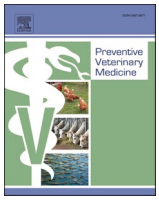
Preventive Veterinary Medicine 214 (2023) 105908

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed



Prevalence of *Babesia* and *Ehrlichia* in owned dogs with suspected tick-borne infection in Hong Kong, and risk factors associated with *Babesia gibsoni*

Daniela Hernandez Muguiro [a,1], Omid Nekouei [b,1], Kei Ying Lee [b], Fraser Hill [a], Vanessa R. Barrs [c,*]

[a] *Veterinary Diagnostic Laboratory, City University of Hong Kong, Kowloon Tong, Hong Kong Special Administrative Region of China*
[b] *Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon Tong, Hong Kong Special Administrative Region of China*
[c] *Department of Veterinary Clinical Sciences, Jockey Club College of Veterinary Medicine and Life Sciences, and Centre for Animal Health and Welfare, City University of Hong Kong, Kowloon Tong, Hong Kong Special Administrative Region of China*

ARTICLE INFO

Keywords:
Babesia
Ehrlichia
Tick-borne infection
Prevalence
Canine

ABSTRACT

Babesiosis and ehrlichiosis are the most clinically significant tick-borne infections in dogs. Although epidemiological investigations of these diseases have been performed in some Asian regions, little data is available in Hong Kong, where competent vector tick species are endemic. The objectives of this study were to determine the molecular prevalence of *Ehrlichia canis* and *Babesia* species (*B. canis, B. gibsoni, B. vogeli*) in owned dogs suspected of tick-borne infection in Hong Kong and to identify risk factors associated with *B. gibsoni* infection. Electronic records from the Veterinary Diagnostic Laboratory of City University of Hong Kong were searched to identify canine blood samples submitted for molecular testing of these pathogens by real time PCR between March 2018 and May 2021. Electronic patient records from the affiliated veterinary hospital were searched to identify a subset of tested dogs to investigate the potential risk factors for *B. gibsoni* infection using logistic regression models. Among 1508 tested dogs for all four pathogens of interest, *Babesia* spp. were detected in 435 (28.8%) and *E. canis* in 112 (7.4%). *Babesia gibsoni* was detected in 408 dogs while *B. vogeli* was detected in 27 dogs. *Babesia canis* was not detected in any dog. Co-infections of different combinations of *B. gibsoni, B. vogeli* and *E. canis* were present in 25 dogs. In multivariable logistic regression, mixed breed dogs were more likely to be infected with *B. gibsoni* than purebreds ($P = 0.005$), while dogs > 10 years of age were less likely to be infected than younger dogs ($P = 0.019$). Hematological abnormalities significantly associated with *B. gibsoni* infection included thrombocytopenia, neutropenia, or pancytopenia. Babesiosis caused by *B. gibsoni* is a common infection in owned dogs suspected of tick-borne infection in Hong Kong. The risk factors reported should be considered in diagnosing dogs suspected of infection with this agent. Furthermore, consideration for testing for *B. gibsoni* infection should be given if the results of a complete blood count show thrombocytopenia even in the absence of anemia, neutropenia or pancytopenia.

1. Introduction

Babesiosis and ehrlichiosis are the most clinically significant tick-borne infections in dogs. Obligate intracellular apicomplexan protozoa in the genus *Babesia* can be classified based on morphological features as large forms (3–5 μm diameter) or small forms (1–3 μm diameter) (Irwin, 2009; Birkenheuer et al., 2022). Based on molecular genotyping, serological testing, and vector specificity, three large and three small *Babesia* species have been identified in dogs (Table 1) (Irwin, 2009; Baneth et al., 2019; Ciuca et al., 2021; Kamani, 2021). Two unnamed large forms of *Babesia* species have been detected in dogs in the USA and the UK (Holm et al., 2006; Lehtinen et al., 2008; Birkenheuer et al., 2004). An intermediate-sized *Babesia* species has also recently been described (Baneth et al., 2020).

* Corresponding author.
E-mail address: vanessa.barrs@cityu.edu.hk (V.R. Barrs).
[1] These authors contributed equally to the manuscript.

https://doi.org/10.1016/j.prevetmed.2023.105908
Received 3 December 2022; Received in revised form 21 March 2023; Accepted 27 March 2023
Available online 29 March 2023

Table 1
Babesia and Ehrlichia species of domestic dogs, associated tick vectors and geographical distribution.

Large *Babesia* species	Main tick vectors	Geographic distribution	References
B. vogeli	*Rhipicephalus sanguineus*	Africa, Asia, Europe, the Americas	All large *Babesia* species: Ayoob et al. (2010), Irwin et al., 2019, Kamani (2021)
B. canis	*Dermacentor reticulatus*	Europe	
B. rossi	*Haemaphysalis elliptica* *H. leachi*	Sub-Saharan Africa, South Africa	
Unnamed (n = 2)	Unknown	USA	
Small *Babesia* species			
B. gibsoni	*H. longicornis* *H. bispinosa* *R. sanguineus*	Asia, North and South America, Africa, Europe, Australia	*B. gibsoni*: Ayoob et al. (2010), Irwin et al., 2019
B. vulpes	*Ixodes hexagonus*	Europe, Russia, North America	Baneth et al. (2019), Ciuca et al. (2021)
B. conradae	Unknown	California and Oklahoma (USA)	Ayoob et al. (2010)
Intermediate *Babesia* species:			
B. negevi sp. nov.	Unknown	Israel	Baneth et al. (2020)
***Ehrlichia* species**			
E. canis	*R. sanguineus* *D. variabilis*	Worldwide USA	Anziani et al. (1990), Groves et al. (1975), Harrus and Waner (2011),Little (2010), Ndip et al., (2005, 2007)
E. chaffeensis	*Amblyomma americanum*	USA	Ndip et al. (2005)
E. ewingii	*A. americanum*	USA	Ndip et al. (2005)

The transmission of *Babesia* is primarily indirect via hard tick (Ixodidae) vectors. The geographic distribution of *Babesia* species is significantly influenced by the ecological ranges of their vector ticks (Table 1). In addition, *B. gibsoni* has been reported to spread via direct dog-to-dog transmission during fighting and biting or transplacentally in regions where endemic tick vectors are absent, including the US and Japan (Birkenheuer et al., 1999; Macintire et al., 2002; Matsuu et al., 2004; Birkenheuer et al., 2005). The presentation of canine babesiosis, or "tick fever" ranges from subclinical chronic infection to severe acute clinical disease characterized by hemolytic anemia, jaundice, splenomegaly, lymphadenopathy and/or vomiting.

Canine ehrlichiosis is caused by three species of rickettsial bacteria transmitted by tick vectors (Table 1) (Anziani et al., 1990; Groves et al., 1975; Ndip et al., 2005; Ndip et al., 2007; Little, 2010). *Ehrlichia canis* causes canine monocytic ehrlichiosis, which can be life-threatening (Little, 2010; Harrus and Waner, 2011).

Although epidemiological studies of canine tick-borne infections have been performed in several regions of Asia, including eastern Asia and southeastern China (Zhang et al., 2017; Colella et al., 2020; Nguyen et al., 2020; Wang et al., 2020), there is a paucity of such studies in Hong Kong. Two molecular surveillance reports from over a decade ago found that *B. gibsoni* was the most common canine tick-borne pathogen in Hong Kong (Wang et al., 2010; Wong et al., 2011).

The objectives of this study were to determine the molecular prevalence of *B. canis*, *B. vogeli*, *B. gibsoni* and *E. canis* among owned dogs with suspected tick-borne infections in Hong Kong and to identify risk factors associated with *B. gibsoni* infection.

2. Materials and methods

2.1. Data collection and study population

To determine the prevalence of *Babesia* spp. and *E. canis* among dogs with suspected tick-borne infections, a cross-sectional study was conducted using all electronic diagnostic testing records from the Veterinary Diagnostic Laboratory (VDL) of City University of Hong Kong, between March 2018 when molecular testing first became available and May 2021. This database was searched to identify records of all canine blood samples submitted for molecular detection of *Babesia* spp., *B. canis*, *B. vogeli*, *B. gibsoni*, and/or *E. canis* during the study period. These blood samples were submitted to VDL by veterinarians from 95 veterinary clinics in Hong Kong who elected to test for tick-borne infections during diagnostic investigations of dogs presenting to them.

Information collected from this original data set of 1731 records included unique identification number, location of submitting veterinary clinic, date of sample collection, breed, sex, age at the time of sampling and the date of birth. Eighty-three samples were not tested for all three *Babesia* species of interest and excluded from the prevalence study, resulting in a final data set of 1648. Of these, 140 samples were only tested for *Babesia* spp. but not for *E. canis*.

To identify potential risk factors associated with *B. gibsoni*-infection, the unique identification number of each record in the original data set from VDL was merged with the unique electronic patient medical records of all dogs identified to match the original tick-borne diagnostic test submission to VDL from the veterinary hospital that is affiliated with the laboratory (CityU Veterinary Medical Centre). Clinical information from the identified dogs was extracted from the medical records, including the geographic location of the dog, history, physical examination findings and hematological data (hematocrit, and platelet, neutrophil and reticulocyte counts). The combined dataset included 425 unique cases with complete matching information and PCR test results for *B. canis, B. vogeli, B. gibsoni*, and *E. canis*. A flowchart summarizing the selection of cases included in the two parts of this study is provided in Fig. 1. All data management and analyses were carried out in Stata v17 (StataCorp LLC, College Station, TX, USA).

2.2. Risk factors associated with B. gibsoni infection

Given the predominance of *B. gibsoni* in the study population (based on historical data and our current findings) and its vector distribution in Hong Kong, the risk factor analysis was restricted to *B. gibsoni* infection alone. Infection was defined as a positive PCR test result. Using the combined data set described above (n = 425), samples/dogs only infected with *B. gibsoni* (case group) were compared with those not infected with any of the four pathogens (comparison group). Independent variables extracted from patient medical records available in the final dataset included: sex, breed, age, history of tick exposure and tick prevention, splenectomy, fighting with other dogs, geographic location, and access to outdoors. Dogs were categorized into four age groups: < 2, 2 to < 5, 5 to < 10, and ≥ 10 years of age. Tick exposure was defined as a dichotomous variable based on the detection of ticks on the dog or in its habitat before visiting the clinic.

Associations between hematological parameters and infection with *B. gibsoni* were assessed. These parameters included anemia, thrombocytopenia, neutropenia, or pancytopenia. Hematological analyses were performed using IDEXX ProCyte DX Hematology Analyzer. Based on the analyzer reference intervals, anemia was defined as hematocrit (HCT) $< 37.3\%$ (reference interval (RI) 37.3 – 61.7%). Thrombocytopenia was defined as a platelet count $< 148 \times 10^9$/L (RI 148–484 $\times 10^9$/L). Neutropenia was defined as a neutrophil count $< 2.95 \times 10^9$/L (RI 2.95–11.64 $\times 10^9$/L). Pancytopenia was defined as concurrent presence of anemia, neutropenia, and thrombocytopenia.

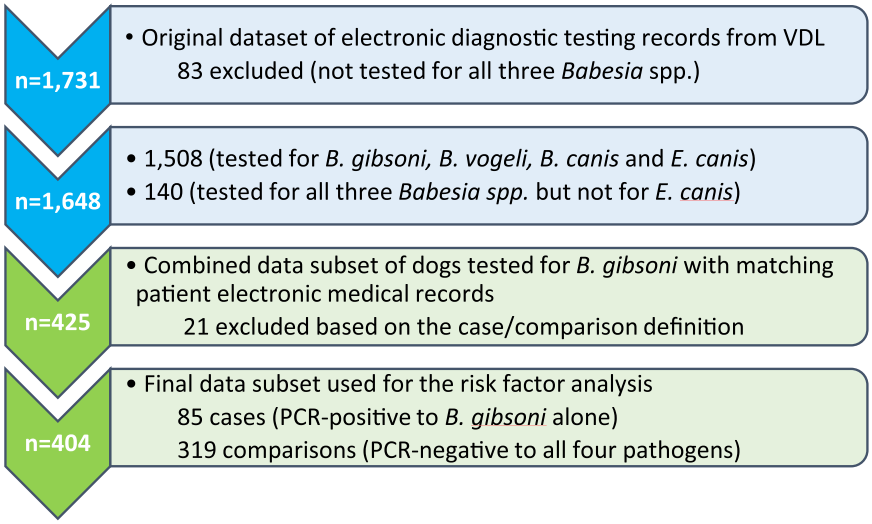


Fig. 1. Flowchart summarizing the selection of cases included in the two parts of this study. For the prevalence study (blue boxes), electronic diagnostic testing records from the Veterinary Diagnostic Laboratory (VDL) matching the inclusion criteria were selected, resulting in a final data set of 1648 cases for determining *Babesia* spp. prevalence and 1508 cases for determining *E. canis* prevalence. For the risk factor analysis for *B. gibsoni* infection (green boxes), the electronic diagnostic testing records from VDL were merged with the patient medical records from a hospital affiliated with the laboratory.

2.3. Statistical modeling

Univariable associations between the outcome variable (being infected with *B. gibsoni* or not) and each independent variable of interest were evaluated using simple logistic regression models. Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were estimated for each variable. Independent variables with a P-value < 0.2 in univariable screening were retained for inclusion in the multivariable model. A backward-elimination strategy was used to build the final multivariable logistic regression model (Dohoo et al., 2009) to determine the risk factors associated with *B. gibsoni*-infection using the significance level of 0.05. The two-way interactions between variables in the multivariable model were also evaluated by adding the interaction term between age and breed to the final model and assessing its statistical significance.

2.4. DNA extraction and PCR for tick-borne pathogens

Blood samples were collected in EDTA (1.3 mL) and transported on ice for analysis. Real-time PCR was used for the detection of the selected tick-borne pathogens. The primer and target gene details for each of the tested tick-borne pathogens are listed in Table 2.

DNA extraction from 200 μL of each blood sample was performed using a robotic automated platform (EZ1 Advanced XL, Qiagen, Germany). Commercial kits including positive controls were used for DNA extraction and PCR amplification of *Babesia* (Qiagen DSP virus extraction kit, Qiagen, Hong Kong) and *E. canis* (Genesig Advanced Kit, Primerdesign Ltd, United Kingdom). The kit for *Babesia* included a primer and a probe to detect an endogenous housekeeping gene (beta-Actin) (Piorkowski et al., 2014) and confirm the extraction of a valid biological template. The commercial kit for *E. canis* included a primer and a probe to detect an endogenous gene, for which nucleotide sequences were not available (Table 2). The detection of *Babesia* and *E. canis* was performed in separate PCR reactions. The *Babesia* and endogenous housekeeping gene control primers were *de novo* synthesized (Techdragon Limited, Hong Kong). The PCR conditions for *Babesia* consisted of an initial three min enzyme activation at 95 °C, followed by 44 cycles of 15 s denaturation at 95 °C, 15 s annealing at 60 °C and 15 s extension at 72 °C. Amplification reactions contained 10 μL SYBR Green Supermix (Bio--Rad, Hong Kong). Fluorescence data were collected after each cycle. Samples with cycle threshold (Ct) values < 40 were considered positive for *Babesia*.

The *E. canis* PCR protocol consisted of a two-step PCR. The amplification reactions contained 10 μL SYBR Green Supermix (Bio-Rad, Hong Kong). The PCR conditions were an initial enzyme activation of 2 min at 95 °C, followed by 50 cycles of 10 s denaturation at 95 °C and extension at 60 °C for 1 min. Fluorescence data were collected after each cycle. The Ct cut-off value for a positive *E. canis* result was < 40. The Ct cut-off values were determined during the initial setup of the PCR protocols using the positive control templates. RNAase/DNAase-free water was used as a negative control in each run.

3. Results

3.1. Prevalence of tick-borne infections

Among all 1648 samples tested for *Babesia* spp., 496 (30.1%) were positive. Among the 496 *Babesia*-positive samples, *B. gibsoni* was the

Table 2
Primers and gene targets for the four vector-borne pathogens in the study.

Pathogen	Primer Name	Gene Target	Primer Sequence (5′ to 3′)	Reference
Babesia spp.	B-lsu-F	*Babesia* lsu5-lsu4	ACCTGTCAARTTCCTTCACTAAMTT	Qurollo et al. (2017)
	B-lsu-R2	*Babesia* lsu5-lsu4	TCTTAACCCAACTCACGTACCA	
	Bmic-F	*B. microti*-like lsu5-lsu4	TTGCGATAGTAATAGATTTACTGC	
B. gibsoni	BG-cox1-F	*B. gibsoni* cox1	CTTCAGCCAATAGCTTTCTGTTTG	
	BG-cox1-R		CCTGAGGCAAGTAAACCAAATAT	
B. canis	BCC-cox1-F2	*B. canis* cox1	GTGCAATGAGTGGAGCAAATTTCA	
	BCC-cox1-R		CCATACAGTTGGTATTAATCTATCC	
B. vogeli	BCV-F	*B. vogeli* 18 S RNA	GTTCGAGTTTGCCATTCGTT	
	BAB722-R		ATGCCCCCAACCGTTCCTATTA	
Ehrlichia canis[a]	-	Major outer membrane protein (p30–10)	-	-

[a] p30-10 genes of *E. canis* GenBank accession number: AF525812, anchor nucleotide: 776, amplicon length: 102 bp (Genesig Advanced Kit, Primerdesign Ltd, United Kingdom)

most common (n = 462), followed by *B. vogeli* (n = 34), while *B. canis* was not detected (Table 3). Among 1508 dogs tested for all four pathogens (*Babesia* spp. and *E. canis),* the prevalence of *Babesia* spp. was 28.8% (435/1508), and the prevalence of *E. canis* was 7.4% (112/1508) (Table 3). Co-infections were detected in 25 dogs, including *B. gibsoni* and *E. canis* (n = 17), *B. vogeli* and *B. gibsoni* (n = 2), and *B. vogeli*, *B. gibsoni*, and *E. canis* (n = 1).

3.2. Risk factors associated with B. gibsoni infection

In the combined dataset, of the 425 dogs tested for *Babesia* and *Ehrlichia* that had complete medical records data available, 85 were positive for *B. gibsoni* alone (case group). Dogs that were negative for all four pathogens formed our comparison group. The final dataset for the risk factor analysis comprised 404 dogs, including 85 cases and 319 comparisons. Amongst this population, age ranged from 21 days to 19 years (median of 10 and inter-quartile range of 6.5–13 years), 185 were female, 219 were male, and over half of all dogs were sexually intact (n = 217) (Table 4). The majority of dogs were purebred (n = 313), comprising 44 different breeds (Table S1). Information about domicile was available for 400 dogs, of which 228 lived in the New Territories and 124 lived in Kowloon. The remainder lived on Hong Kong Island (n = 34) or Lantau Island (n = 14). Twenty-four dogs were splenectomized, of which three were infected with *B. gibsoni*. No dogs had a history of bite wounds or fights with other dogs. Information about access to outdoors was described for 69 dogs, of which 18 had no outdoor access. Variables tested for an association with *B. gibsoni* infection in univariable analysis are listed in Table 4. Of these, only breed and age (P < 0.2) were eligible for inclusion in the multivariable model.

Based on the output of the final multivariable logistic model (Table 5), mixed-breed dogs had approximately two times higher odds of being infected compared to purebreds (P = 0.005). A negative association was observed between infection status and age (P = 0.019). As age increased, the odds of infection decreased, but this trend was only statistically significant for dogs older than 10 years compared to younger age groups (Table 5). The interaction between age and breed was not statistically significant (P = 0.996); therefore, it was not included in the final model.

Of dogs for which hematological parameters were available, the majority were anemic (251/306) and thrombocytopenic (200/277). Dogs infected with *B. gibsoni* were more likely to be thrombocytopenic, neutropenic, or pancytopenic (Table 4; all with P < 0.05).

4. Discussion

This study represents the largest molecular survey for *Babesia* and *Ehrlichia* species in owned dogs in Hong Kong. Although the prevalence of babesiosis among the dogs suspected of tick-borne infection was high overall (28.8 – 30.1%), it was lower than reported previously in two small studies, which identified *Babesia* DNA in blood samples from 30/64 (46.9%) and 33/100 (33%) owned dogs as well as in 48/100 (48%) stray dogs in Hong Kong (Wang et al., 2010; Wong et al., 2011). In contrast, we found a low proportion of detection of *E. canis* DNA of 7.4%, which was similar to the previously reported prevalence of 6.5% in 2011 (Wong et al., 2011). While the prevalences reported in the previous studies are not directly comparable to our findings due to differences in sampling, testing methodology and the background of study animals, it appears that the relative prevalence of *B. gibsoni* and *B. vogeli* among *Babesia* positive dogs, has remained constant over the last decade, with *B. gibsoni* comprising 93% of *Babesia* spp. in 2022, compared to 92.6% in 2011 (Wong et al., 2011).

The *Babesia* and *Ehrlichia* species and their prevalence reported here are not surprising given what is known about the ecological niche and

Table 3
PCR test results of blood samples from 1648 owned dogs subjected to screening for *Babesia canis, B. vogeli* and *B. canis* alone or together with *E. canis*.

	No. of PCR positive samples (%)			
No. of samples tested	***B. canis***	***B. vogeli***	***B. gibsoni***	***E. canis***
1508	0	27 (1.79)	408 (27.06)	112 (7.43)
140	0	7 (5.00)	54 (38.57)	Not tested
1648 (total)	0	34 (2.06)	462 (28.03)	-

Table 4
Univariable associations between *Babesia gibsoni* infection status (PCR positive/negative) and the independent variables of interest for 404 study dogs (from simple logistic regressions).

Variable	No. *B. gibsoni* PCR negative (%)	No. *B. gibsoni* PCR positive (%)	Odds Ratio	95% Confidence Interval	P-value
Sex					
Female	150 (81.1)	35 (18.9)	-		
Male	169 (77.2)	50 (22.8)	1.27	0.78–2.06	0.337
Reproductive Status					
Intact	170 (78.3)	47 (21.7)	-		
Desexed	149 (79.7)	38 (20.3)	0.92	0.57–1.49	0.742
Breed					
Purebred	261 (83.4)	52 (16.6)	-		
Mixed	58 (63.7)	33 (36.3)	2.85	1.69–4.81	< 0.001
Age group (years)					
< 2	9 (56.2)	7 (43.8)	-		
2 to < 5	30 (63.8)	17 (36.2)	0.73	0.23–2.31	0.590
5 to < 10	85 (75.2)	28 (24.8)	0.42	0.14–1.24	0.118
≥ 10	195 (85.5)	33 (14.5)	0.22	0.08–0.62	0.005
Tick exposure					
ND[a]	294		-		
No	64 (80.0)	16 (20.0)	-		
Yes	23 (76.7)	7 (23.3)	1.22	0.44–3.33	0.702
Tick prevention					
ND	234		-		
No	56 (77.8)	16 (22.2)	-		
Yes	80 (81.6)	18 (18.4)	0.79	0.37–1.67	0.535
Living location					
New Territories	179 (78.5)	49 (21.5)	-		
Kowloon	98 (79.0)	26 (21.0)	0.97	0.57–1.66	0.909
Other	38 (79.2)	10 (20.8)	0.96	0.45–2.06	0.920
Outdoor access					
ND	335				
No	16 (88.9)	2 (11.1)	-		
Yes	41 (80.4)	10 (19.6)	2.25	0.49–10.39	0.299
Hematological parameters					
Anemia					
ND	98				
No	45 (82.0)	10 (18.0)	-		
Yes	191 (76.1)	60 (23.9)	1.41	0.67–2.97	0.362
Thrombocytopenia					
ND	127				
No	64 (83.1)	13 (16.9)	-		
Yes	143 (71.5)	57 (51.5)	1.96	1.01–3.84	0.049
Neutropenia					
ND	268				
No	100 (93.4)	7 (6.5)	-		
Yes	23 (79.3)	6 (20.7)	3.73	1.14–12.14	0.029
Pancytopenia					
ND	297				
No	81 (91.0)	8 (9.0)			
Yes	13 (72.2)	5 (27.8)	3.89	1.11–13.75	0.035

[a] ND: not determined

Table 5
Output of the final multivariable logistic regression model evaluating the association between *B. gibsoni*-infection status (PCR positive/negative) and risk factors in the 404 study dogs.

Variable	Odds Ratio	95% Confidence Interval	P-value
Mixed breed			
No	-		
Yes	2.22	1.28–3.86	0.005
Age group (years)[a]			
< 2	-		
2 to < 5	0.83	0.26–2.69	0.759
5 to < 10	0.56	0.18–1.71	0.310
≥ 10	0.31	0.11–0.94	0.039

[a] Overall P-value = 0.01.

geographical distribution of the vector ticks of these pathogens. *Rhipicephalus sanguineus* and *H. longicornis,* tick-vectors of *B. vogeli*, *B. gibsoni* and *E. canis,* are found in tropical and subtropical regions (Irwin, 2009). In addition, several epidemiological studies of ticks and tick-borne pathogens in pet dogs in East and Southeast Asia have shown that *Rh. sanguineus* and *H. longicornis* are the most common ticks identified on owned dogs (Zhang et al., 2017; Colella et al., 2020; Nguyen et al., 2020; Wang et al., 2020). Hong Kong's climate is subtropical, tending towards temperate for six months of the year and is suitable for the survival of *Rh. sanguineus* and *H. longicornis* ticks (Lin et al., 2015). Despite a lack of reported surveillance of tick species in Hong Kong, the Food, Environment and Health Department of the Hong Kong government has recorded the presence of tick species of four genera, including *Rhipicephalus, Haemaophysalis, Ixodes* and *Hyalomma* (Chan et al., 2011). *Amblyomma americanum*, the main tick vector of *E. ewingii* and *E. chaffeensis,* has not been reported to be present in Hong Kong.

The absence of *B. canis* identified here and in previous reports in Hong Kong, likely reflects the absence of a competent vector tick. *Babesia canis* is transmitted by *D. reticulatus* (Table 1), which is endemic to Europe and Central Asia and generally occurs in mild, cooler climates (Rubel et al., 2016). In China, Shanxi province was previously considered the eastern limit of its distribution (Foldvari et al., 2016). However, *B. canis* has recently been identified in blood samples from dogs in Hunan province in South-Central China (Wang et al., 2020), suggesting the geographical expansion of *D. reticulatus*. Hunan province is less than 700 km north of Hong Kong and both have similar climates, thus ongoing surveillance for *B. canis* and for *D. reticulatus* is warranted in Hong Kong. The vectors of other *Babesia* species that infect dogs appear to be restricted to specific, distant geographical areas (Table 1).

Of the risk factors evaluated in this study, only breed and age were significantly associated with *B. gibsoni* infection. In other studies, some pure-breed fighting dogs were over-represented for *B. gibsoni* infection included American pit bull terriers, Staffordshire bull terriers, and bull terriers, due to horizontal transmission through biting (Birkenheuer et al., 1999; Macintire et al., 2002; Birkenheuer et al., 2005). Despite a predominance of pure-bred dogs (77.5%) within the cohort evaluated in our risk-factor analysis, less than 1% were "pit-bull" or bull terriers and mixed-breed dogs were more likely to be infected with *B. gibsoni* than pure-bred dogs. Although we could not confirm the origin of the dogs, due to our study design, it is possible that mixed-breed dogs may have originated from environments where tick exposure is more likely, since adoption of stray dogs from villages and rural areas with dense foliage is common in Hong Kong. Similarly, stray dogs are likely to have had more outdoor access than owned dogs, many of which live in apartments and have more restricted outdoor access. Outdoor access was not identified as a risk factor for *B. gibsoni* infection, likely because information about outdoor access was only available for a small proportion of dogs in this study (69/404). Further, two owned dogs with no outdoor access were PCR positive for *B. gibsoni,* demonstrating the potential for transfer of tick vectors from their owners or visitors as suggested in another study (Piesman and Eisen, 2008).

Our results indicated that dogs older than 10 years were significantly less likely to be infected than dogs in other age groups. Other studies found the highest prevalence of infection in dogs aged 3–5 years with declining prevalence in dogs > 5 years of age (Hornok et al., 2006; Obeta et al., 2020). This finding can be attributed to decreased roaming activity and thus reduced exposure to vector ticks. We consider this is also a likely contributing factor in this population of dogs, as it is common in Hong Kong for older dogs to stay at home or be carried (in arms or strollers) during outings. Similar to some other studies, we found no association between sex and the risk of infection (Hornok et al., 2006; Li et al., 2020). However, in some studies, being male has been associated with an increased risk of babesiosis in dogs, possibly due to increased roaming behavior, or genetic and hormonal influences (Mellanby et al., 2011; Obeta et al., 2020).

The majority of the dogs tested for *B. gibsoni* resided in the New Territories, a region of Hong Kong characterized by wetlands, mountains, and wilderness parks. However, the risk of infection was not significantly different from dogs residing in Kowloon, which is more urban. Whilst a greater abundance of infected ticks and higher risk of exposure might be expected in less urbanized areas, it is possible that infected dogs may have originated from or traveled to less urban areas. Prospective studies of tick species and their environmental abundance are warranted.

Although the proportions of infection with *B. gibsoni* were higher in dogs with the history of tick exposure and outdoor access, these associations were not statistically significant. These findings are likely explained by the large number of missing data in the medical records of these dogs (a type of selection bias) where veterinarians did not collect or report information about tick-exposure or outdoor access. In addition, the information collected was based upon owners' responses, which could be prone to recall bias (a type of information bias). Similarly, data on whether dogs had been splenectomized or not was only available for a small number of dogs precluding the risk factor analysis. Splenectomy is, however, a well-known major risk factor for severe babesiosis in both dogs and humans (Camacho et al., 2002; Vannier and Krause, 2012). The absence of a history of fighting in dogs in this study combined with the known presence of an endemic competent tick vector for *B. gibsoni* is consistent with tick transmission as the major route of infection for dogs in Hong Kong.

In the present study, tick prevention measures taken did not significantly contribute to decreased *B. gibsoni* infection. Dogs may have been infected before they were acquired by their owners, or the frequency of application of acaricides may have been inadequate. Investigations of the efficacy of acaricidal products to prevent experimental and natural *Babesia* infection have focused on *B. canis* and *B. rossi* transmission. The efficacy of the same acaricides in the prevention of *B. gibsoni* transmission is assumed, but prospective studies to determine optimal acaricide protocols can be beneficial (Fourie et al., 2013; Beugnet et al., 2014; Jongejan et al., 2015; Taenzler et al., 2015).

In the current study, hematological abnormalities significantly associated with *B. gibsoni* infection were thrombocytopenia, neutropenia, or pancytopenia. Thrombocytopenia is the most common hematological abnormality in canine babesiosis, and is often, but not always, accompanied by anemia, as demonstrated by our data (Matsuu et al., 2004; Ayoob et al., 2010; Birkenheuer et al., 2022; Karasová et al., 2022). The lack of a statistical association between *B. gibsoni* and anemia here, may simply represent that the finding of anemia in a complete blood count is a trigger for molecular testing for babesiosis, but that babesiosis is only one of many causes of anemia in dogs. Although changes in the leukogram associated with *B. gibsoni* infection are generally regarded as non-specific, similar to our findings, neutropenia has been previously reported (Meinkoth et al., 2002; Brown et al., 2015; Yogeshpriya et al., 2018).

As with most cross-sectional studies, discerning the true associations between some independent variables (e.g. blood parameters and tick prevention measures in this study) and a disease can be difficult due to

the unclear temporal sequence of events. Therefore, those associations must be interpreted with caution.

Here we estimated the prevalence of infection with the four pathogens of interest among dogs with suspected tick-borne infections in Hong Kong as our intended target population. The actual prevalence of infection with these pathogens in the general population of dogs in Hong Kong could be lower or higher depending on the effect of selection bias for cases with a clinical presentation suggestive of tick-borne infection and on the prevalence of subclinical infections, which were not screened for in our study. Future investigations are warranted to confirm the tick reservoirs of *B. gibsoni* and *E.canis* in Hong Kong, their geographical distribution and their role in the dynamics of infections in the region.

5. Conclusions

In conclusion, babesiosis is common among dogs with suspected tick-borne infections in Hong Kong and it is most frequently caused by *B. gibsoni* followed by *B. vogeli.* In comparison, ehrlichiosis is much less common, and when it does occur, is caused by *E. canis.* The absence of history of dog-fighting and the known presence of competent tick vectors suggest tick-borne transmission as the primary route of infection of *B. gibsoni* in this geographic region. Veterinary practitioners in Hong Kong should have a high index of suspicion for babesiosis in dogs with consistent clinical signs, particularly if they are of mixed-breed and younger than 10 years of age. Further, consideration for testing for *B. gibsoni* infection should be given if the results of a complete blood count show thrombocytopenia even in the absence of anemia, neutropenia or pancytopenia.

Funding

This study was funded by an SGP grant (Project no. 9380113, One-health Pathogen Surveillance and Discovery) to Prof. Vanessa Barrs from City University of Hong Kong.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors thank the Executive Director and veterinary clinicians of the Veterinary Medical Centre of City University of Hong Kong and staff at the Veterinary Diagnostic Laboratory, City University of Hong Kong for assistance with this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.105908.

References

Anziani, O.S., Ewing, S.A., Barker, R.W., 1990. Experimental transmission of a granulocytic form of the tribe Ehrlichieae by *Dermacentor variabilis* and *Amblyomma americanum* to dogs. Am. J. Vet. Res 51 (6), 929–931 (Jun).

Ayoob, A.L., Hackner, S.G., Prittie, J., 2010. Clinical management of canine babesiosis (Feb). J. Vet. Emerg. Crit. Care (San. Antonio) 20 (1), 77–89. https://doi.org/10.1111/j.1476-4431.2009.00489.x.

Baneth, G., Cardoso, L., Brilhante-Simões, P., Schnittger, L., 2019. Establishment of *Babesia vulpes n.* sp. (Apicomplexa: Babesiidae), a piroplasmid species pathogenic for domestic dogs. Parasites Vectors 12 (1). https://doi.org/10.1186/s13071-019-3385-z.

Baneth, G., Nachum-Biala, Y., Birkenheuer, A.J., Schreeg, M.E., Prince, H., Florin-Christensen, M., Schnittger, L., Aroch, I., 2020. A new piroplasmid species infecting dogs: morphological and molecular characterization and pathogeny of *Babesia negevi n.* sp. Apr 21 Parasit. Vectors 13 (1), 130. https://doi.org/10.1186/s13071-020-3995-5.

Beugnet, F., Halos, L., Larsen, D., Labuschagne, M., Erasmus, H., Fourie, J., 2014. The ability of an oral formulation of afoxolaner to block the transmission of *Babesia canis* by *Dermacentor reticulatus* ticks to dogs. Jun 23 Parasit. Vectors 7, 283. https://doi.org/10.1186/1756-3305-7-283.

Birkenheuer, A., Levy, M., Savary, K., Gager, R., Breitschwerdt, E., 1999. *Babesia gibsoni* infections in dogs from North Carolina. J. Am. Anim. Hosp. Assoc. 35 (2), 125–128. https://doi.org/10.5326/15473317-35-2-125.

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Oct 5 Vet. Parasitol. 124 (3–4), 151–160. https://doi.org/10.1016/j.vetpar.2004.07.008.

Birkenheuer, A.J., Correa, M.T., Levy, M.G., Breitschwerdt, E.B., 2005. Geographic distribution of babesiosis among dogs in the United States and association with dog bites: 150 cases (2000-2003). Sep 15 J. Am. Vet. Med Assoc. 227 (6), 942–947. https://doi.org/10.2460/javma.2005.227.942.

Birkenheuer, A.J., Sykes, J.E., Greene, C.E., 2022. Babesiosis. In: Sykes, J.E. (Ed.), Infectious Diseases of the Dog and Cat., Chapter 97. Elsevier - Health Sciences Division, Philadelphia, United States, pp. 1223–1237.

Brown, A.L., Shiel, R.E., Irwin, P.J., 2015. Clinical, haematological, cytokine and acute phase protein changes during experimental *Babesia gibsoni* infection of beagle puppies (Oct). Exp. Parasitol. 157, 185–196. https://doi.org/10.1016/j.exppara.2015.08.002.

Camacho, A.T., Pallas, E., Gestal, J.J., Guitián, F.J., Olmeda, A.S., 2002. *Babesia canis* infection in a splenectomized dog. Bull. Soc. Pathol. Exot. 95 (1), 17–19 (Mar).

Chan, C., Chan, F., Ip, K., Kong, W., Kung, K., Lee, M., Leung, Y., Que, T., Borwein, S., Wong, K., Wu, T., Yeung, L., 2011. Prevention of tick-borne encephalitis in Hong Kong, Scientific Committee on Vector-Born Diseases. Centre for Health Protection. Department of Health,, Hong Kong SAR (Available from). ⟨https://www.chp.gov.hk/files/pdf/prevention_of_tick_borne_encephalitis_in_hong_kong_r.pdf⟩.

Ciuca, L., Martinescu, G., Miron, L.D., Roman, C., Acatrinei, D., Cringoli, G., Rinaldi, L., Maurelli, M.P., 2021. Occurrence of *Babesia* Species and Co-Infection with *Hepatozoon canis* in Symptomatic Dogs and in Their Ticks in Eastern Romania. Pathogens 10 (10), 1339. https://doi.org/10.3390/pathogens10101339.

Colella, V., Nguyen, V.L., Tan, D.Y., Lu, N., Fang, F., Zhijuan, Y., Wang, J., Liu, X., Chen, X., Dong, J., Nurcahyo, W., Hadi, U.K., Venturina, V., Tong, K.B.Y., Tsai, Y.L., Taweethavonsawat, P., Tiwananthagorn, S., Le, T.Q., Bui, K.L., Watanabe, M., Rani, P., Annoscia, G., Beugnet, F., Otranto, D., Halos, L., 2020. Zoonotic Vectorborne Pathogens and Ectoparasites of Dogs and Cats in Eastern and Southeast Asia (Jun). Emerg. Infect. Dis. 26 (6), 1221–1233. https://doi.org/10.3201/eid2606.191832.

Foldvari, G., Siroky, P., Szekeres, S., Majoros, G., Sprong, H., 2016. *Dermacentor reticulatus*: a vector on the rise. In: Parasites & Vectors., 9.

Fourie, J.J., Stanneck, D., Jongejan, F., 2013. Prevention of transmission of *Babesia canis* by *Dermacentor reticulatus* ticks to dogs treated with an imidacloprid/flumethrin collar. Feb 18 Vet. Parasitol. 192 (1–3), 273–278. https://doi.org/10.1016/j.vetpar.2012.10.017.

Groves, M.G., Dennis, G.L., Amyx, H.L., Huxsoll, D.L., 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. 36 (7), 937–940 (Jul).

Harrus, S., Waner, T., 2011. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview (Mar). Vet. J. 187 (3), 292–296. https://doi.org/10.1016/j.tvjl.2010.02.001.

Holm, L.P., Kerr, M.G., Trees, A.J., McGarry, J.W., Munro, E.R., Shaw, S.E., 2006. Fatal babesiosis in an untravelled British dog. Aug 5 Vet. Rec. 159 (6), 179–180. https://doi.org/10.1136/vr.159.6.179.

Hornok, S., Edelhofer, R., Farkas, R., 2006. Seroprevalence of canine babesiosis in Hungary suggesting breed predisposition (Nov). Parasitol. Res. 99 (6), 638–642. https://doi.org/10.1007/s00436-006-0218-8.

Irwin, P.J., 2009. Canine babesiosis: from molecular taxonomy to control. Mar 26 Parasit. Vectors (2 Suppl 1), S4. https://doi.org/10.1186/1756-3305-2-S1-S4.

Jongejan, F., de Vos, C., Fourie, J.J., Beugnet, F., 2015. A novel combination of fipronil and permethrin (Frontline Tri-Act(R)/Frontect(R)) reduces risk of transmission of *Babesia canis* by *Dermacentor reticulatus* and of *Ehrlichia canis* by *Rhipicephalus sanguineus* ticks to dogs. Nov 19 Parasit. Vectors 8, 602. https://doi.org/10.1186/s13071-015-1207-5.

Kamani, J., 2021. Molecular evidence indicts *Haemaphysalis leachi* (Acari: Ixodidae) as the vector of *Babesia rossi* in dogs in Nigeria, West Africa, 07 Ticks Tick. Borne Dis. 12 (4), 101717. https://doi.org/10.1016/j.ttbdis.2021.101717.

Karasová, M., Tóthová, C., Grelová, S., Fialkovičová, M., 2022. The Etiology, Incidence, Pathogenesis, Diagnostics, and Treatment of Canine Babesiosis Caused by *Babesia gibsoni* Infection. Animals 12 (6), 739. https://doi.org/10.3390/ani12060739.

Lehtinen, L.E., Birkenheuer, A.J., Droleskey, R.E., Holman, P.J., 2008. In vitro cultivation of a newly recognized *Babesia* sp. in dogs in North Carolina. Feb 14 Vet. Parasitol. 151 (2–4), 150–157. https://doi.org/10.1016/j.vetpar.2007.10.022.

Li, X.W., Zhang, X.L., Huang, H.L., Li, W.J., Wang, S.J., Huang, S.J., Shao, J.W., 2020. Prevalence and molecular characterization of *Babesia* in pet dogs in Shenzhen, China (Jun). Comp. Immunol. Microbiol Infect. Dis. 70, 101452. https://doi.org/10.1016/j.cimid.2020.101452.Cited.

Lin, G., Fu, J., Jiang, D., Wang, J., Wang, Q., Dong, D., 2015. Spatial Variation of the Relationship between PM 2.5 Concentrations and Meteorological Parameters in China. Biomed. Res Int. 2015, 684618 https://doi.org/10.1155/2015/684618.

Little, S.E., 2010. Ehrlichiosis and anaplasmosis in dogs and cats (Nov). Vet. Clin. North Am. Small Anim. Pract. 40 (6), 1121–1140. https://doi.org/10.1016/j.cvsm.2010.07.004.

Macintire, D.K., Boudreaux, M.K., West, G.D., Bourne, C., Wright, J.C., Conrad, P.A., 2002. *Babesia gibsoni* infection among dogs in the southeastern United States. J. Am. Vet. Med. Assoc. 220 (3), 325–329. https://doi.org/10.2460/javma.2002.220.325.

Matsuu, A., Kawabe, A., Koshida, Y., Ikadai, H., Okano, S., Higuchi, S., 2004. Incidence of Canine *Babesia gibsoni* Infection and Subclinical Infection among Tosa dogs in Aomori Prefecture. Jpn. J. Vet. Med. Sci. 66 (8), 893–897. https://doi.org/10.1292/jvms.66.893.

Meinkoth, J.H., Kocan, A.A., Loud, S.D., Lorenz, M.D., 2002. Clinical and hematologic effects of experimental infection of dogs with recently identified *Babesia gibsoni*-like isolates from Oklahoma. Jan 15 J. Am. Vet. Med Assoc. 220 (2), 185–189. https://doi.org/10.2460/javma.2002.220.185.

Mellanby, R.J., Handel, I.G., Clements DN, C., Bronsvoort, B.M., Lengeling, A., Schoeman, J.P., 2011. Breed and Sex Risk Factors for Canine Babesiosis in South Africa. J. Vet. Intern. Med. 25 (5), 1186–1189. https://doi.org/10.1111/j.1939-1676.2011.00779.x.

Ndip, L.M., Ndip, R.N., Esemu, S.N., Dickmu, V.L., Fokam, E.B., Walker, D.H., McBride, J. W., 2005. Ehrlichial infection in Cameroonian canines by *Ehrlichia canis* and *Ehrlichia ewingii*. Nov 30 Vet. Microbiol. 111 (1–2), 59–66. https://doi.org/10.1016/j.vetmic.2005.08.010.

Ndip, L.M., Ndip, R.N., Ndive, V.E., Awuh, J.A., Walker, D.H., McBride, J.W., 2007. *Ehrlichia* species in *Rhipicephalus sanguineus* ticks in Cameroon (eng.). Vector Borne Zoonotic Dis. 7 (2), 221–227. https://doi.org/10.1089/vbz.2006.0608.

Nguyen, V.L., Colella, V., Greco, G., Fang, F., Nurcahyo, W., Hadi, U.K., Venturina, V., Tong, K.B.Y., Tsai, Y.L., Taweethavonsawat, P., Tiwananthagorn, S., Tangtrongsup, S., Le, T.Q., Bui, K.L., Do, T., Watanabe, M., Rani, P., Dantas-Torres, F., Halos, L., Beugnet, F., Otranto, D., 2020. Molecular detection of pathogens in ticks and fleas collected from companion dogs and cats in East and Southeast Asia. Aug 15 Parasit. Vectors 13 (1), 420. https://doi.org/10.1186/s13071-020-04288-8.

Obeta, S.S., Ibrahim, B., Lawal, I.A., Natala, J.A., Ogo, N.I., Balogun, E.O., 2020. Prevalence of canine babesiosis and their risk factors among asymptomatic dogs in the federal capital territory, Abuja, Nigeria (Nov). Parasite Epidemiol. Control. 11, e00186. https://doi.org/10.1016/j.parepi.2020.e00186.

Piesman, J., Eisen, L., 2008. Prevention of tick-borne diseases. Annu Rev. Entomol. 53, 323–343. https://doi.org/10.1146/annurev.ento.53.103106.093429.

Piorkowski, G., Baronti, C., de Lamballerie, X., de Fabritus, L., Bichaud, L., Pastorino, B. A., Bessaud, M., 2014. Development of generic Taqman PCR and RT-PCR assays for the detection of DNA and mRNA of β-actin-encoding sequences in a wide range of animal species (Jun). J. Virol. Methods 202, 101–105. https://doi.org/10.1016/j.jviromet.2014.02.026.

Qurollo, B.A., Archer, N.R., Schreeg, M.E., Marr, H.S., Birkenheuer, A.J., Haney, K.N., Thomas, B.S., Breitschwerdt, E.B., 2017. Improved molecular detection of *Babesia* infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. Mar 7 Parasit. Vectors 10 (1), 128. https://doi.org/10.1186/s13071-017-2064-1.

Rubel, F., Brugger, K., Pfeffer, M., Chitimia-Dobler, L., Didyk, Y.M., Leverenz, S., Dautel, H., Kahl, O., 2016. Geographical distribution of *Dermacentor marginatus* and *Dermacentor reticulatus* in Europe (Feb). Ticks Tick. Borne Dis. 7 (1), 224–233. https://doi.org/10.1016/j.ttbdis.2015.10.015.

Taenzler, J., Liebenberg, J., Roepke, R.K., Heckeroth, A.R., 2015. Prevention of transmission of *Babesia canis* by *Dermacentor reticulatus* ticks to dogs treated orally with fluralaner chewable tablets (Bravecto). Jun 4 Parasit. Vectors 8, 305. https://doi.org/10.1186/s13071-015-0923-1.

Vannier, E., Krause, P.J., 2012. Human babesiosis. Jun 21 N. Engl. J. Med. 366 (25), 2397–2407. https://doi.org/10.1056/NEJMra1202018.

Wang, C., Ahluwalia, S.K., Li, Y., Gao, D., Poudel, A., Chowdhury, E., Boudreaux, M.K., Kaltenboeck, B., 2010. Frequency and therapy monitoring of canine *Babesia* spp. infection by high-resolution melting curve quantitative FRET-PCR. Feb 26 Vet. Parasitol. 168 (1–2), 11–18. https://doi.org/10.1016/j.vetpar.2009.10.015.

Wang, J., Wang, X., Sun, H., Lv, Z., Li, Y., Luo, J., Guan, G., Yin, H., 2020. Molecular evidence of piroplasm infection in companion animals in Hunan Province, China. Aug 17 BMC Vet. Res. 16 (1), 297. https://doi.org/10.1186/s12917-020-02500-6.

Wong, S.S., Teng, J.L., Poon, R.W., Choi, G.K., Chan, K.H., Yeung, M.L., Hui, J.J., Yuen, K.Y., 2011. Comparative evaluation of a point-of-care immunochromatographic test SNAP 4Dx with molecular detection tests for vector-borne canine pathogens in Hong Kong (Sep). Vector Borne Zoonotic Dis. 11 (9), 1269–1277. https://doi.org/10.1089/vbz.2010.0265.

Yogeshpriya, S., Sivakumar, M., Saravanan, M., Venkatesan, M., Veeraselvam, M., Jayalakshmi, K., 2018. Selvaraj P. Clinical, haemato-biochemical and ultrasonographical studies on naturally occurring *Babesia gibsoni* infection in dogs. J. Entomol. Zool. Stud. 6 (1), 1334–1337.

Zhang, J., Liu, Q., Wang, D., Li, W., Beugnet, F., Zhou, J., 2017. Epidemiological survey of ticks and tick-borne pathogens in pet dogs in south-eastern China. Parasite 24, 35.