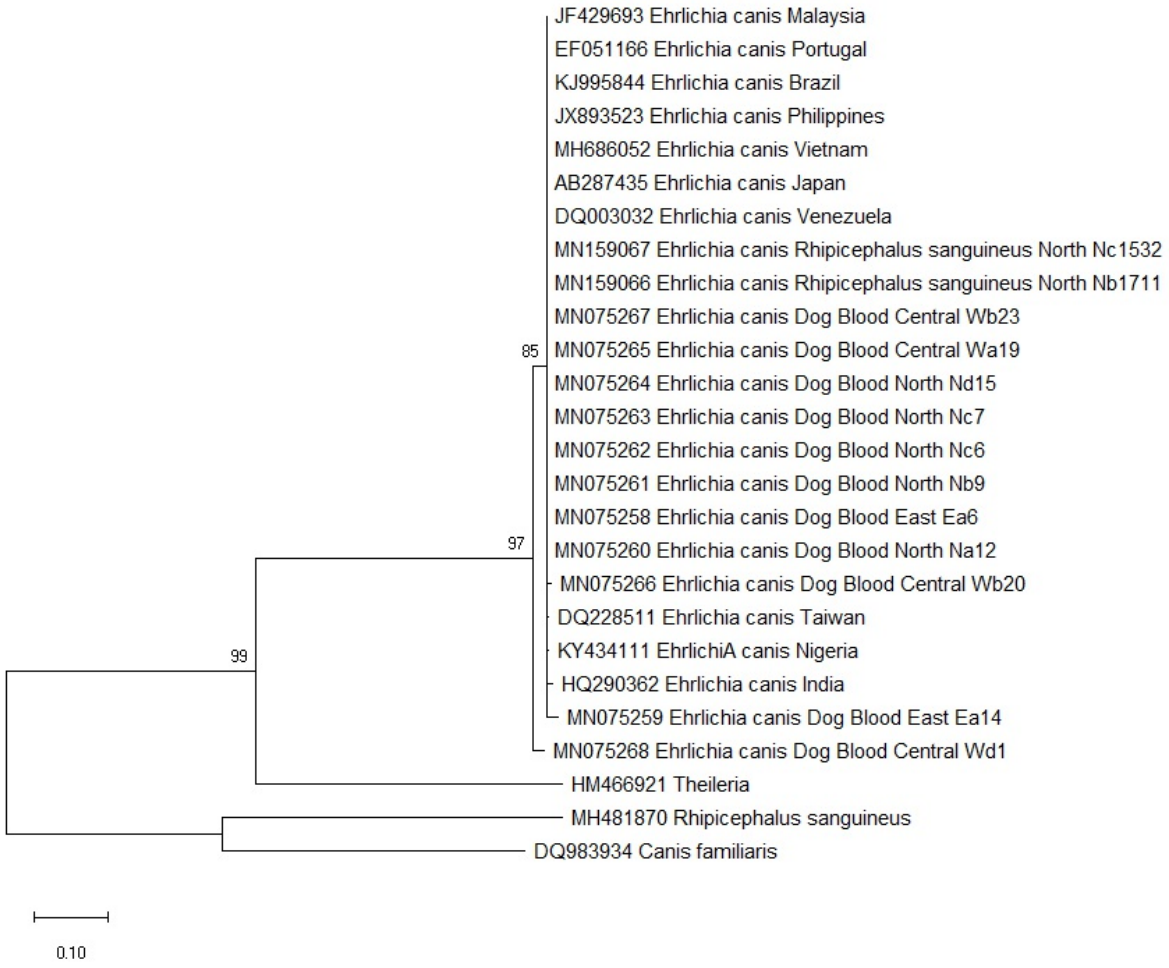

Figure 4

Phylogenetic tree of E. canis constructed using 16S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.

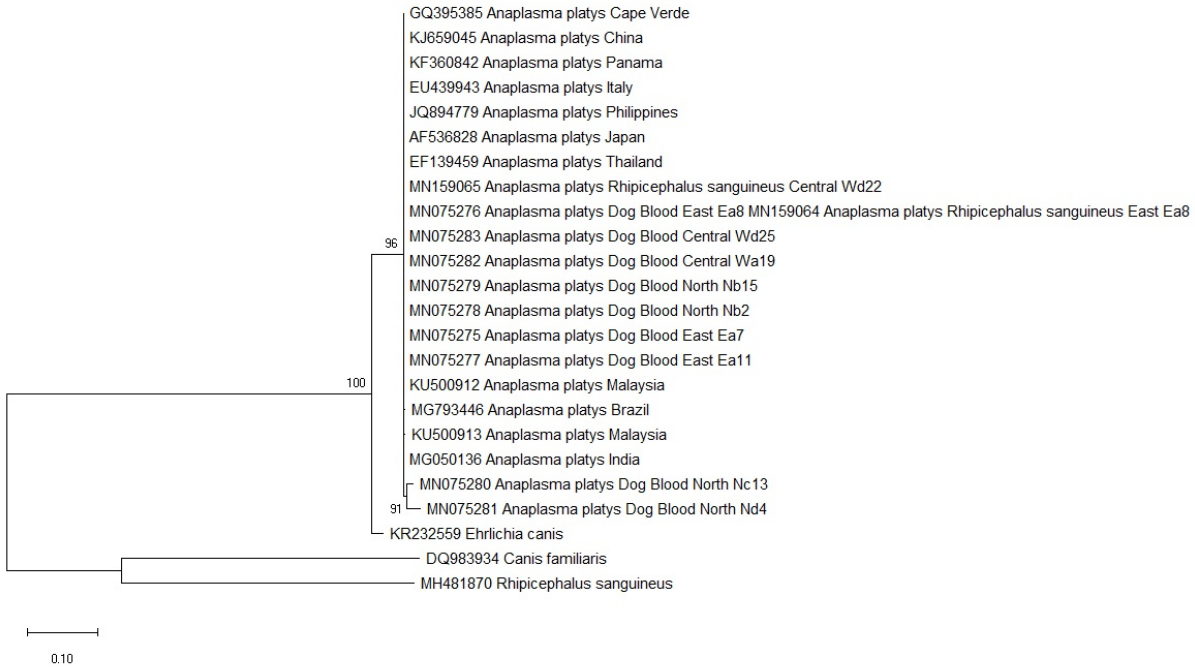

Figure 5

Phylogenetic tree of A. platys constructed using 16S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.

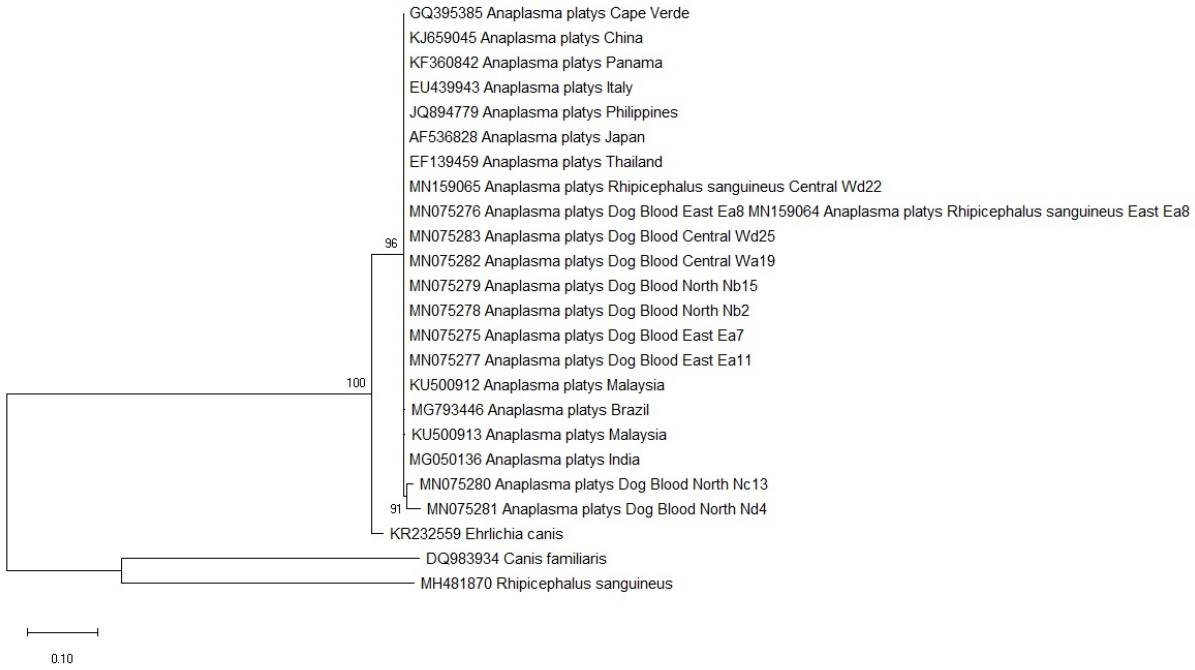

Figure 5

Phylogenetic tree of A. platys constructed using 16S rRNA gene and inferred using the Maximum Likelihood algorithm. Numbers above branches represent bootstrap percentages of 1000 replicates. The scale bar represents the number of nucleotide substitutions per site.