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First molecular confirmation of multiple zoonotic vector-borne diseases in pet dogs and cats of Hong Kong SAR

Hussain, Sabir; Hussain, Abrar; Aziz, Muhammad Umair; Song, Baolin; Zeb, Jehan; SPARAGANO, Olivier Andre; Hasib, F M Yasir; Almendros, Angel ; Cabezas-Cruz, Alejandro; George, David; Sparagano, Olivier

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Original article

First molecular confirmation of multiple zoonotic vector-borne diseases in pet dogs and cats of Hong Kong SAR

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ABSTRACT

In recent years, the incidence of vector-borne diseases (VBDs) has increased throughout the globe. In particular, tick-borne diseases (e.g., caused by *Ehrlichia canis, E. ewingii, Anaplasma phagocytophilum, A. platys, Borrelia burgdorferi sensu stricto* (s.s.) and *Babesia gibsoni*) and mosquito-borne diseases (e.g., caused by *Dirofilaria immitis*) diseases pose a burden on animal health. Nevertheless, there have been no studies undertaken on the occurrence of VBDs in pet dogs and cats in Hong Kong SAR. This study fills this gap, and is the first to determine the seroprevalence of major VBDs, such as those caused by *D. immitis, E. canis, E. ewingii, A. phagocytophilum, A. platys* and *B. burgdorferi* s.s, in dogs and cats through commercially available SNAP 4Dx plus testing. Infection by all these pathogens and *Babesia* sp. was further assessed through PCR and DNA sequencing. A total of 224 blood samples were collected from domestic dogs ($n = 159$) and cats ($n = 65$) in Hong Kong SAR during summer 2022. Hematocrit and platelet counts were determined in each blood sample and other hematological parameters were assessed using an automatic hematology analyzer and vortex the specimen for one to two minutes at or near the highest setting to minimize the clumping. All cat sera samples were negative for tested pathogens, but antibodies against some of the pathogens were detected in dog sera samples. Here, the highest figures were recorded for seroprevalence of *E. canis/E. ewingii* (10.7%), followed by *D. immitis* (5.7%), and *A. phagocytophilum/A. platys* (2.5%). No *B. burgdorferi* s.s. antibodies were detected in any of the dogs tested. Through molecular diagnostics, we detected the presence of *B. gibsoni* (3.7%), *E. canis* (3.1%), *D. immitis* (5.7%), and *A. phagocytophilum* (1.3%). Neighbor-Joining phylogenetic trees for vector-borne pathogens (i.e., genus *Anaplasma* sp.) showed 100% clustering to Japan, the USA and Germany, whereas genus *Ehrlichia* sp. showed 100% clustering to China, Turkey, Cuba, and Greece. Similarly, genus *Babesia* sp. clustered 100% to India, Sri Lanka and Austria, while *D. immitis* clustered in Iraq, South Korea, Portugal, France, the USA and Italy. This study provides the first evidence on the occurrence of tick-borne pathogens in pet dogs in Hong Kong SAR. Based on these findings, it is recommended that appropriate screening should be undertaken in domestic dogs to evaluate the prevalence of these pathogens and promote the timely control of VBDs.

1. Introduction

Vector-borne diseases, particularly those that are tick and mosquito-borne, are continuously emerging and globally distributed (WHO 2004; Jones et al., 2008). Each year, tick-borne pathogens (TBPs) alone resulted in over 100,000 cases of human illness throughout the world

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(de la Fuente et al., 2008). Ticks are considered to be the most important vector of disease-causing pathogens in domestic and non-domestic animals (Jongejan and Uilenberg, 2004), posing a significant threat to both animal and human health in the environments where they are found (Hussain et al., 2021). Here, ticks act to spread vector-borne diseases, including Anaplasmosis, Babesiosis, Ehrlichiosis, Borreliosis, that are emerging concerns for veterinarians and physicians. Canine and feline populations are susceptible to most of the TBPs commonly known to infect various other mammals, including humans, with dogs previously reported as potential reservoirs and sentinels for various infectious and zoonotic diseases (McCown et al., 2014). The most commonly diagnosed vector-borne diseases in dogs and cats include ehrlichiosis (*E. canis / E. ewingii*), anaplasmosis (*A. phagocytophilum / A. platys*), Lyme disease (*B. burgdorferi*), babesiosis, and heartworm disease (*Dirofilaria immitis*). In addition to their veterinary significance, pathogens are also of concern to public health. Veterinary investigations have revealed the impact of the above diseases in susceptible dogs and cats in different countries- where causal agents have been reported, though no work has been undertaken in this area in other countries, including Hong Kong SAR-

Lyme borreliosis is a bacterial disease caused by the gram-negative spirochete *B. burgdorferi sensu lato* in dogs (Bowman et al., 2009). *B. burgdorferi sensu lato* is the most common TBP in temperate regions of the Northern Hemisphere (Hussain et al., 2021b), where ticks play a vital role in the host-to-host transmission and consequent geographic expansion of this TBP (Margos et al., 2019). This pathogen is mainly vectored by *Ixodes* tick species, such as *Ixodes scapularis, Ixodes ricinus, I. persulcatus* and *I. pacificus*, but other members of this genus also contribute to transmission (Hussain et al., 2021b; Margos et al., 2019). Lyme borreliosis in dogs is characterized by fever, anorexia, lameness, lethargy, and lymphadenopathy, also causing arthritis and glomerulonephritis (Krupka and Straubinger, 2010).

Rickettsial microorganisms, such as *A. phagocytophilum* (infects neutrophilic and eosinophilic granulocytes), and *A. platys* (infect the platelets) (Rikihisa, 1991) cause symptoms, including fever, anorexia, myalgia, and thrombocytopenia, which can lead to severe manifestations such as epistaxis, or even death in extremely severe cases (Rikihisa, 1991). Domestic and wild dogs may also host pathogens of the genus *Ehrlichia*, which have worldwide distribution (Groves et al., 1975; Harrus and Waner, 2011). *Rhipicephalus sanguineus*, the brown dog tick, is the main vector of *E. canis*, the causal agent of canine monocytic ehrlichiosis (Groves et al., 1975; Harrus and Waner, 2011), while *Amblyomma americanum* is the main vector of *E. ewingii*, the causal agent of canine granulocytic ehrlichiosis (Springer et al., 2014). (Rikihisa, 1991; Bown et al., 2003). *Anaplasma platys* is responsible for causing canine cyclic thrombocytopenia, infects dog platelets, and is mainly transmitted by *R. sanguineus* ticks (Harvey and Greene, 2006; Harvey et al., 1978). *Anaplasma phagocytophilum*, which causes human granulocytic anaplasmosis (HGA), is conversely transmitted by *I. scapularis* and *I. pacificus* (Rikihisa, 2006). *Anaplasma phagocytophilum* and *B. burgdorferi* can share their reservoir hosts and can subsequently be transmitted by the same vector, *I. scapularis* (Daniels et al., 1998).

Dirofilaria immitis (heartworm) is a disease-causing nematode transmitted as third-stage (L3) larvae during blood feeding by culicid mosquitoes, including *Aedes aegypti*. It is the most important helminth affecting dogs in numerous countries, including Hong Kong SAR (Bowman et al., 2009). Adult *D. immitis* reside in the pulmonary arteries and the right heart of definitive hosts, where mature viviparous females release unsheathed first-stage larvae (microfilariae) into the bloodstream (Kume and Itagaki, 1955; Kotani and Powers, 1982; Muñoz-Caro et al., 2018). The microfilariae can cause right heart / pulmonary disease in adult dogs, with canines considered a natural host for *D. immitis*. Nevertheless, heartworms can also be found in cats, ferrets and coyotes (Bowman, 2020).

Babesiosis is a tick-borne protozoan disease caused by globally widespread parasites of the genus *Babesia* (Solano-Gallego and Baneth, 2011; Hussain et al., 2021a). In dogs and cats this intraerythrocytic protozoan disease can vary in presentation from subclinical to severe and potentially fatal (Solano-Gallego and Baneth, 2011). The most common signs and symptoms of infection include anorexia, fever, splenomegaly and hypotension (Matijatko et al., 2012). Canine babesiosis is caused by *B. canis, B. vogeli, B. gibsoni* and *B. microti*-like parasites (Matijatko et al., 2012; Solano-Gallego and Baneth, 2011), where the most common vectors include *R. sanguineus* (*B. vogeli*), *Dermacentor reticulatus* (*B. canis*) and *I. hexagonus* (for *B. microti*-like parasites) (Matijatko et al., 2012).

There is currently no data available regarding the presence of vector-borne diseases (VBDs) in pet dogs and cats in Hong Kong SAR, which is surprising given the potential public health significance of these diseases and especially important due to the high density of dogs and cats per unit area, which looks to be very high. The current study addresses this knowledge gap, utilizing serological and molecular techniques to provide confirmation of the presence or absence of multiple vector-borne diseases infecting cats and dogs in Hong Kong SAR. VBDs that pose a particular risk of zoonotic spread were selected as targets for study (i.e. *B. burgdorferi, A. phagocytophilum, B. gibsoni* and *D. immitis*), with additional results presented to characterize the degree of coinfection with multiple tick-borne pathogens in the sample populations, as well as to determine risk factors associated with infection.

2. Materials and methods

2.1. Ethical approval

This study was conducted under ethical approval provided by the Institutional Research Ethics Committee of the City University of Hong Kong (internal reference number A-0672), and was conducted in accordance with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidance and relevant regulation.

2.2. Study design and samples collection

A total of 224 blood samples (159 from dogs and 65 from cats) were collected from animals at the City University Veterinary Medical center (CityU VMC) between December 2021 and April 2022. Animals were visiting the CityU VMC for a variety of reasons (including regular checkups, vaccinations, respiratory and cardiovascular problems), from locations across three regions of Hong Kong SAR, namely the New Territories (NT), Kowloon (KLN) and Hong Kong Island (HK). Samples were taken from animals by practicing veterinarians at CityU VMC, irrespective of presentation of clinical signs and symptoms. Of the 224 animals initially sampled, 18 were excluded from the study due to missing metadata (e.g. on geographic origin of the animals and their demographics). Of the remaining 206 samples, 80 were from animals in KLN, 100 from animals in NT, and 26 from animals in HK Island. Data was also obtained from each of these animals pertaining to their breed, age, neutered status, and any signs and symptoms of illness on visiting the CityU VMC.

2.3. Hematological analysis

Hematological parameters, i.e. hematocrit (HCT) and platelet counts, were assessed as soon as possible after the collection of blood samples in the EDTA vacutainer to avoid morphological alterations. Blood samples were vortexed for one to two minutes at the highest setting to minimize the clumping. Analysis was undertaken at the CityU VMC using an Auto Hematology Analyzer, MINDRAY, BC-2800 Vet (Shenzhen, China), following the manufacturer's instructions. Hematocrit was selected to determine anemic condition, and platelet counts were done to assess thrombocytopenia. On the basis of hematocrit and platelet counts, animals were divided into two groups; the first containing dogs and cats presenting within normal ranges (i.e. platelet

count: 175,000–500,000 and HCT% = 35–55) and the second containing individuals with lower levels of platelets and HCT.

2.4. SNAP 4Dx plus test

All blood samples were analyzed for four vector-borne diseases: anaplasmosis, ehrlichiosis, borreliosis and dirofilariasis, through use of Snap® 4Dx® Plus test kits (Westbrook, United States). The details for each assay are given in Supplementary Table S1 (RE et al., 2014).

A single analyte was used with the test kit that detected antibodies to a peptide from the MSP2/p44 major surface protein of two distinct *Anaplasma* sp.: *A. phagocytophilum* and *A. platys*, where the detection of *A. platys* was included in recognition of significant cross-reactivity (SNAP® 4Dx® Test kit insert, IDEXX Laboratories, Inc.) (B. B. Stillman et al., 2014). This kit was also used to detect antibodies of two *Ehrlichia* species, namely *E. canis* and *E. ewingii*, responding to the p30 and p30–1 proteins of the former, and the p28 protein of the latter (in one reaction so not species specific), species (IDEXX Laboratories, Inc.) (O'Connor et al., 2002; B. A. B.A. Stillman et al., 2014). Detection of antibodies against surface lipoproteins of *B. burgdorferi sensu stricto* (s.s.) was also undertaken with this kit, where the analyte utilized for the this assay was the C6 peptide (O'Connor et al., 2004). Lastly, *D. immitis* detection as part of this assay was derived from -heartworm antigen in canid serum, plasma, or whole blood.

2.5. DNA extraction

DNA was extracted from EDTA whole blood samples (using QIAgen kit, Hilden, Germany) according to the manufacturer's instructions. Positive control group samples for both species of *Anaplasma* (*A. phagocytophilum* and *A. platys*), both species of *Ehrlichia* (*E. canis* and *E. ewingii*), *B. burgdorferi, D. immitis*, two species of *Babesia* (*B. canis* and *B. gibsoni*) of all five VBDs (anaplasmosis, ehrlichiosis, borreliosis, babesiosis and dirofilariasis) were taken from the Veterinary Diagnostic Laboratory, City University of Hong Kong, and run along with other samples when they were tested for their respective pathogens. The concentration of extracted DNA was determined using a Nanodrop (Thermo Fisher, Waltham, USA). All the samples were analyzed through PCR using primers specific for *Anaplasma* sp., *Ehrlichia* sp., *D. immitis, Babesia* sp. and *Theileria* sp. (Table 1)

2.6. DNA amplification, and sequencing

All the amplified PCR products were purified through QIAquick Gel Extraction Kits (Hilden, Germany). The purified PCR products were sent to BGI Tech solutions (Hong Kong Co. Limited, 202 SAR China) for amplicon sequencing.

2.7. Phylogenetic analysis

Phylogenetic analyses were performed for the five targeted VBPs using Mega X software (Kumar et al., 2018; Tamura and Nei, 1993). 16S RNA representative sequences for *A. phagocytophilum, E. canis* and corresponding sequences available from GenBank were included. *E. canis* 16S rRNA gene partial sequences were included as an outgroup, where for *A. phagocytophilum* and *A. centrale* 16S rRNA gene partial sequences were included as outgroup for *E. canis*. 18S RNA representative sequence for *B. gibsoni*, and corresponding sequences available from GenBank, were included for tree construction, as were *Theileria lestoquardi* 18S rRNA gene partial sequences. Lastly, the cytochrome oxidase subunit 1 (COI) gene for *D. immitis*, and corresponding sequences available from GenBank, were also included, with *Toxocara canis* included as outgroup. Phylogenetic tree construction was inferred in MEGA X, using Neighbor-Joining with the P-distance method for 1000 bootstrap replications. Where trees are shown, only bootstrap values higher than 50% are included.

2.8. Statistical analysis

All the collected data were compiled in comma-separated values (csv) file and imported into R (open-source software version 4.2.1). After calculating the prevalence of all target VBPs (*Anaplasma* sp., *Ehrlichia* sp., *Borrelia* sp. and *D. immitis*) considered by the SNAP Kit used, risk factor association was explored through univariate analysis for each disease with the cut off value of $p < 0.05$ for significance All the statistical analysis was done by taking the seroprevalence through SNAP 4DX plus as a outcome variable and Kappa test was run to check its agreement with PCR.

3. Results

3.1. Sero-prevalence of VBPs in pet animals

We tested 159 dogs and 65 cats for the detection of *Anaplasma,*

Table 1
Pathogens targeted in blood samples from pet dogs and cats in Hong Kong SAR and the list of primers used in their detection.

Pathogen	Target gene	Primer	PCR conditions	Fragment length (bp)	References
Ehrlichia/ Anaplasma spp.	16S rRNA	Forward Ehr-F2 (5′-AGA GTT TGA TCC TGG CTC AG-3′) Reverse: Ehr-R2 (5′-biotin-GAG TTT GCC GGG ACT TYT TCT-3′)	Initial denaturation: 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 7 min.	450-500	(Rehman et al., 2019)
Borrelia spp.	16S rRNA	Forward: B16S_FL, 5′-GAC TCG TCA AGA CTG ACG CTA AGT C-3′, Reverse: r B16S_R, 5′–GCA CAC TTA ACA CGT TAG CTT CGG TAC TAAC-3′	Initial denaturation: 95 °C for 2 min, followed by 95 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s in 40 cycles, and final extension at 72 °C for 7 min	131	(Wilhelmsson et al., 2010)
Babesia spp.	18S rRNA	Forward RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′), Reverse: RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′)	Initial denaturation: 95 °C for 3 min followed by 32 cycles of denaturation at 94 °C for 1 min, -, annealing at 54 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min	460–540	(Hailemariam et al., 2017, 2017)
Dirofilaria immitis	Cytochrome c oxidase subunit-1 (Cox-1)	Forward: DI-COI-F1 (5′-AGTGTAGAGGGTCAGCCTGAGTTA-3′ Reverse: DI-COI-R1 (5′-ACAGGCACTGACAATACCAAT-3′)	Initial denaturation: 94 °C for 2 min, 32 cycles of denaturing 94 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min	203	(Torres-Chable et al., 2018)

Dirofilaria, Borrelia and *Ehrlichia* through SNAP kits. No samples from cats ($n = 65$) were positive for any of the targeted VBPs and no samples were found to be positive for *B. burgdorferi* in dogs. Other VBPs were, however, detected in dogs, where the prevalence of *Anaplasma* was 2.5% (4/159), *Ehrlichia* 10.7% (17/159) and *D. immitis* 5.7% (9/159) (Table 2).

3.2. Coinfections

Coinfection with *Dirofilaria immitis* and *E. canis*- was detected in one dog, and a further four dogs were found to be coinfected with *A. phagocytophilum* and *E. canis*.

3.3. Molecular prevalence of VBPs in pet animals

Molecular testing of blood samples was performed using PCR, which detected the prevalence of *A. phagocytophilum* at 1.2% (2/159), *D. immitis* at 5.7% (9/159), *Ehrlichia* sp. (*E. canis*-) at 3.1% (5/159) and found no *B. burgdorferi* to be present (above the detection level). All screened positive samples were also tested for *Babesia*, where a prevalence of 3.7% (6/159) was recorded (Table 3).

In case of PCR, two positive samples of *A. phagocytophilum* were among the four positive samples of *A. phagocytophilum/A. platys* tested through SNAP 4DX, similarly five PCR positive samples of *E. canis* were among the 17 SNAP 4DX positive samples of *E. canis/E. ewingii*. All the samples of *D. immitis* that were positive in SNAP 4DX were also found positive through PCR as well. Moreover, we run the Kappa test to check the agreement between SNAP and PCR which turn out 0.82 showing almost perfect agreement (McHugh, 2012).

3.3.1. Anaplasma phagocytophilum

DNA of *A. phagocytophilum* was detected using PCR in blood from dogs collected in Hong Kong SAR, with the corresponding sequences submitted in NCBI GenBank with assigned accession numbers OP236686 and OP236687. The sequence result was 100% identical to *A. phagocytophilum* detected in dogs' blood from the USA (NR_044762), 99.61% dog's blood from Germany (JX173651) 99.6% match to *Canis lupus familiaris* blood from Japan (LC435049) 99.9% similar to samples taken from dog ticks in Iran (MN795150), and 99.44% similar to samples taken from *Ixodes ovatus* tick from Japan (AY969012) (Supplementary Table S2). A representative sequence that was deposited in GenBank with accession numbers (OP236686 and OP236687), phylogenetic tree (Fig. 1) was constructed using MEGA X (Kumar et al., 2018) that was showing high similarities. When constructing a phylogenic tree, the *A. phagocytophilum* DNA sequence from the present study formed a well-defined branch, which was supported by a significant bootstrap value. Neighbor-Joining demonstrated that this sequence was 100% identical to others from Japan (AY969010), the USA (NR044762), and Germany (JX173651). The bootstrap values are indicated at each node in Fig. 1, where an outgroup has also been shown.

3.3.2. Ehrlichia canis

The sequences of *E. canis* DNA detected in samples were submitted to NCBI GenBank and the accession numbers OP236551, OP236552, OP236553, OP236554, OP236555 were assigned. These were 100% identical to *E. canis* DNA detected in dogs' blood from Turkey (KJ513194), dogs' blood Thailand (EF139458), *Canis familiaris* blood Italy (EU439944), Canis *lupus familiaris* -blood (MK507008), and dogs' blood India (JX861392), tick *R. microplus* China (KX987326), dog with spontaneous acute (non-myelosuppressive) monocytic ehrlichiosis Greece (EF011110), (Supplementary Table S3). The best sequences, with the accession numbers OP236551, OP236553 and OP236555, were included in the present study, and formed a well-defined branch when constructing a phylogenic tree, which was supported by a significant bootstrap value. This phylogenetic tree (Fig. 2) was again constructed using MEGA X [35] Neighbor-Joining found The sequence was 100% identical to sequences obtained in China (KX987326), Turkey (KY594915), Cuba (MK507008), and Greece (EF011110). The bootstrap values are indicated at each node in Fig. 2, where an outgroup has also been shown.

Table 2
Seroprevalence of vector-borne pathogens in pet dogs in Hong Kong SAR.

Pathogens	Positive/ Tested	Prevalence %	95% CI	*p*-value
Anaplasma phagocytophilum/A. platys	4/159	2.5	0.8–6.7	<0.001
Ehrlichia canis/E. ewingii	17/159	10.7	6.5–16.8	<0.001
Borrelia burgdorferi	0	0	0	0
Dirofilaria immitis	9/159	5.7	2.8–10.9	<0.001

Table 3
Molecular confirmation of vector-borne pathogens in pet dogs in Hong Kong SAR.

Pathogens	Positive/ Tested	Prevalence %	95% CI	*p*-value
Anaplasma phagocytophilum	2/159	1.2	0.20–4.90	0.012
Ehrlichia canis	5/159	3.1	1.16–7.57	0.031
Borrelia burgdorferi	0/159	0	0	0
Dirofilaria immitis	9/159	5.6	2.78–10.79	0.056
Babesia gibsoni	6/159	3.7	1.54–8.39	0.037

3.3.3. Babesia gibsoni

DNA of *B. gibsoni* was detected from dog blood samples collected in Hong Kong SAR, and the sequence for *B. gibsoni* was submitted to NCBI GenBank with the accession numbers OP231200, OP231201, OP231202, OP231203, OP231204 assigned. The sequence result submitted was 100% identical to *B. gibsoni* detected in dogs' blood from India (MH718810) and *R. annulatus* engorged on cattle in India (OM462365), and a 99.47% match to dogs' blood from Austria (MZ293793), Japan (LC012799 andLC602469), China (MW276038) and the West Indies JX112784 (Supplementary Table S4). The sequences identified in the present study formed a well-defined branch of the phylogenetic tree (Fig. 3) constructed using MEGA X [35], as supported by a significant bootstrap value. Neighbor-Joining showed that this sequence was 100% identical to others from India (OM462365), Sri Lanka (MN988993) and Austria (MZ293793). The bootstrap values are indicated at each node in Fig. 3 and an outgroup has also been shown.

3.3.4. Dirofilaria immitis

The sequence of *D. immitis* DNA detected in the blood of dogs from Hong was submitted to NCBI GenBank and was assigned the accession numbers OP218723, OP218724, OP218725, OP218726, OP218727, OP218728 and OP218729. The sequence submitted was 100% identical to *D. immitis* detected in dog's blood in France (MT193088), Romania (MN650648) and Thailand (MK250760) (Supplementary Table S5). The sequences with the accession numbers OP218723, OP218724, OP218725, OP218726, OP218727, OP218728, OP218729 formed a well-defined branch, supported by a high bootstrap value, and the phylogenetic tree (Fig. 4) constructed using MEGA X [35] Neighbor-Joining found this sequence to be 100% identical to sequences from dog blood from Iraq (MZ619056), South Korea (KF918394), Portugal (MW246129), France (MT230086), the USA (MN945948) and Italy (AM749229). The bootstrap values are indicated at each node in Fig. 4, where an outgroup has also been shown.

4. Risk factor assessment

Univariate analysis was performed to explore measures of association between risk factors derived from available animal histories and their (SNAP) seropositivity for VBPs. We divided the dogs into three

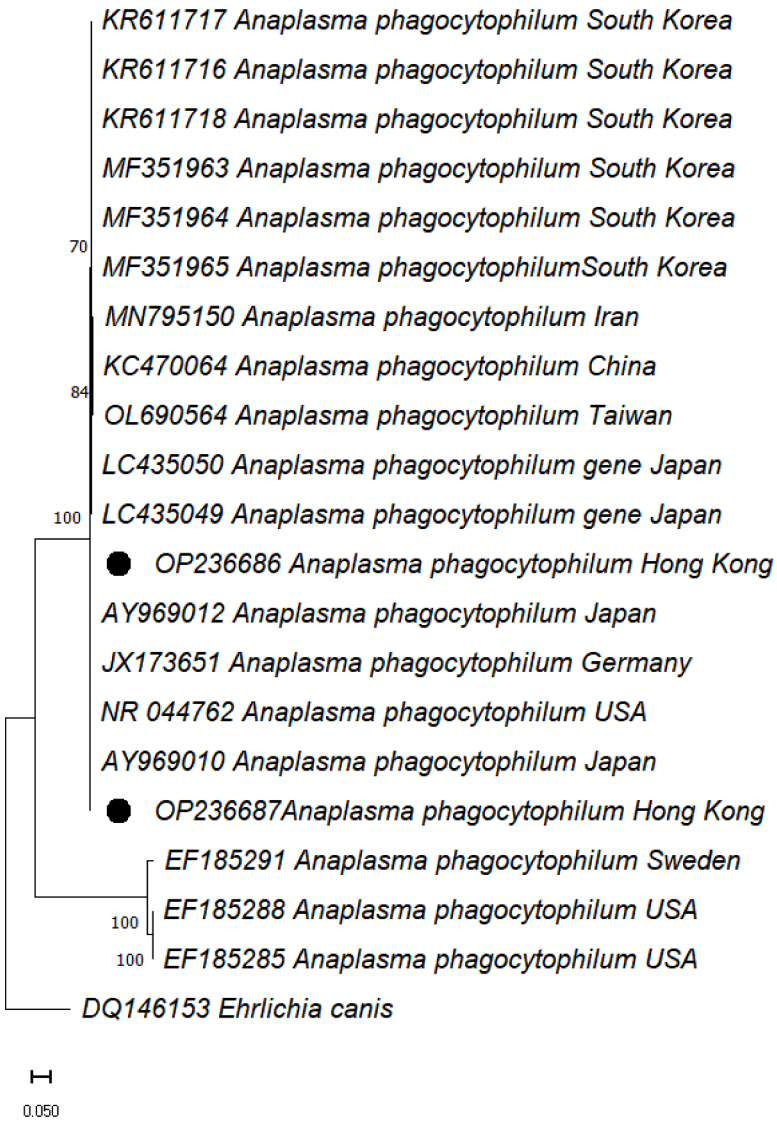

Fig. 1. 16S rRNA based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Anaplasma phagocytophilum* collected from dogs in the present study (black circles) related to other similar representative studies available in the NCBI GenBank. The partial sequence of 16S rRNA gene was aligned and the phylogenetic tree was inferred in MEGA X using Neighbor-Joining with the P-distance method with 1000 bootstrap replications. Only bootstrap values higher than 50% are shown. *Ehrlichia canis* 16S rRNA gene from USA partial sequences was included as outgroup. The scale bar is shown.

groups: puppies (1–5 months), juveniles (6–11 months), adults (11 months or above) (Harvey, 2021).

4.1. Anaplasma

There was neither a significant association between the sex (OR=3.54; 95%CI = 0.44–72.72, $p = 0.510$) and seropositivity of *Anaplasma* in dogs from Hong Kong SAR, nor between their neutered status and seropositivity (OR=2.12; 95%CI=0.10–17.51, $p = 1.000$). Low levels of hematocrit (HCT%) (OR=2.13; 95%CI=0.10–17.52, $p = 1.000$) were similarly not significantly associated with SNAP seropositivity either, and there was no association between dog age (OR = 1.13; 95%CI =0.16–2.75, $p = 0.362$) and SNAP seropositivity, where the geographical distribution of cases in different areas of Hong Kong SAR (OR=0.74; 95%CI= 0.07–16.5, $p = 0.475$) was not significant either (Table 4).

4.2. Ehrlichia canis, E. ewingii

There was no significant association between the sex (OR=1.13; 95% CI=0.48–3.73, $p = 0.762$) or age (OR=0.98; 95%CI=0.29–3.88, $p = 0.726$) of dogs and SNAP seropositivity of *Ehrlichia* sp., and the neutered status of animals was not associated with seropositivity either (OR=2.10; 95%CI=0.55–6.78, $p = 0.391$). A decreased level of hematocrit (HCT%) (OR=0.54; 95%CI=0.19–1.58, p-<0.001) was significantly associated with SNAP seropositivity, however, with a significant effect of geographic location also recorded and dogs being more likely to test positive in Kowloon, Hong Kong SAR (OR=6.17; 95% CI=1.10–16.23, p<0.001) (Table 5).

4.3. Dirofilaria immitis

There was no significant association between the sex of dogs (OR = 2.4; 95%CI = 0.61–11.76, $p = 0.367$) and SNAP seropositivity of *D. immitis*, and their neutered status was not associated with seropositivity either (OR = 0.7; 95%CI = 0.004–4.50, $p = 1.000$). Decreased hematocrit (HCT) (OR = 23.5; 95%CI= 4.13–44.46, p< 0.001) was significantly associated with SNAP seropositivity. There was also no association between the age of dogs (OR=2.7; 95%CI= 0.18–4.98, $p = 0.252$) and SNAP seropositivity, which was also unaffected by geographical distribution of cases in the different areas of Hong Kong SAR sampled (OR=2.2; 95%CI=1.04–4.38, $p = 0.093$) (Table 6).

5. Discussion

Prompt and accurate detection of VBPs is paramount to ensure the health and welfare of companion animals such as dogs and cats, and to

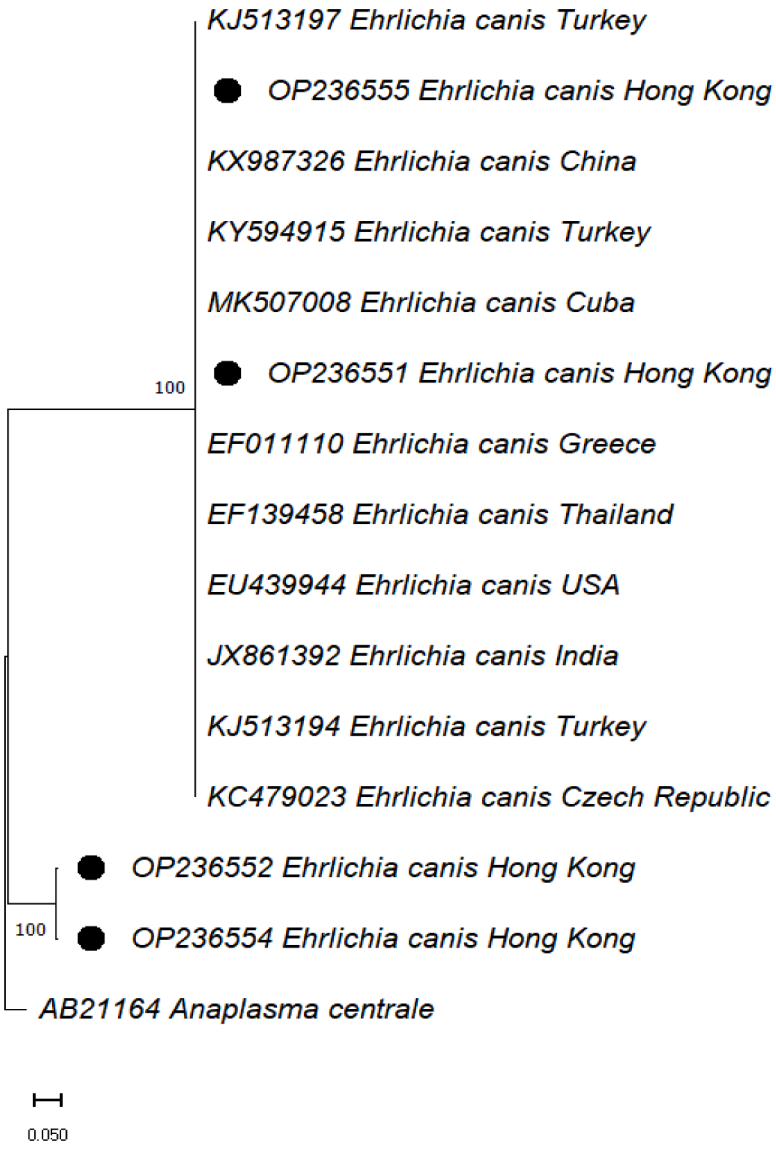

Fig. 2. 16S rRNA based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Ehrlichia canis* collected from dogs in the present study (black circles) related to other similar representative studies available in the NCBI GenBank. The partial sequence of 16S rRNA gene was aligned and the phylogenetic tree was inferred in MEGA X using Neighbor-Joining with the P-distance method with 1000 bootstrap replications. Only bootstrap values higher than 50% are shown. *Anaplasma centrale* 16S rRNA (Pakistan) gene partial sequences was included as outgroup. The scale bar is shown.

reduce potential problems associated with their empirical treatment. In particular, early detection of these pathogens is crucial to curtailing their spread, and subsequently reducing risk of further exposure and possible outbreaks, both in conspecifics and humans (Cunningham et al., 2017; Hussain et al., 2021). This is the first study conducted in pet dogs and cats to determine seropositivity through commercially-available SNAP testing kits, although a similar study, sampling only dogs, was conducted in 2011 (Wong et al., 2011). SNAP positive cases were further confirmed through PCR in the current study, providing strong evidence of several significant VBPs circulating in Hong Kong SAR. This study provides baseline data to inform improved pet management practices for the prevention, identification and control of these VBPs.

The samples used in the present study was obtained from the Veterinary Medical center, City University of Hong Kong SAR, to determine the five most common VBPs circulating in dogs and cats in Hong Kong SAR. Based on SNAP testing, the highest value of circulating antibodies was for *E. canis/E. ewingii* (present in 10.6% of dogs), followed by antigen for *D. immitis* (5.6%) and antibodies for *A. phagocytophilum/A. platys* (2.5%). PCR confirmed the highest prevalence value was for *D. immitis* (5.6%), followed by *E. canis* (3.1%), and *A. phagocytophilum* (1.2%.). In addition, we also performed PCR testing for *B. gibsoni*, which found this VBP to have a prevalence of 3.7% in dogs in Hong Kong SAR.

Our study indicated that the prevalence of *A. phagocytophilum* varied according to the diagnostic approach used, being 2.5% when a serology test was employed and 1.2% through PCR. PCR was conducted for the confirmation of species and cross reactivity that can happen between *A. platys* and *A phagocytophilum.* A similar study conducted in Hong Kong SAR in 2011, reported the prevalence of anaplasmosis in both stray and pet dogs, finding this to be 8% and 0%, respectively (Wong et al., 2011). These results combined indicate that anaplasmosis in stray dogs might be higher than in their domestic counterparts, with the former potentially presenting an infection risk for the latter, particularly in areas such as Hong Kong SAR where the density of dogs *per se* per unit area is high, encouraging interaction between animals. Nevertheless, prevalence of anaplasmosis was still relatively low in the currently study, which may be commensurate with the wider geographic region. Figures from neighboring Shenzhen, for example, support 0.50% (Xia et al., 2012) prevalence of this pathogen in dogs, but with higher rates reported elsewhere in countries including the USA (5.5–11.6%) (Bowman et al., 2009) and Italy (8.7%) (Ebani et al., 2008). Regardless, the presence of *A. phagocytophilum* in Hong Kong SAR is supported by the current study, and is likely being facilitated by the local climate and presence of haematophagous arthropods. The phylogenetic profile of *A. phagocytophilum* in Hong Kong SAR was inferred in the current study by multi-locus analysis, using 16S rRNA as a phylogenetic marker. Molecular phylogeny revealed that the *A. phagocytophilum* detected in

Fig. 3. 18S rRNA based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *Babesia gibsoni* collected from dogs in the present study (black circles) related to other similar representative studies available in the NCBI GenBank. The partial sequences of 18S rRNA gene was aligned and the phylogenetic tree was inferred in MEGA X using Neighbor-Joining with the P-distance method with 1000 bootstrap replications. Only bootstrap values higher than 50% are shown. *Theileria lestoquardi* (Scotland) 18S rRNA gene partial sequences was included as outgroup. The scale bar is shown.

the current study were genetically consistent with samples obtained in Japan (AY969010), the USA (NR04462) and Germany (JX173651).

There was no significant association between age or sex of dogs and seropositivity for *A. phagocytophilum*, in agreement with a similar study conducted in Germany which found the same to be true (Jensen et al., 2007). Additionally, no significant association between hematocrit level and seropositivity of *A. phagocytophilum* was found in the current study, supporting work conducted elsewhere in 2010 which found no significant difference in hematocrit levels between infected and non-infected dogs (Gaunt et al., 2010)

Studies conducted in different countries around the world have reported prevalence of *E. canis* in dogs that diverge from the 10.7% recorded in the current study from Hong Kong SAR. In nearby Korea, for example, a prevalence of 1.4% was observed in canines using SNAP testing (Miranda et al., 2022), though this might be explained by this study sampling from a pool of randomly selected dogs, rather than dogs that were visiting veterinary facilities (and thus may have been more likely to be carrying VBPs. In a study adopting a similar sampling approach to our own, 11.2% *E. canis* prevalence was recorded, this being more comparable to the results of the current work, despite being conducted further afield in Egypt (Selim et al., 2021). In Brazil, *E. canis* prevalences may be much higher in dogs 59.1% (Paula et al., 2021), where this may reflect generally neglected tick control practices in the sample areas. A study conducted in Thailand also reported notably high prevalence of *E. canis* in dogs (32.5%), as detected through PCR, though elevated figures here can be at least partially explained by samples being taken exclusively from dogs displaying hematological abnormalities (Mitpasa et al., 2022). Another molecular-based study conducted in Pakistan reported 28% prevalence of *E. canis* in dogs, though as all dogs in this study were infested with ticks a relatively higher probably of them harboring VBPs could have been expected (Malik et al., 2018). A similar study, conducted by Wong et al. in 2011, reported lowered presence of *E. canis* in both stray (8%) and pet (6%) dogs in Hong Kong SAR (Wong et al., 2011). Our study reported still lower prevalence (3.1%) in pet dogs in Hong Kong SAR, which may be explained by improvements in VBP control in this country over the last decade leading to a decrease in disease prevalence.

Ehrlichia canis is the only species found in Hong Kong SAR, presumably due to the absence of the tick *A. americanum*, which is the vector for *E. ewingii* and *E. chaffeensis*. In the current study multi-locus analysis, using 16S rRNA as a phylogenetic marker, revealed that our findings are consistent with, and support, other molecular studies

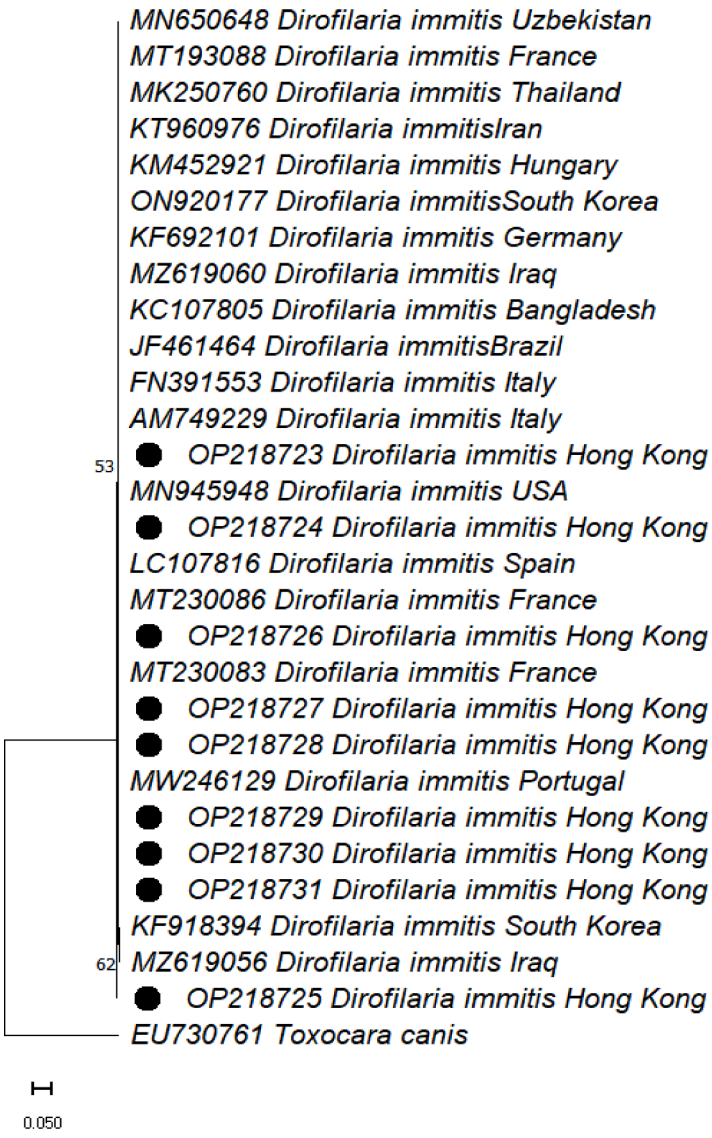

Fig. 4. Cytochrome oxidase subunit 1 (COI) gene based phylogenetic analysis of genotypes identified in this study. Phylogenetic tree highlighting the position of *D. immitis* collected from dogs in the present study (black circles) related to other similar representative studies available in the NCBI GenBank. The partial sequence of cytochrome oxidase subunit 1 (COI) gene was aligned and the phylogenetic tree was inferred in MEGA X using Neighbor-Joining with the P-distance method with 1000 bootstrap replications. Only bootstraps values higher than 50% are shown. *Toxocara canis* (Australia) was included as outgroup. The scale bar is shown.

Table 4
Risk factor analysis of *Anaplasma phagocytophilum / Anaplasma platys* in pet dogs in Hong Kong SAR.

Variables	Categories	Positive/Tested	Prevalence% (95% CI)	Odds Ratio	95% CI	*p*-value
Sex	Male	3/74	4.05 (1.05–12.18)	3.54	0.44–72.62	0.510
	Female	1/85	1.18 (0.06–7.29)	Ref.		
Neutered status	Yes	3/137	2.10 (0.56–6.75	Ref.	0.10–17.51	1.000
	No	1/22	4.54 (0.23–24.88)	2.12		
Hematocrit (HCT%)	Normal	2/113	1.77 (0.30–6.87)	Ref.	0.10–17.52	1.000
	Low	2/46	4.34 (0.75–16.03)	2.13		
Age (Months)	1–5	1/33	3.03(0.15–17.51)	Ref		0.362
	6–10	3/75	4.00(1.03–12.02)	1.33	0.16–2.75	
	11 and above	0/51	0.00(0.00–0.00)	0.00		
					0.00–2.49	
Area in Hong Kong SAR	Kowloon	2/53	3.77(0.65–14.08)	0.74	0.07–16.5	0.475
	New Territories	1/86	1.16(0.06–7.21)	0.23	0.00–5.82	
	Hong Kong Island	1/20	5.00(0.26–26.94)	Ref		

reported in China, Turkey, Cuba and Greece. Results elsewhere have also shown that the sex and age of dogs are not associated with seropositivity for *E. canis* (Abd Rani et al., 2011; Aktas, 2014; Malik et al., 2018; Miranda et al., 2022), which aligns with the risk factor analysis of *E. canis* undertaken in the present work. A correlation appeared to be present between reduced HCT and seropositivity, which is also in agreement with other research, where dogs with low hematocrit levels tested positive for *E. canis* (Gaunt et al., 2010; Rawangchue and Sungpradit, 2020). But the number of positive samples are too small to make the results valid. The present study also indicated that those domestic dogs that were living in the Kowloon area of Hog Kong are six times more likely to contract *E. canis* when compared to those in the New Territory and Hong Kong Island. The larger size and density of dog population Kowloon might be the reason for this.

Table 5
Risk factor analysis of *Ehrlichia canis / E. ewingii* in pet dogs in Hong Kong SAR.

Variables	Categories	Positive/Tested	Prevalence% (95% CI)	Odds Ratio	95% CI	*p*-value
Sex	Male	9/74	12.2 (6.05–22.32)	1.33	0.48–3.73	0.762
	Female	8/85	9.4 (4.44–18.20)	Ref.		
Neutered status	Yes	13/137	9.5 (5.35–15.99)	2.10	0.55–6.78	0.391
	No	4/22	18.1 (5.99–41.00)			
Hematocrit (HCT%)	Normal	1/113	0.9 (0.04–5.54)	0.54	0.19–1.58	<0.001
	Low	8/46	17.3 (8.32 −31.95)			
Age (Months)	1–5	4/33	12.1 (3.96–29.14)	Ref		0.726
	6–10	9/75	12.0 (5.97–22.04)	0.98	0.29–3.88	
	11 and above	4/51	7.8 (2.54–19.74)	0.61		
					0.13–2.79	
Area in Hong Kong SAR	Kowloon	13/53	24.5 (14.19–38.57)	6.17	1.10–16.23	0.000
	New Territories	3/86	3.5 (0.90–10.56)	0.68	0.08–14.29	
	Hong Kong Island	1/20	5.0 (0.26–26.94)	Ref		

Table 6
Risk factor analysis of *Dirofilaria immitis* in pet dogs in Hong Kong SAR.

Variables	Categories	Positive/Tested	Prevalence% (95% CI)	Odds Ratio	95% CI	*p*-value
Sex	Male	6/74	8.1 (3.33–17.42)	2.4	0.61–11.76	0.367
	Female	5/85	3.5 (0.91–10.68)	Ref.		
Neutered status	Yes	8/137	5.8 (2.74–11.56	Ref.	0.04–4.50	1
	No	1/22	4.5 (0.23–24.89)	0.7		
Hematocrit (HCT%)	Normal	10/113	8.8 (6.51–19.21)	Ref.	4.13–44.46	<0.001
	Low	7/46	15.2 (6.83 −29.48)	23.5		
Age (Months)	1–5	0/33	0.0 (0.00–0.00)	Ref		0.252
	6–10	6/75	8.0 (3.29–17.20)	2.7	0.18–4.98	
	11 and above	3/51	5.9 (1.53- 17.22)	1.9		
					0.58–3.34	
Area in Hong Kong SAR	Kowloon	1/53	1.9 (0.09–11.3)	2.2	1.04–4.38	0.093
	New Territories	8/86	9.3 (2.86–15.13)	1.1	0.55–3.97	
	Hong Kong Island	0/20	0.0 (0.00–0.00)	Ref		

Globally, the prevalence of *D. immitis* is highly variable, ranging between 0.2% to over 50% in different countries (Lee et al., 2010). The prevalence of *D. immitis* in domestic dogs in Hong Kong SAR was 5.7%, with this low-moderate figure potentially explained by circulating mosquito vector presence, and closely matching prevalence levels of 5.5% reported from dogs in Albania (Anvari et al., 2020). A similar study conducted by Wong *et al.* in Hong Kong SAR in 2011 reported that *D. immitis* was found in 12 dogs (10 strays and 2 pets) (Wong et al., 2011). This translates to a lower prevalence than observed in the current study, which might be explained by increased mosquito populations in Hong Kong SAR.

The accurate diagnosis of canine heartworm is imperative, as this condition can cause severe cardiopulmonary disease. Diagnosis of this dirofilariasis can be undertaken through blood smear examination (modified Knott's method), or through antigen detection which can be done using SNAP kits or PCR. The current study found that all dogs that were positive on SNAP were also positive on PCR, supporting the accuracy of SNAP testing to detect heartworm infestation. –

The phylogenetic profile of *D. immitis* was inferred by multi-locus analysis, using the cytochrome oxidase subunit 1 (COI) gene as a phylogenetic marker. This molecular phylogeny revealed that our findings are consistent with and support other studies reported in Iraq (MZ619056), South Korea (KF918394), France (MZ230083), Spain (LC107816) and Italy (AM749229). The present study also reported that low levels of hematocrit in dogs was significantly associated with *D. immitis*. This aligns with published work considering HCT and dirofilariasis (Volgina et al., 2013), though contrasts with the results of some studies (Kryvoruchenko, 2022).

Babesia gibsoni has been reported in numerous East Asian countries, including Japan, India, Bangladesh, Korea, Taiwan and Malaysia (Bora et al., 2022; Inokuma et al., 2004; Lee et al., 2009; Mandal et al., 2014; Miyama et al., 2005; Mokhtar et al., 2013; Terao et al., 2015). The presence of *B. gibsoni* was also confirmed in Hong Kong SAR by the present study, with a prevalence in pet dogs of 3.77%. Higher prevalence (10.5%) of *B. gibsoni* has been reported in United Kingdom, though figures may have been exacerbated by only sampling ticks in the study, which are known as major vectors of *B. gibsoni*. Prevalences that are higher still have been recorded in India (28.6%) through nested-PCR testing, potentially as a result of large populations of street dogs being present in close proximity to their domestic counterparts (Singh et al., 2016). Higher figures for *B. gibsoni* prevalence have also been reported in Hong Kong SAR by Wong in 2011, these being 48% in stray dogs and 33% in pet dogs (Wong et al., 2011). The much lower prevalence seen in the current work (3.8%) might be due to decreasing tick pressure, and improved disease control and prophylaxis, leading to disease prevalence declines.

The phylogenetic profile of *B. gibsoni*, inferred by multi-locus analysis using 18S rRNA gene as a phylogenetic marker, revealed that our findings are consistent with and support other studies reported in China (HG328235), India (OM462365) and Sri Lanka (MN988993). These studies reported and characterized the same pathogen, using the same the 18S rRNA gene as a phylogenetic marker.

In the present study, samples were obtained from dogs and cats that visited veterinary hospitals in Hong Kong SAR for various reasons (including conditions related to respiratory, gastric, cardiac and nervous distress, lymphoma, pyometra, toxicity and seizures). This approach to sample acquisition could be expected to introduce bias, increasing the chances of study animals having disease by only selecting from those already receiving veterinary attention. Consequently, to ascertain VBD prevalence in the wider population further study on a more representative sample population could be recommended. Inclusion of both stray and pet dogs could also be worthwhile here, as undertaken by Wong *et al.* where 100 individuals of each were considered (Wong et al., 2011). This could be especially important given that, despite no clinical signs being seen in either, the highest rates of infection were seen in stray animals in Wong *et al.*'s work (although relatively high infection rates were also reported in pet dogs in this study). Despite potential sampling bias, however, prevalence in the current study was lower than in Wong

et al.'s work. It can be speculated that over the decade that separates these studies, improved awareness and treatment of VBDs in companion animals has led to a general decline in their prevalence in Hong Kong SAR.

6. Conclusions

This is the first study exploring the seroprevalence and molecular confirmation of multiple VBDs in pet dogs and cats of Hong Kong SAR. As such, this study provides baseline data on these diseases and offers valuable insight into the major contributing risk factors for these VBDs in domestic pet dogs (where no VBPs were detected in cats in the current study). Based on these findings, it can be recommended that proper screening of pet dogs should be undertaken to evaluate the cause of any disease symptoms, where early detection of pathogens is crucial in curtailing their spread, and subsequently in reducing risk of exposure and potential outbreaks in conspecifics or humans. This, and other measures, should reduce the burden of these VBDs in the pet dog population in Hong Kong SAR. Appropriate vaccinations, timely check-ups, follow-ups and good tick management practice, achieved through acaricide use or other means, may also play a vital role. Implementation of good hygienic practices *per se* (such as timely tick removal) may further reduce these VBPs and their spread, thus minimizing the risk of VBDs occurring in pet dogs of Hong Kong SAR.

Author contributions

S.H. conceived the study, gathered and entered data, performed the statistical analysis and drafted the initial version of the manuscript. S.H., O.S. and A.H. revised the initial manuscript. FMYH., U.A., J.Z., B.S., A. R., A.A., and O.S. provided intellectual inputs, O.S., A.R., D.G., and A.C. C. critically revised the manuscript to create the final version. All authors read and approved the final manuscript.

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Informed Consent Statement: Not applicable.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102191.

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