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RESEARCH ARTICLE

Molecular detection, risk factors, and phylogenetic analysis of tick-borne pathogens in dogs from northern Vietnam

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ABSTRACT

In tropical regions, numerous tick-borne pathogens (TBPs) play a crucial role as causative agents of infectious diseases in humans and animals. Recently, the population of companion and pet dogs has significantly increased in Vietnam; however, information on the occurrence of TBPs is still limited. The objectives of this investigation were to determine the occurrence rate, risk factors, and phylogenetic characteristics of TBPs in dogs from northern Vietnam. Of 341 blood samples tested by PCR, the total infection of TBPs was 73.9% (252/341). *Babesia vogeli* (*18SrRNA* gene – 30.5%) was detected most frequently in studied dogs followed by *Rickettsia* spp. (*OmpA* gene – 27%), *Anaplasma platys* (groEL gene – 22%), *Bartonella* spp. (*16SrRNA* – 18.8%), *Mycoplasma haemocanis* (*16SrRNA* – 9.4%) and *Hepatozoon canis* (*18SrRNA* gene – 1.2%), respectively. All samples were negative for *Ehrlichia canis* and *Anaplasma phagocytophylum.* Co-infection was detected in 31.4% of the samples (107/341) of which, *A. platys*/*Bartonella* spp. (34/94,10%), *Rickettsia* spp./*B. vogeli* (19/94, 5.6%), and *M. haemocanis/B. vogeli* (19/94, 5.6%) were recorded as the three most frequent two species of co-infection types. Statistical analysis revealed a significant correlation between TBP infection and several host variables regarding age, breed, and living area in the current study. The recent findings reported herein, for the first time in Vietnam, are essential for local veterinarians when considering the appropriate approaches for diagnosing these diseases. Furthermore, this data can be used to establish control measures for future surveillance and prevention strategies against canine TBPs in Vietnam.

Keywords: Dogs; tick-borne pathogens; risk factors; phylogeny; Vietnam.

INTRODUCTION

Vector-borne pathogens refer to numerous pathogens, including bacteria, protozoa, and viruses, which are known to be spread by vectors such as ticks, fleas, mosquitoes, and lice, causing a variety of infectious illnesses in both people and animals (Otranto *et al*., 2009). Tick-borne pathogens (TBPs), including protozoan agents like*Babesia* spp., and *Hepatozoon* spp., as well as bacterial agents like *Anaplasma* spp., *Mycoplasma* spp., *Rickettsia* spp., *Bartonella* spp., have been increasing detected in dogs from tropical areas (Chomel, 2011; Kaewkong *et al*., 2014).

Piroplasmosis, also known as babesiosis, is one of the most imperative canine tick-borne diseases that have a worldwide distribution. *Babesia* species that infect dogs can be categorized into two groups including small form (1.0-2.5 µm) or large form (2.5-5.0 µm) (Petra *et al*., 2018). The large form of *Babesia* spp., primarily reported in Europe, Africa, and Southeast Asia, includes *B. canis*, *B. rossi*, and *B. vogeli* which were previously classified as *B. canis* (Chomel, 2011; Petra *et al*., 2018). The small *Babesia* spp. includes *B. gibsoni* (mainly detected in Japan and Korea), *B. conradae* (reported in the western USA), and *B. annae* (found in Croatia) (Chomel, 2011). Hepatozoon infection in dogs is widespread across Asia, Europe, Africa, and South merica (Ivanov & Tsachev, 2008). The manifestations of canine hepatozoonosis, caused by *Hepatozoon canis* and *Hepatozoon americanum,* can range from subclinical to severe and critical diseases (Baneth *et al*., 2003). Dogs usually contract *Hepatozoon* spp. through the ingestion of an infected tick containing mature oocysts, rather than through the bite of the vector (Baneth *et al*., 2001).

Among the tick-borne intracellular bacteria, *Anaplasma* and *Ehrlichia* species belonging to the order Rickettsiale and the family *Anaplasmataceae*, are commonly reported in dogs worldwide (Ogbu *et al*., 2018). Dogs might get infected with both *Anaplasma platys* and *Anaplasma phagocytophilum*. *Anaplasma platys* is primarily associated with cyclic thrombocytopenia in dogs, while the latter is known to cause granulocytic anaplasmosis in patients (Ogbu *et al*., 2018). Common species of *Ehrlichia* found in dogs include *E. canis, E. chaffeensis,* and *E. ewingii*, of which *E. canis* is primarily

responsible for causing monocytic ehrlichiosis in dogs (Little, 2010). Other emerging bacteria in dogs include *Rickettsia, Mycoplasma,* and *Bartonella.* The causative agents of rickettsial diseases (order Rickettsiales, family *Rickettsiaceae*) are categorized into three primary groups: the spotted fever group (SFG), the scrub typhus group (STG), and the typhus group (TG) based on their intracellular localization and growth conditions (Abdad *et al*., 2018). Canine can serve as reservoirs for several *Rickettsia* species, such as *R. rickettsii* , *R. Conorii,* and *R. felis*, all of which belong to the spotted fever group and are known to be zoonotic (Chomel, 2011; Ng-Nguyen *et al*., 2020). Hemotropic mycoplasmas are epi-erythrocytic bacteria without cell walls that can be found in the blood of mammals, including dogs. The causative agents of Mycoplasmosis in dogs are *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum. These pathogens can induce clinical signs ranging from mild to severe hemolytic syndrome, especially in immunocompromised dogs (Valle *et al*., 2014). In recent years, *Bartonella* infection in cats and dogs has been commonly reported in studies around the world, and it is considered an emerging infectious threat to humans (Chomel, 2011). Dogs are assumed to be reservoirs for several *Bartonella* species known as the causative agents of endocarditis in humans such as *B. henselae* and *B. vinsonii berkhoffii* (Ávarez-Fernández *et al*., 2018).

In Southeast Asian countries, including Vietnam, the tropical climate represented by the high levels of rainfall and warm temperatures, along with the presence of stray animals, creates a favorable environment for the development of various vectors, including ticks, leading to the increased spread of TBPs (Irwin & Jefferies, 2004). Improved socio-economic status and living standards in Vietnam have resulted in a growing number of pets. While TBPs in dogs have been sporadically reported in Hanoi and TP. Ho Chi Minh with a rate of 25% approximately (Colella *et al*., 2020), there is still a lack of detailed information on canine tick-borne diseases and their potential threats to public health in this region. In this study, we investigated the occurrence, associated risk factors, and phylogeny of common TBPs in dogs from different areas in northern Vietnam.

MATERIALS AND METHODS

Sampling areas and data on sample collection

Samples were randomly collected between June 2021 and October 2022 at veterinary clinics/hospitals or households located in Ha Noi (n = 304), Hung Yen (n = 27), and Thanh Hoa (n = 10) in the north of Vietnam (Figure 1). The involved dogs were humanely restrained, and 1-2 mL of blood samples were taken and transferred into an anticoagulant EDTA-contained tube by a qualified veterinary technician. Subsequently, the samples were stored in a freezer at -20°C at Vietnam Academy of Agriculture for further laboratory investigations.

Data from the investigated dogs were also collected during the sampling and classified into different categories including age (≤ 1 year/ 1–5 years/ ≥ 5 years, considered as puppies/ juveniles/ adults), breed (domestic/ exotic), sex (male/ female), living area (urban/ suburban/ rural) and lifestyle (Indoor/ outdoor/ semi-outdoor), respectively.

Nucleic acid preparation and detection

Genomic DNA concentration was determined by employing a Nanodrop 2000 Spectrometer at 260/230 nm after extraction from blood samples using a Blood DNA Mini Kit (Quiagen, Hilden, Germany). Extracted DNA was kept at -20°C for further molecular screening.

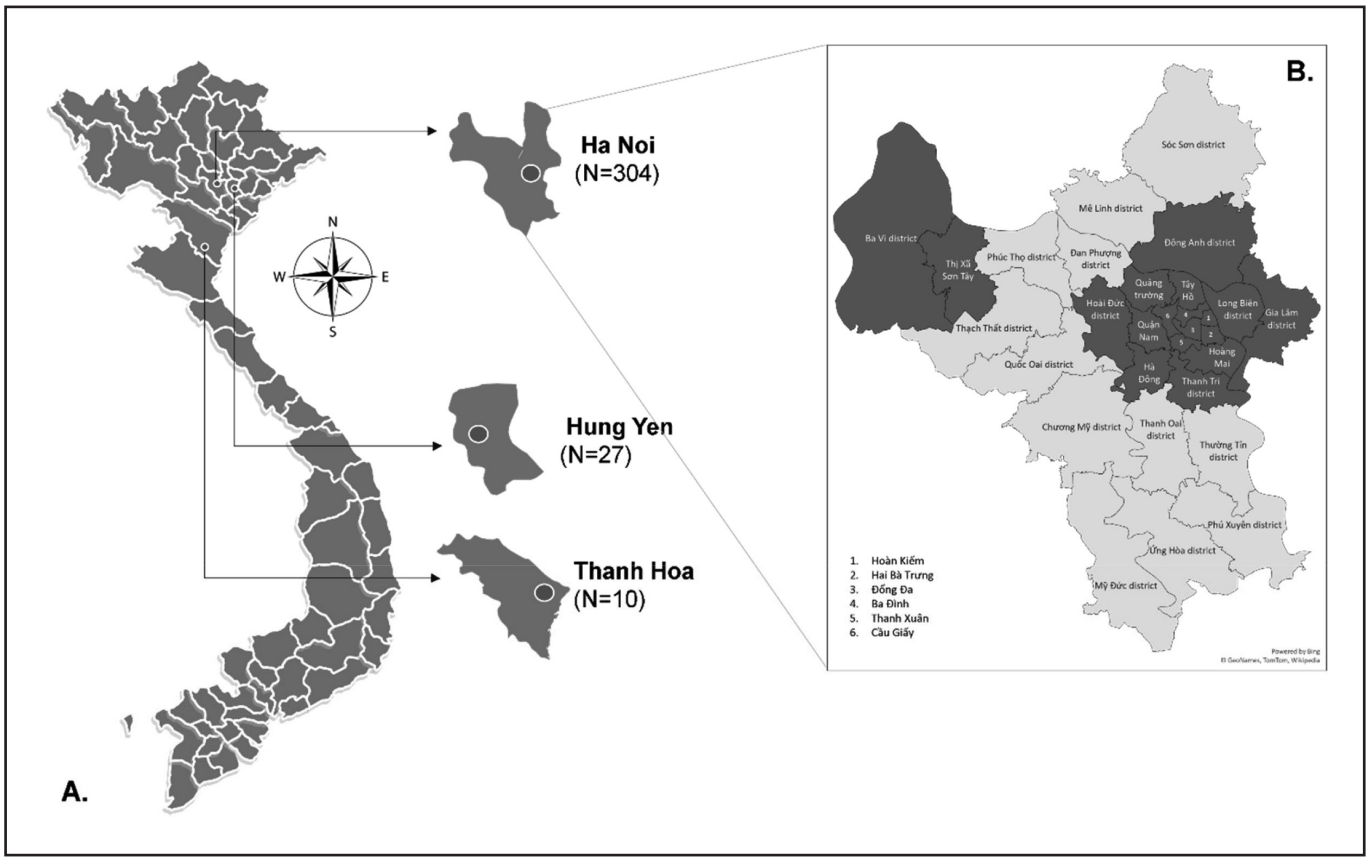

Figure 1. 1-A. Map of the study area in Nothern Vietnam (red circles represent sampling sites in each province); 1-B. The red highlighting 18 districts of Hanoi indicate the sampling areas.

Firstly, targeting sequences of the genus of *Ehrlichia*/*Anaplasma* spp. (16S rRNA gene) and *Babesia*/*Hepatozoon* spp. (18S rRNA gene) was amplified by PCR. Subsequently, positive samples for the aforementioned genera were further examined by using species-specific primers. Additionally, conventional PCR with universal primer sets was used for the detection of *Mycoplasma* spp. *Bartonella* spp. and *Rickettsia* spp. (Table 3). The reaction mixture of all DNA amplifications consisted of 1 µL of DNA template (20-50 ng/uL), 0.2 µM of each reverse and forward primer, 200 µM of mixed deoxynucleotide triphosphate, 2 µL of 5X buffer (final concentration 1X), 0.05 unit of Taq DNA polymerase and distilled deionized water up to 10 µL (Takara Bio, Kyoto, Japan). Subsequently, target sequences were amplified using a VeritiPro Thermal Cycler following previously reported conditions with some modifications (Thermo Fisher Scientific, Waltham, MA, USA) (Table 1). Finally, the amplified product was read under a UV transilluminator (ATTO, Tokyo, Japan) after electrophoresis in 1.5% agarose gel.

Cloning and sequencing

After amplification by PCR, DNA was extracted from the positive bands using a Gel Extraction Kit (QIAGEN, Germany). The target sequences were then ligated into a plasmid (pGEM-T Easy, Promega, Madison, USA). Later, the vector was inserted into competent cells (*Escherichia coli* DH5α calcium) for cloning. The inserted cells were then transferred on LB agar plates containing ampicillin (50ug/ml) and incubated for 15 – 18 hours at 37°C. To confirm if vectors were successfully inserted into the cells, the positive colonies were subjected to do colony PCR using a primer set of pUC/M13. Plasmids then were extracted using Plasmid QuickPure Kit (Macherey-Nagel, Nordrhein-Westfalen, Germany). The purified plasmids containing the target sequences were then sequenced using the ABI PRISM 3100 Genetic Analyzer with specific forward and reverse primers (Applied Biosystem, USA).

Phylogenetic analysis

Chromatograms of the sequences were viewed and processed using the BioEdit software version 7.5.2. The similarity between the complete length sequences found in this study and other published isolates were assessed and compared by using the Basic Local Alignment Search Tool (BLAST) of the U.S. National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The phylogeny of *OmpA* gene-based 212bp *Rickettsia*, the 16S rRNA gene-based 666bp *Mycoplasma haemocanis,* the groEL gene-based 724bp *Anaplasma platys* and the 18S rRNA gene-based 208 bp *Babesia vogeli* isolates found in this investigation were analyzed with other reported sequences worldwide using MEGA X software. The maximum likelihood method with 1000 no. of bootstrap replications was chosen to construct the phylogenetic trees of each pathogen. Each sequence presents its Genbank accession number and countries of origin in the phylogenetic tree.

Statistical analysis

The occurrence rate of different species and confidence intervals of a mean for a population proportion were determined using OpenEpi program (https://www.openepi.com/Proportion/Proportion.htm). The relationship between the detection rate of TBPs and the various host attributes considered in the current study including age, breed, sex, living area and lifestyle, was tested using a chi-square test for the independent analysis. If a significant association was found

Table 1. Sets of primer used in this study

Target pathogen (Target gene)	Primer sequences (F/R) 5'-3'	Product size (bp)	Annealing temperature (°C)	Reference
Rickettsia spp. spotted fever group (*OmpA*)	GCTTTATTCACCACCTCAACTR(G/A)ATCACCACCGTAAGTAAAT	212	55	(Kidd *et al.*, 2008)
Rickettsia spp. spotted fever group (*gltA*)	GCAAGTATCGGTGAGGATGTAATGCTTCCTTAAAATTCAATAAATCAGGAT	401	52	(Hii *et al.*, 2011)
Mycoplasma spp. (*16S rRNA*)	ATACGGCCCATATTCCTACGTGCTCCACCACTTGTTCA	595-618	60	(Criado-Fornelio *et al.*, 2003)
Anaplasma/Ehrlichia (*16S rRNA*)	GGTACCTACAGAAGAAGTCCTAGCACTCATCGTTTACAGC	345	52	(Inokuma *et al.*, 2000)
Anaplasma platys (*groEL*)	AAGGCGAAAGAAGCAGTCTTACATAGTCTGAAGTGGAGGAC	724	54	(Inokuma *et al.*, 2002a)
Ehrlichia canis (*gltA*)	TTATCTGTTTATGTTATATAAGCCAGTACCTATGCATATCAATCC	1372	50	(Inokuma *et al.*, 2001)
Anaplasma phagocytophilum (*16S rRNA*)	GCTGAATGTGGGGATAATTTATATGGCTGCTTCCTTTCGGTTA	641	50	(Kawahara *et al.*, 2006)
Bartonella spp. (*16S-23S rRNA*)	(C/T)CTTCGTTTCTCTTTCTTCAAACCAACTGAGCTACAAGCC	260	55	(Jensen *et al.*, 2000)
Babesia/Hepatozoon (*18S rRNA*)	CCAGCAGCCGCGGTAATTCCTTTCGCAGTAGTTYGTCTTTAACAAATCT	350–400	57	(Tabar *et al.*, 2008)
Babesia canis (*18S rRNA*)	GCW(A/T)TTTAGCGATGGACCATTCAAGCCTGTATTGTTATTTCTTGTCACTACCTC	208	60	(Kordick *et al.*, 1999)
Hepatozoon canis (*18S rRNA*)	ATACATGAGCAAAATCTCAAC CTTATTATTCCATGCTGCA	666	57	(Inokuma *et al.*, 2002b)

Abbreviations: F: Forward, R: Reverse, ompA: outer membrane protein A, groEL: heat shock protein gene, gltA: citrate synthase gene.

between any host attribute and TBP infection, the odds ratio (OR) was calculated to assess the strength of the relationship between the two events. The significance level and the confidence level used in the study were set at $p \leq 0.05$ and 95%, respectively. Data analysis was conducted sing the Statulator program (https://statulator.com/stat/chisq.html).

RESULTS

Sample information and TBP detection

A total of 341 dogs were involved in the study, comprising of 132 (38.7%) males and 125 (36.7%) females. Regarding age groups, the studied population included 83 (24.3%) puppies, 118 (34.6%) juveniles, and 65 (19.1%) adults, respectively. Most of the dogs in the current study belonged to exotic breeds (41.1%), resided in suburban areas (49.9%) and had indoor lifestyles (49%) (Table 2).

Of the 341 samples screened, 252 (73.9%) showed positive for TBPs. *Babesia vogeli, Rickettsia* spp., *A. platys*, *Bartonella* spp., *M. haemocanis,* and *H. canis* were detected in 30.5% (n=104), 27% (n=92), 22% (n=75), 18.8% (n=64), 9.4% (n=32) and 1.2% (n=4) of the samples, respectively (Table 2). All samples tested negative for *E. canis* and *A. phagocytophylum*. Single-pathogen infections were found in 42.5% (145/252) of the positive samples, of which *Rickettsia* spp. and *B. vogeli* were the most frequently detected bacterial and protozoan pathogens in the current study. Co-infections were detected in 107 samples (31.4%). Among the co-infections, 94 (27.6%) involved two pathogens, and 13 samples (3.8%) involved three pathogens. Notably, concurrent infections of *A. platys*/*Bartonella* spp. (34/94,10%), *Rickettsia* spp./*B. vogeli* (19/94, 5.6%), and *M. haemocanis/B. vogeli* (19/94, 5.6%) were the three most frequent two-species of co-infection types (Table 2).

Risk factors associated with TBP detection

The relationship between PCR-positive TBPs with several host attributes was assessed in the present study. Samples lacking information were excluded from the analysis. Statistical analysis indicated that the detection rate of TBPs was higher in puppies (81.9%, 68/83) and juveniles (75.4%, 89/118) compared to older dogs (55.4%, 36/65, $\chi^2 = 13.77$, $df = 2$). Specifically, puppies and the juvenile group had 3.65 times ($p < 0.001$) and 2.47 times ($p = 0.005$) the odds of contracting TBP infections than the older dogs, respectively. The infection rate of TBPs was considerably higher in domestic breed dogs (82.3%, 79/96) than exotic breed dogs (69.3%, 97/140, $\chi^2 = 5.08$, $df = 1$). Domestic dogs were 2.06 times more likely to contract TBP infection than exotic dogs ($p = 0.024$). Moreover, a significant difference was found between different categories of living areas of study dogs and the TBP detection rate in this study. Particularly, dogs living in urban areas were less likely to contract TBP infection than those living in suburban areas ($\chi^2 = 8.04$, $df = 1$, OR = 0.48, $p = 0.005$) and rural areas ($\chi^2 = 6.75$, $df = 1$, OR = 0.28, $p = 0.009$) (Table 3). However, the correlation between TBP infection and factors related to lifestyle ($p = 0.1$) and sex ($p = 0.094$) was not statistically significant in the current study.

Table 2. The occurrence rate of tick-borne pathogens (N=341)

Pathogen detected	No. infected samples	Detection rate (%)	95% CI
CTBP total	**252**	**73.9**	**69.2–78.6**
Anaplasma platys	75	22.0	17.6–26.4
Bartonella spp.	64	18.8	14.6–22.9
Rickettsia spp.	92	27.0	22.3–31.7
Mycoplasma haemocanis	32	9.4	6.3–12.5
Babesia vogeli	104	30.5	25.6–35.4
Hepatozoon canis	4	1.2	0–2.3
1 CTBP species	**145**	**42.5**	**37.3–47.8**
A. platys	28	8.2	5.3–11.2
Bartonella spp.	8	2.3	0.7–4
Rickettsia spp.	58	17.0	13–21
M. haemocanis	7	2.1	0.5–3.6
B. vogeli	44	12.9	9.3–16.5
H. canis	0	0	0
2 CTBP species	**94**	**27.6**	**22.8–32.3**
A. platys + Bartonella spp.	34	10.0	6.8–13.2
A. platys + Rickettsia spp.	1	0.3	0–0.9
A. platys + B. vogeli	5	1.5	0.2–2.7
Bartonella spp. *+ Rickettsia* spp.	5	1.5	0.2–2.7
Bartonella spp. *+ M. haemocanis*	3	0.9	0–1.9
Bartonella spp. *+ B. vogeli*	6	1.8	0.4–3.2
Rickettsia spp. *+ M. haemocanis*	1	0.3	0–0.9
Rickettsia spp. *+ B. vogeli*	19	5.6	3.1–8
Rickettsia spp. *+ H. canis*	1	0.3	0–0.9
M. haemocanis + B. vogeli	19	5.6	3.1–8
3 CTBP species	**13**	**3.8**	**1.8–5.8**
A. platys + Bartonella spp. *+ Rickettsia* spp.	2	0.6	0–1.4
A. platys + Bartonella spp. *+ B. vogeli*	4	1.2	0–2.3
A. platys + Rickettsia spp. *+ B. vogeli*	1	0.3	0–0.9
Bartonella spp. *+ Rickettsia* spp. *+ B. vogeli*	1	0.3	0–0.9
Bartonella spp. *+ M. haemocanis + B. vogeli*	1	0.3	0–0.9
Rickettsia spp. *+ M. haemocanis + B. vogeli*	1	0.3	0–0.9
Rickettsia spp. *+ B. vogeli + H. canis*	3	0.9	0–1.9
Total co-infection	**107**	**31.4**	**26.5–36.3**

Table 3. The associations of host parameters and tick-borne pathogen occurrence

Variables	No. tested dogs	No. TBP positive dogs (%)	OR (CI 95%)	*p-value*	No. positive dogs (%)					
					Anaplasma platys	*Bartonella* spp.	*Rickettsia* spp.	*Mycoplasma haemocanis*	*Babesia vogeli*	*Hepatozon canis*
Age (year)										
≤1	83	68 (81.9)	3.65 (1.74 – 7.68)	0.001	23 (27.7)	19 (22.9)	22 (26.5)	5 (6)**	23 (27.7)**	1 (1.2)
1-5	118	89 (75.4)	2.47 (1.30 – 4.71)	0.005	21 (17.8)	21 (17.8)	23 (19.5)	21 (17.8)	46 (39)	1 (0.8)
≥5	65	36 (55.4)	Ref.		21 (32.3)	15 (23.1)	11 (16.9)	2 (3.1)	11 (16.9)	0
Unknown	75	59 (78.7)			10 (13.3)	9 (12)	36 (48)	4 (5.3)	24 (32)	2 (2.7)
Breed										
Domestic	96	79 (82.3)	2.06 (1.09 – 3.89)	0.024	8 (8.3)	11 (11.5)	22 (22.9)	23 (24)**	48 (50)**	1 (1.0)
Exotic	140	97 (69.3)	Ref.		47 (33.6)	35 (25)	32 (22.9)	4 (2.9)	26 (18.6)	0
Unknown	97	70 (72.2)			17 (17.5)	16 (16.5)	37 (38.1)	4 (4.1)	28 (28.9)	2 (2.1)
Sex										
Female	125	96 (76.8)	1.6 (0.92 – 2.78)	0.094	28 (22.4)	24 (19.2)	27 (21.6)	15 (12)	36 (28.8)	0
Male	132	89 (67.4)	Ref.		28 (21.2)	24 (18.2)	26 (19.7)	15 (11.4)	43 (32.6)	2 (1.5)
Unknown	84	67 (79.8)			19 (22.6)	16 (19)	39 (46.4)	2 (2.4)	25 (29.8)	2 (2.4)
Living area										
Urban area	134	86 (64.2)	Ref.		35 (26.1)	25 (18.7)	36 (26.9)**	2 (1.5)	23 (17.2)**	1 (0.7)
Suburban area	170	134 (78.8)	0.48 (0.29 – 0.80)	0.005	36 (21.2)	39 (22.9)	35 (20.6)	30 (17.6)	64 (37.6)	2 (1.2)
Rural area	37	32 (86.5)	0.28 (0.10 – 0.77)	0.009	4 (10.8)	0	21 (56.8)	0	17 (45.9)	1 (2.7)
Lifestyle										
Indoor	167	117 (70.1)	0.55 (0.27 – 1.12)	0.1	53 (31.7)	28 (16.8)	46 (27.5)	2 (1.2)**	34 (20.4)**	2 (1.2)
Outdoor	63	51 (81)	Ref.		4 (6.3)	13 (20.6)	3 (4.8)	26 (41.3)	34 (54)	0
Unknown	111	84 (75.7)			18 (16.2)	23 (20.7)	43 (38.7)	4 (3.6)	36 (32.4)	2 (1.8)

Abbreviations: OR = Odds ratio; CI = confidence intervals; Ref. = reference used; No. = number; ** = $p < 0.001$.

Based on statistical analysis, associations between host parameters and different specific pathogens, including *Rickettsia* spp., *M. haemocanis,* and *B. vogeli* were identified in the present study. Significantly associations with *B. vogeli* positivity were found for age (χ^2 = 9.51, p = 0.002), breed (χ^2 = 26.13, p < 0.001), living area (χ^2 = 15.39, p < 0.001), and lifestyle (χ^2 = 24.81, p < 0.001). In addition, statistical analysis revealed that age group (χ^2 = 12.23, p < 0.001), breed (χ^2 = 25, p < 0.001), and lifestyle (χ^2 = 68.1, p < 0.001) were significantly correlated with the detection of *Mycoplasma haemocanis.* Furthermore, there were significantly increased odds of *Rickettsia* infection in dogs residing in rural areas compared to those in suburban areas (χ^2 = 20, OR = 0.2, p < 0.001) and urban areas (χ^2 = 11.66, OR = 0.28, p <0.001) (Table 3).

Sequencing identities and phylogenetic analysis

Positive amplicons of each TBP detected were subjected to sequencing and subsequently submitted to the Genbank database to compare with reported isolates for sequencing identity analysis. The obtained sequences for *Mycoplasma, Anaplasma, Babesia* and *Hepatozoon* species shared 100% identity with each other and shared 100% identity with previously reported isolates of *M. haemocanis* (GenBank: KX641903, OP101175, ON980770), *A. platys* (GenBank: KU765205, KR011926, LC556384), *B. vogeli* (GenBank: MT386936, MN823219, MK881091) and *H. canis* (GenBank: KU765210, KT267953, OP699205). For *Rickettsia*, all sequences shared approximately 100% identity with the reported sequences of *Rickettsia raoultii* (GenBank: KU961537). In the case of *Bartonella,* the obtained sequences showed 98.07-98.48% identity with the published isolates of *Bartonella bovis* (GenBank: AY116638, EF418060, EF418061). Representative sequences from this investigation, which exhibited strong and clear signals after sequencing each pathogen reported in this investigation, were then submitted to Genbank. The issued accession numbers were OQ944894-OQ944895 (*M. haemocanis*), OQ932918-OQ932919 (*A. platys*), OQ836196-OQ836198 (*B. vogeli*), OQ891170-OQ891171 (*H. canis*), OQ992942-OQ992944 (*Rickettsia* spp.), and OQ909074-OQ909076 (*Bartonella* spp.).

Representative sequences of TBPs based on the *18S rRNA* gene (*Babesia*), the *OmpA* gene (*Rickettsia*), the *16S rRNA* gene (*Mycoplasma*), and the *groEL* gene (*Anaplasma*) collected from this study and other publications reported from different geographic regions, were used to establish phylogenetic trees. In the analysis, *Plasmodium falciparum, Anaplasma marginale, Mycoplasma pneumonia,* and *Rickettsia typhi* were used as outgroups in the analysis. In the phylogenetic tree, the isolates of each pathogen from the current study were clustered within a single clade with high bootstrap values, indicating a single genotype of each pathogen circulating in the studied areas. The selected amplified sequence regions in the present study showed high variation, allowing for clear differentiation of the studied species from other species within the same family (Figure 2-5).

DISCUSSION

These findings update the list of tick-borne pathogens affecting dogs and suggest that dogs might serve as carriers of TBPs in these studied areas in Vietnam. Recently, TBPs detected in this investigation have commonly emerged in animals and humans, becoming a significant concern for public health worldwide (Chomel, 2011). Dogs often have close contact with humans due to sharing the same living environment. As a consequence, humans face a high risk of getting infections from zoonotic pathogens. Furthermore, a significant number of dogs annually illegally transported into Vietnam for meat trade without receiving any medical care, thereby establishing an additional source for introducing and spreading exotic infectious diseases (Ngo, 2011; Shaharul Nizim *et al*., 2019). A high detection rate of TBPs in dogs (73.9%) in this study highlights the significant risk of TBP infection to both animal and human health in the areas. In this regard, the involvement of the government in implementing the control measures and strategies, such as enacting a ban on dog meat trading and consumption in Vietnam, would be crucial and necessary to protect public health from exposure to these pathogens.

Our results indicate that selected TBPs are prevalent in the studied area, with an occurrence rate of 73.9%. The bacterial pathogens *E. canis, A.platys,* and the protozoan pathogen *H. canis* have been previously found in northern Vietnam (Nguyen *et al*., 2019; Colella *et al*., 2020). In contrast, *B. vogeli, M. haemocanis*, *Rickettsia* spp., and *Bartonella* spp. have been reported here for the first time in dogs. These TBPs are also reported commonly in other Southeast Asian countries, with varying detection rates such as 76.4%, 71.3%, 35%, and 30.2% in Thailand (Do *et al*., 2021), Cambodia (Inpankaew *et al*., 2016), Malaysia (Sipin *et al*., 2020) and Philippines (Galay *et al*., 2018), respectively. In this study, *Babesia vogeli* was the most common pathogen (104/252, 30.5%) contracted by canines. *Babesia vogeli* and *Hepatozoon canis* have shown different occurrence rates in dogs across Southeast Asian countries (Galay *et al*., 2018; Sipin *et al*., 2020). The differences in TBP occurrence rates between the studies in this area could be attributed to variations in sample size, the sampling season, or the target gene selected (Do *et al*., 2021). *Babesia vogeli* and *Hepatozoon canis* are known to be less virulent, causing mild clinical illness. However, they may co-infect with other blood-borne bacterial infections, leading to the development of severe and potentially fatal hemolytic anemia, especially in puppies (Salem & Farag, 2014).

Surprisingly, the most prevalent bacterium detected in dogs in this study was *Rickettsia* spp. (27%). The positive amplicons amplified from both genes (*OmpA* and *gltA* gene) classified the *Rickettsia* spp. as *R. raoultii*, sharing a high identity (99.5 – 100%) with the previously reported sequences (Figure 3). *Rickettsia raoultii* belongs to the spotted fever group, which has been detected in animals and multiple tick species worldwide (Chang *et al*., 2022). However, knowledge about this pathogen remains limited. Investigations of *R. raoultii* in dogs have been scarce in Asia, including this study in Vietnam (Galay *et al*., 2018; Shao *et al*., 2021). This result should raise concern about the risk of infection for the residents in the studied areas, as all *Rickettsia* species infecting dogs are known to be able to transmit to humans (Chomel, 2011; Tariq *et al*., 2021). The clinical manifestations of *R. raoultii*-infected patients range from mild to moderate or severe illness, including fever, malaise, myalgia, lymphadenopathy, nausea, rash, and eschar (Li *et al*., 2018). In Vietnam, among different groups of rickettsial diseases, the epidemiological information regarding spotted fever group rickettsial infections in humans is the least recognized (Hamaguchi *et al*., 2015). In the latest study on rickettsial infection in humans by Trung *et al*. (2022), scrub typhus was the most commonly reported rickettsial infection in humans, followed by murine typhus and spotted fever. Nevertheless, the authors could not identify the specific rickettsial species within the spotted fever group in this research due to the lack of clinical sample material (Trung *et al*., 2022).

Despite the increasing number of studies on canine haemoplasma in Southeast Asia, particularly in Cambodia (Inpankaew *et al*., 2016), Thailand (Liu *et al*., 2016; Do *et al*., 2021), and China (Shi *et al*., 2022), veterinarians and researchers in Vietnam have paid relatively less attention to the diagnosis and investigation of this pathogen. Phylogenetic analysis of a partial 16S rRNA gene shows that our sequences are in the same clade as *Mycoplasma haemocanis*, which is distinct from other clades of *Candidatus* Mycoplasma haematoparvum and *Candidatus* Mycoplasma haemominutum (other common species of *Mycoplasma* spp. in dogs) (Figure 4). The findings of this study emphasize to local veterinarians the importance of diagnosing this pathogen, as it is considered an opportunistic pathogen that poses new health threats to dogs. Bartonellosis is also recognized as an emerging infectious threat to humans, with varying impacts depending

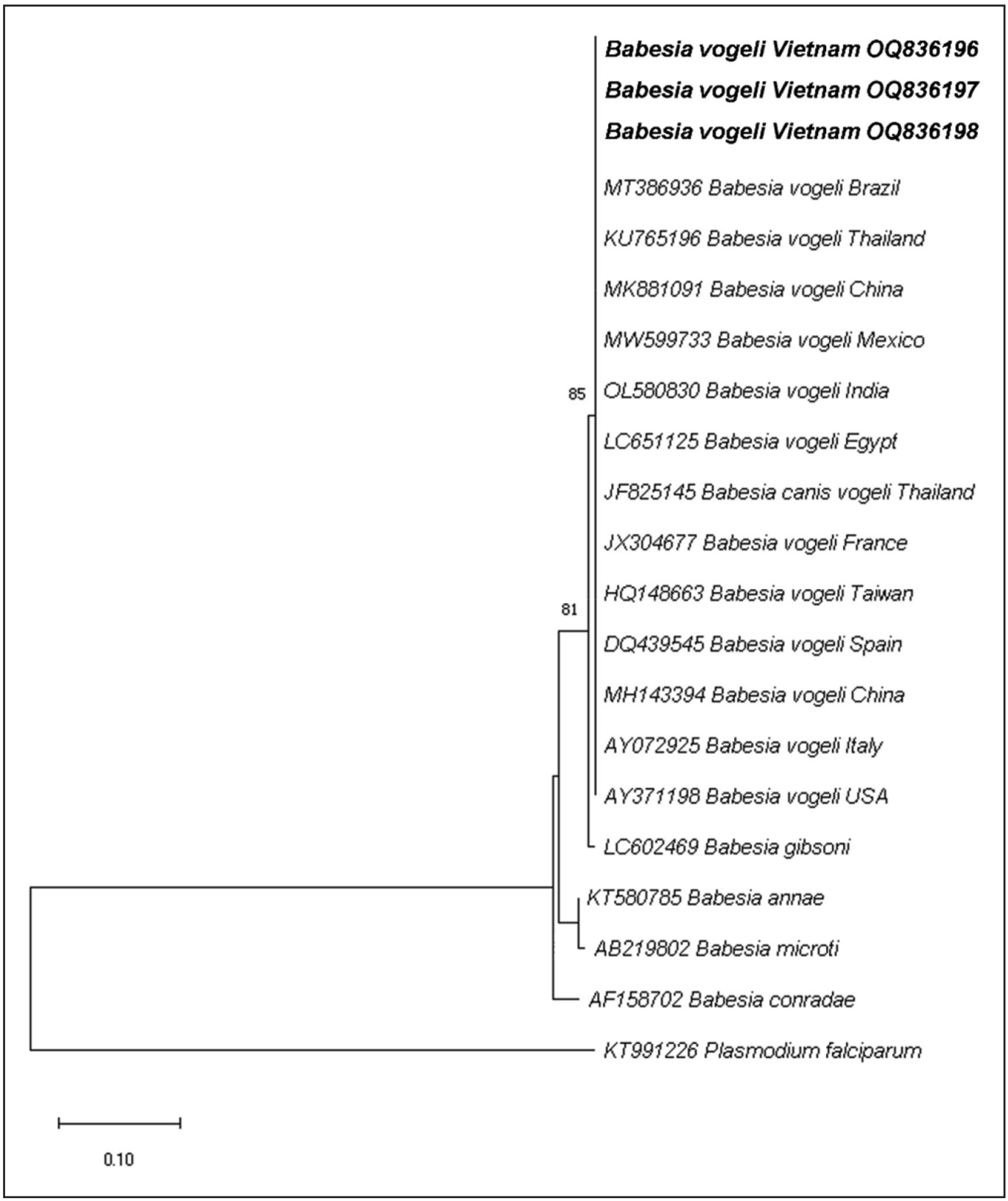

Figure 2. Phylogenetic analysis of *Babesia vogeli* based on the nucleotide sequences of a 208 bp fragment of 18S rRNA gene using Kimura 3 parameter model. Isolates obtained from this study are presented in bold text.

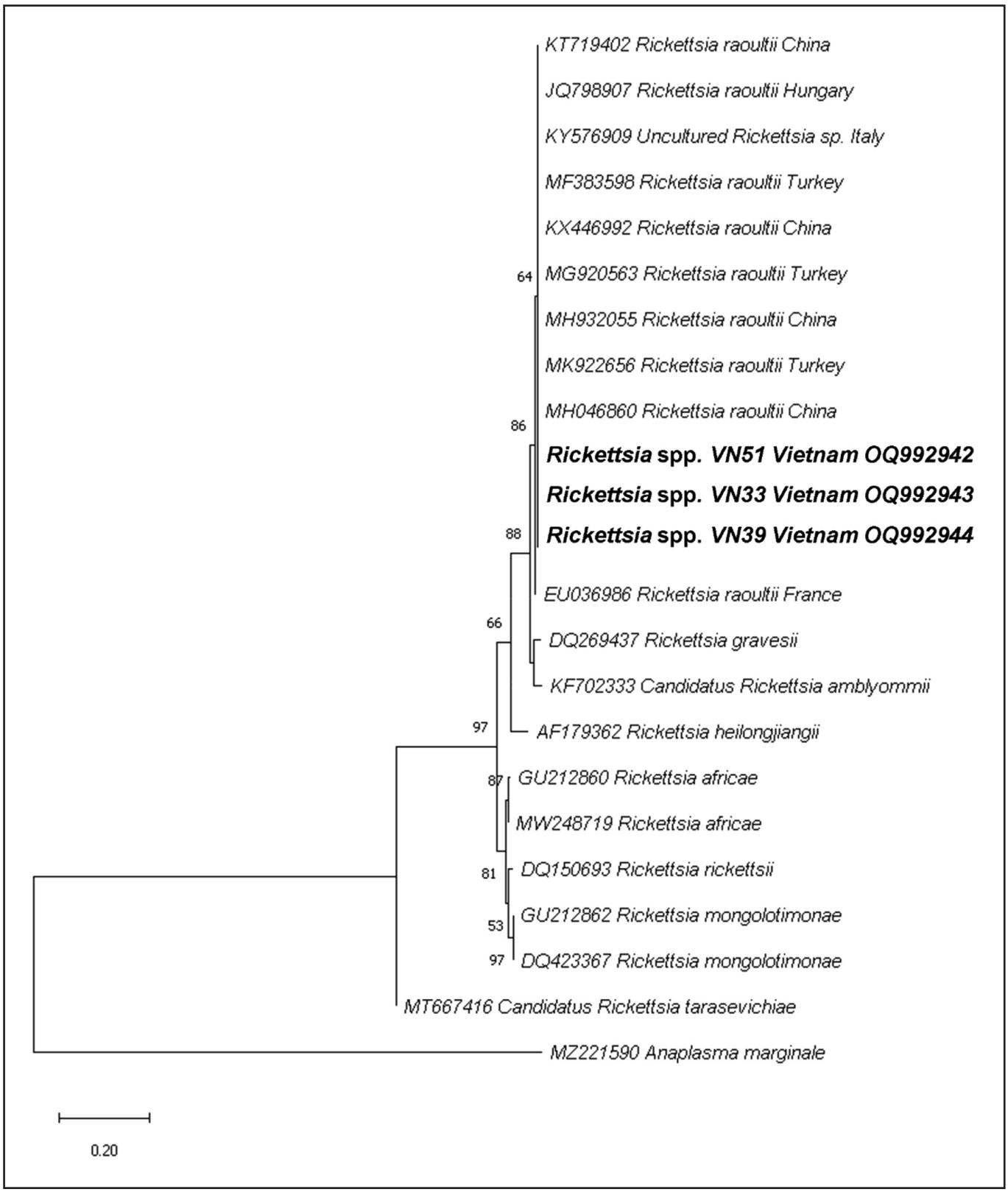

Figure 3. Phylogenetic analysis of *Rickettsia* spp. based on the nucleotide sequences of a 212 bp fragment of OmpA gene using Kimura 3 parameter model (Gamma distributed G). Isolates obtained from this study are presented in bold text.

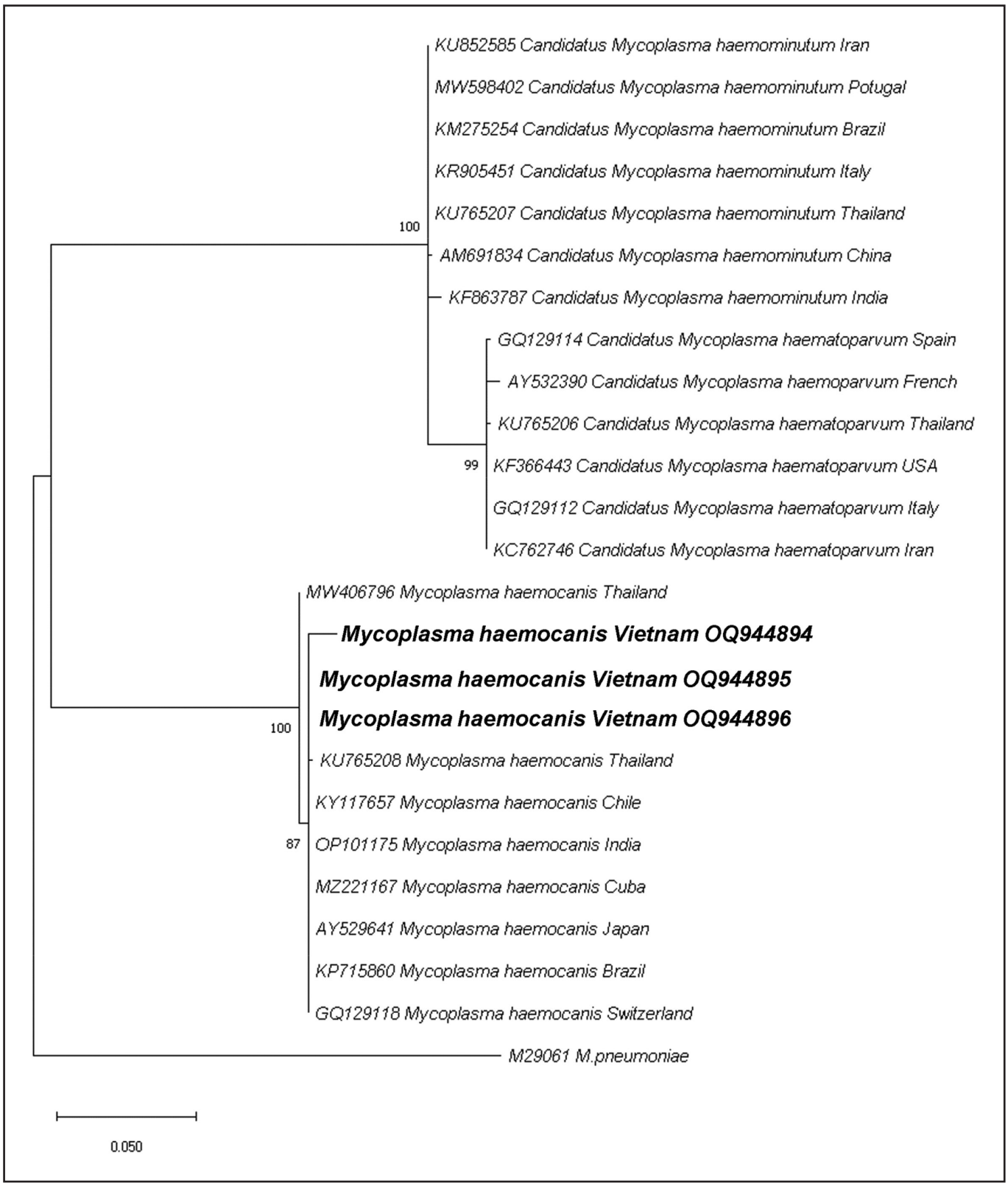

Figure 4. Phylogenetic analysis of *Mycoplasma haemocanis* based on the nucleotide sequences of a 595 bp fragment of 16S rRNA gene using Kimura 3 parameter model. Isolates obtained from this study are presented in bold text.

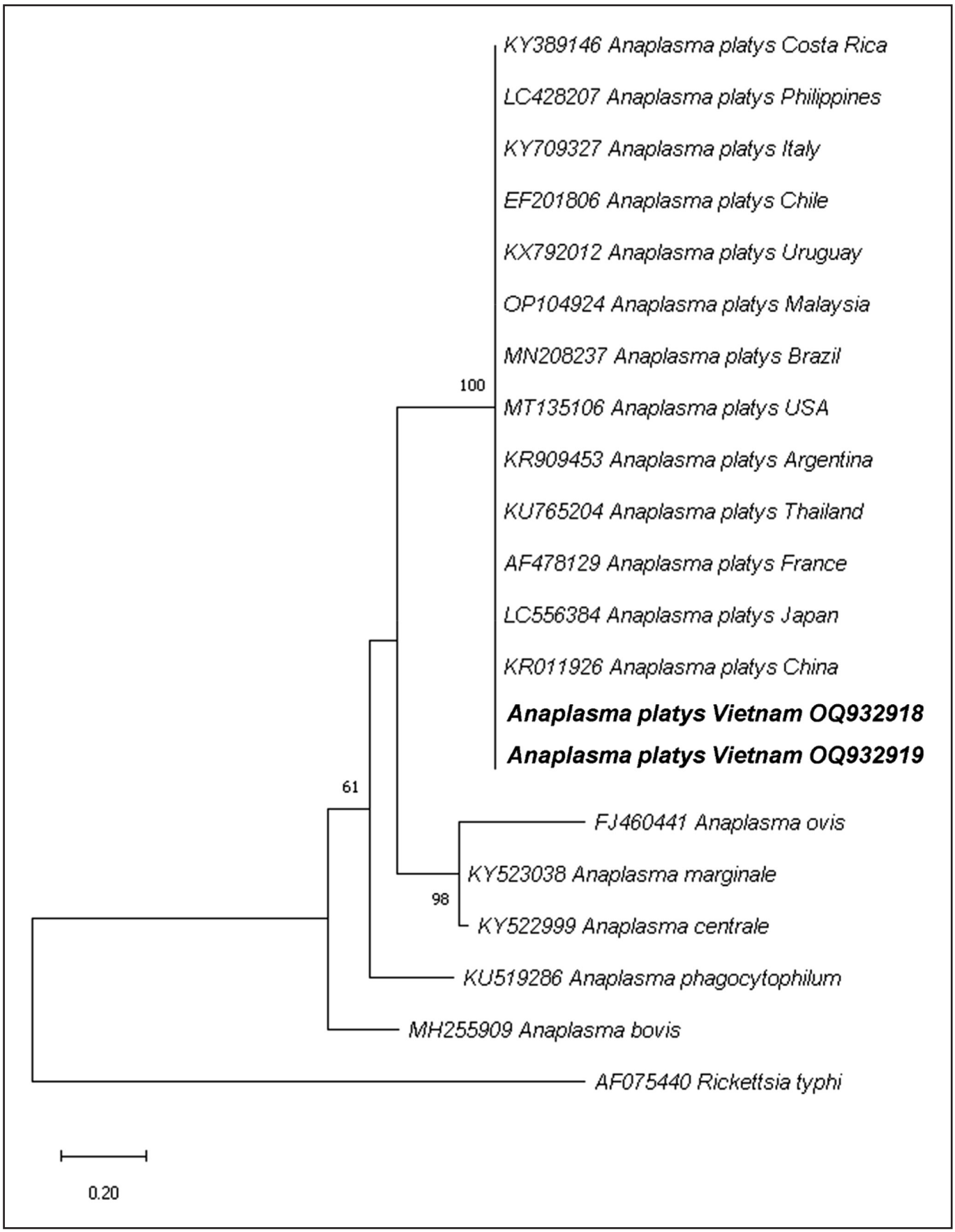

Figure 5. Phylogenetic analysis of *Anaplasma platys* based on the nucleotide sequences of a 724 bp fragment of groEL gene using Kimura 3 parameter model (Gamma distributed with invariant sites G+I). Isolates obtained from this study are presented in bold text.

on *Bartonella* species (Deng *et al*., 2018). This pathogen can be transmitted through blood-sucking arthropods (sand flies, fleas, lice, and ticks) or through animal scratches and bites (Chomel, 2011). Dogs are highly likely to serve as accidental hosts and carriers for *Bartonella* spp. Interestingly, the positive amplicons detected in the current study classified *Bartonella* spp. as *Bartonella bovis,* whose primary host is domestic cattle (Ávarez-Fernández *et al*., 2018). In this investigation, a large number of dogs that tested positive for *Bartonella* spp. resided on farms where they shared their habitat with other livestock, including cattle. It is possible that these dogs were accidentally infected by this pathogen through vectors like fleas or ticks that may have acquired the pathogens from infected hosts. However, further research on TBPs in on-host ticks should be conducted to confirm this point. Moreover, this pathogen was also found in some dogs living in urban areas; however, the transmission routes of these pathogens in such environments are still unknown.

Co-infection was tested in 107 (31.4%) individuals, with concurrent infection of *A. platys* and *Bartonella* spp. being the most frequently detected in this study. In tropical areas, it has been previously reported that due to the high diversity of vectors and TBPs (Irwin & Jefferies, 2004), dogs are more likely to get infected with multiple infectious agents (Galay *et al*., 2018; Low *et al*., 2018). Dogs infected with multiple pathogens could result from transmission with multiple pathogens by the same vector or the transmission of individual pathogens by different vectors. The common diagnostic methods used to detect suspected tick-borne pathogens in veterinary clinics are blood smears, hematological parameters, or clinical signs. Although these methods may be the most accessible diagnostic tests for veterinarians, they have limitations, such as being time-consuming and requiring a certain level of technical expertise. Furthermore, dogs affected by TBP co-infection may exhibit a wider range of clinical symptoms, making diagnosis more challenging. Molecular-based diagnostic methods, such as PCR, could overcome these limitations. Therefore, the establishment of veterinary services, such as regional veterinary pathology laboratories and molecular-based diagnostic laboratories for companion animals is essential to provide diagnostic support for small animal clinics. Our findings also indicated that concurrent infections with both a bacterial and one protozoan pathogen, like *Rickettsia* spp./*B. vogeli,* or *M. haemocanis/B. vogeli* were often detected. This emphasizes the necessity of examining more than one TBP in suspected cases because the treatment course for bacterial and protozoan pathogens differs (Kordick *et al*., 1999; Irwin & Jefferies, 2004).

The statistical analysis showed that certain host attributes exhibited a higher likelihood of contracting tick-borne diseases. Specifically, puppies, domestic breeds, and dogs living in rural areas had higher odds of being infected with TBPs compared to other groups (Table 3). A significant number of domestic breed dogs were located in suburban and rural areas, where they often served as guard dogs and were allowed to roam freely outdoors. These dogs might get infested with ticks from the environment or tick-infested roaming animals in the neighborhoods. This could explain the high prevalence of TBPs among dogs in these area. A further study on TBPs in ticks collected from the surrounding environment could be conducted to reinforce this statement. In agreement with previously reported publications, puppies are at higher risk of TBP infection, likely due to their increased vulnerability to tick infestations compared to aged dogs (Galay *et al*., 2018; Do *et al*., 2021). Unfortunately, questionnaires regarding tick infestation in dogs returned to us from the veterinary hospitals involved in the study were incomplete, limiting our analysis. Future research warrants more comprehensive epidemiological surveys of TBPs in ticks from Vietnam to confirm the observations in our study.

CONCLUSION

This study provides updated and pertinent information on the presence of tick-borne pathogens in dogs and the potential risks of spreading zoonotic pathogens to people residing in the studied areas. The findings of our study could assist policymakers, epidemiologists, researchers, and technicians for the establishment of control measures and shaping future strategies for the surveillance and prevention of canine tick-borne pathogens in Vietnam.

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Conflict of interest statement

The author declares that they have no conflict of interests.

Ethical approval

All materials and procedures for animal-originated sample use in the study were consulted and consented with the ethics committee of Obihiro University of Agriculture and Veterinary Medicine (the animal test: 21-25; DNA test: 1725-5)

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