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Comparative Evaluation of a Point-of-Care Immunochromatographic Test SNAP 4Dx with Molecular Detection Tests for Vector-Borne Canine Pathogens in Hong Kong

Samson S.Y. Wong,[1,2,*] Jade L.L. Teng,[1,2,*] Rosana W.S. Poon,[1,2] Garnet K.Y. Choi,[1,2]
Kwok-Hung Chan,[1,2] Michelle L. Yeung,[3] Janet J.Y. Hui,[4] and Kwok-Yung Yuen[1,2]

Abstract

There are no comprehensive studies on the performance of commonly used point-of-care diagnostic enzyme immunoassay for common arthropod-borne canine pathogens. A comparative evaluation of an immunochromatographic test for these infections with a comprehensive polymerase chain reaction (PCR) test panel was performed on 100 pet dogs and 100 stray dogs without obvious clinical symptoms. Of the 162 positive test results from both immunochromatographic test and PCR, there was 85.2% concordance. The 24 discordant results between serology and PCR occurred in tests involving *Ehrlichia canis* (14) and *Anaplasma platys* (10), which may be related to the time of infection. No positive cases of borreliosis or rickettsiosis were detected. One important limitation of the immunochromatographic test was its lack of testing for babesiosis and hepatozoonosis. The former is the most prevalent arthropod-borne canine infection in our cohort (41%). Coinfections were found in 19% stray dogs and 6% of pet dogs with both tests ($p < 0.01$). Seventeen and 8 samples from stray and pet dogs, respectively, were initially positive in the PCR test for *Ehrlichia*. However, on sequencing of the PCR amplicon, 10 from stray and 2 from pet dogs were found to be *Wolbachia* sequences instead, with 100% nucleotide identity to the 16S rRNA sequence of *Wolbachia* endosymbiont of *Dirofilaria immitis*. The presence of *Wolbachia* DNAemia (6%) correlated well with the molecular test and immunochromatographic antigen test for *D. immitis*.

Key Words: *Anaplasma platys—Babesia—Dirofilaria immitis—Ehrlichia—Wolbachia.*

Introduction

Ehrlichiosis, anaplasmosis, borreliosis, and dirofilariasis are important arthropod-borne canine infections that are often diagnosed on the basis of the results from point-of-care testing at the veterinary practice. Immunochromatographic assays such as SNAP 3Dx and 4Dx (IDEXX Laboratories, Westbrook, ME) are commonly used for rapid diagnostic purposes. Most of the tests detect antibodies that may indicate past exposure instead of active infection. Only the test for *Dirofilaria immitis* detects specific parasitic circulating antigen. There are, however, few comparative evaluations on the performance of these assays against other diagnostic techniques. Previous studies on zoonotic pathogens suggested that nucleic acid amplification tests are both sensitive and specific for active infection (Littman 2003, Lau et al. 2005, 2008, 2010, Woo et al. 2009, Ayoob et al. 2010, Gioia et al. 2010, Irwin 2010, Little 2010, Veir and Lappin 2010). Although some pathogens may not cause symptomatic disease, accurate diagnosis is still important, as the infected animals may serve as transport hosts for dissemination of the pathogens or infected ectoparasitic vectors. The primary aim

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[1] State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, Hong Kong.
[2] Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong.
[3] Agriculture, Fisheries and Conservation Department, Hong Kong Special Administrative Region, Hong Kong.
[4] PathLab Medical Laboratories Ltd., Hong Kong.

These two authors contributed equally to this work and are co-first authors.

Table 1. Sequences of Primers Used in the Study

Pathogen	*Gene*	*Sequence*	*Amplicon size (bp)*	*Reference*
Ehrlichia spp.	16S rRNA	Forward 5′ GTTAGTGGCAGACGGGTGA Reverse 5′ TCATCTAATAGCGATAAATC	120	
Ehrlichia canis	16S rRNA	Forward 5′ CAATTATTTATAGCCTCTGGCTATAGGA Reverse 5′ TATAGGTACCGTCATTATCTTCCCTAT	400	Murphy et al. (1998)
Babesia spp.	18S rRNA	Forward 5′ GTCTTGTAATTGGAATGATGG Reverse 5′ CCAAAGACTTTGATTTCTCTC	560	Beck et al. (2009)
Babesia canis	18S rRNA	Forward 5′ GTTTATTAGTTTGAAACCCGC Reverse 5′ GAACTCGAAAAAGCCAAACGA	650	Inokuma et al. (2004)
Babesia gibsoni	18S rRNA	Forward 5′ CTCGGCTACTTGCCTTGTC Reverse 5′ GCCGAAACTGAAATAACGGC	650	Inokuma et al. (2004)
Hepatozoon spp.	18S rRNA	Forward 5′ AAGGCAGTTAAAATGCT Reverse 5′ AAGCAGACCGGTTACTTT	183	
Anaplasma phagocytophilum	*msp2* (outer membrane protein-2)	Forward 5′ CCGGCGCATGTGTAAGGTGAAA Reverse 5′ GGTTACATAAGGGCCGCAAAGGTG	334	Lin et al. (2004), Massung and Slater (2003), M′Ghirbi et al. (2009)
Anaplasma platys	*groEL*	Forward 5′ AGCGTAGTCCGATTCTCCAGTTTT Reverse 5′ TCGCCGTTAGCAGAGATGGTAG	516	Beall et al. (2008)
Borrelia burgdorferi sensu lato	*ospA* (outer surface protein A)	Forward 5′ AATAGGTCTAATAATAGCCTTAATAGC Reverse 5′ CTAGTGTTTTGCCATCTTCTTTGAAAA	308	Demaerschalck et al. (1995), Shaw et al. (2005)
Spotted fever group rickettsiae	*gltA* (rickettsial citrate synthase)	Forward 5′ GGGGGCCTGCTCACGGCGG Reverse 5′ ATTGCAAAAAGTACAGTGAACA	382	Regnery et al. (1991), Satoh et al. (2002)
	rompA (SFG rickettsial 190-kDa surface antigen)	Forward 5′ ATGGCGAATATTTCTCCAAAA Reverse 5′ AGTGCAGCATTCGCTCCCCCT	533	Regnery et al. (1991), Satoh et al. (2002)
Orientia tsutsugamushi	*groEL*	Forward 5′ AGATGATTACCGAATTTGAAAAT Reverse 5′ AACTCTATTTGCAGTTCCTA	344	
Wolbachia spp.	16S rRNA	Forward 5′ AAACGGCAGCTAATACCGTATA Reverse 5′ACGCTAGCCCTCTCTGTATTA	353	
Dirofilaria immitis	ITS (internal transcribed spacer)	Forward 5′ATGATGATTGCTCAATTAAGTAGAC Reverse 5′ GATAATCTGATCGATATTGACCCT	290	Thanchomnang et al. (2010)

of our study is to evaluate the performance of the SNAP 4Dx kit against a panel of newly developed rapid molecular diagnostic tests for important canine vector-borne infections in Hong Kong. The secondary aim of our study is to determine the prevalence of these zoonotic infections in Hong Kong, which has not been systematically defined earlier. In addition to *Ehrlichia*, *Anaplasma*, *Borrelia*, and *D. immitis*, we also tested for *Babesia*, *Hepatozoon*, *Rickettsia*, and *Orientia tsutsugamushi*, for which there are currently no commercial point-of-care tests available.

Materials and Methods

Animals

Two groups of dogs were included in the study. The first group were pet dogs ($n = 100$) brought to the care of veterinarians for routine health checking from March to July 2010. After clinical evaluation, relevant specimens were sent to a veterinary pathologist for laboratory investigations. Peripheral EDTA blood was taken, and the plasma was tested by the SNAP 4Dx for antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Borrelia burgdorferi*, and *D. immitis* antigen. The second group were stray dogs ($n = 100$) captured by the government Agriculture, Fisheries, and Conservation Department from June 2009 to January 2010. EDTA blood samples were collected by veterinarians in the kennels during euthanasia for polymerase chain reaction (PCR) and SNAP 4Dx testing.

Immunochromatographic assay

SNAP 4Dx test was performed according to the manufacturer's instructions by the same investigator.

PCR and DNA sequencing for blood pathogens

DNA was extracted from EDTA whole blood samples using EZ1 mini kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in elution buffer and was used as the template for PCR. The primer sequences are listed in Table 1. The sequences of the PCR products were compared with known sequences by BLAST analysis against the NCBI database (Yuen et al. 2001).

Quantitative PCR

Quantitative PCR was performed on the PCR-positive samples (14 for *E. canis* and 81 for *Babesia*) using TaqMan Universal PCR Master Mix with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) (Lau et al. 2009). The primers and probes are listed in Table 2. This set of quantitative PCR primers and probe for *Ehrlichia* does not cross amplify *Wolbachia*. Two plasmids containing the target sequences were used for generating the standard curves. The limit of detection for either species is 10 plasmid copies per reaction.

Phylogenetic characterization

Phylogenetic tree was constructed by the neighbor-joining method using Kimura's two-parameter correction with ClustalX 1.83. The 316, 292, 338, 150, 264, and 171 bp of amplicons from the 16S rRNA of *E. canis*, 16S rRNA of *Wolbachia* endosymbioint of *D. immitis*, 18S rRNA of *Babesia*, 18S rRNA of *Hepatozoon canis*, *groEL* of *Anaplasma platys*, and ITS of *D. immitis*, respectively, from all positive samples were included in the analysis.

Data analysis

The chi-square test and Student's *t*-test were used where appropriate to assess whether data obtained in the two groups of dogs significantly differed. A p-value of < 0.05 is considered statistically significant.

Nucleotide sequence accession numbers

Partial nucleotide sequences of 16S rRNA gene (*E. canis* and *Wolbachia* endosymbiont *of D. immitis)*, 18S rRNA gene (*Babesia gibsoni*, *Babesia canis*, and *H. canis)*, *groEL* gene (*A. platys)*, and ITS (*D. immitis)* obtained in this study have been lodged within the GenBank sequence database under accession numbers HQ718601 to HQ718730.

Results

Comparison between SNAP 4Dx and PCR

There were 162 positive test results from both SNAP 4Dx and PCR with 85.2% concordance between the two tests. The results were summarized in Table 3. Concordance between SNAP 4Dx and PCR was 100% for *D. immitis*. Discrepancies between serologic and PCR results were observed for 24 test results (Table 4) involving *E. canis* and *A. platys*. Thirteen samples had positive serology but negative PCR for the respective pathogens, whereas 3 *A. platys* and 8 *E. canis* samples had positive PCR but negative serology results.

Prevalence of individual pathogens

We did not find any *B. burgdorferi*, *Rickettsia*, and *O. tsutsugamushi* infection in our samples. The commonest pathogen detected is *Babesia*, which is present in 48% and 33% of stray

TABLE 2. PRIMERS AND PROBES USED FOR QUANTITATIVE POLYMERASE CHAIN REACTION OF *EHRLICHIA*-AND *BABESIA*-POSITIVE SAMPLES

Pathogen (gene)	*Primers/probes*	*Sequences*	*Product length (bp)*
Ehrlichia spp. (16S rRNA)	Forward	5′CGGGTGAGTAATGCGTAGGAAT	82
	Reverse	5′CCCKCRGGGATTATACAGTATTACC	
	Probe	5′ [FAM]TACCTAGTAGTAYGGAATAGCCAT [MGB]	
Babesia spp. (18S rRNA)	Forward	5′GACTAGDGATTGGAGGTCGTCRT	79
	Reverse	5′TCCCCCCAGAACCCAAAG	
	Probe	5′ [FAM] CCTTCAGSAVCTTGAGAGA [MGB]	

Table 3. Results of SNAP 4Dx and Polymerase Chain Reaction for Canine Arthropod-Borne Pathogens

	Stray dogs (n = *100)*			*Pet dogs* (n = *100)*		
	Number positive for tests	*Speciation by sequencing*	*DNA load in blood (mean, [range] copies/mL)*	*Number positive for tests*	*Speciation by sequencing*	*DNA load in blood (mean, [range] copies/mL)*
PCR						
Borrelia burgdorferi	0			0		
Rickettsia spp.	0			0		
O. tsutsugamushi	0			0		
Anaplasma spp.	8	*A. platys* (8)		0		
Ehrlichia spp.	8	*E. canis* (7)	2.25×10^5 [2.50×10^1 to 1.43×10^6]	6	*E. canis* (6)	9.08×10^4 [6.88×10^2 to 4.03×10^5]
Wolbachia spp.	10			2		
Babesia spp.	48	*B. canis* (4), *B. gibsoni* (44)	2.26×10^6 [1.40×10^4 to 2.05×10^6] [8.86×10^3 to 1.75×10^7]	33	*B. canis* (2), *B. gibsoni* (31)	4.8×10^7 [7.74×10^5 to 5.08×10^6] [1.61×10^4 to 4.29×10^8]
Hepatozoon spp.	2	*H. canis* (2)		1	*H. canis* (1)	
D. immitis	10			2		
SNAP 4Dx						
D. immitis	10			2		
E. canis + *A. phagocytophilum*	1			3		
E. canis only	1			7		
A. phagocytophilum only	6			0		
A. phagocytophilum + *D. immitis*	2					
B. burgdorferi	0			0		
Co-infections[a]	19			6		
Babesia + *E. canis*	3			1		
Babesia + *D. immitis*				1		
Babesia + *H. canis*				1		
E. canis + *Anaplasma*	7			3		
D. immitis + *H. canis*	2					
Babesia + *E. canis* + *Anaplasma*	2					
Anaplasma + *D. immitis* + *H. canis*	1					

[a] Based on both serology and PCR studied. Co-detection of *Wolbachia* and *D. immitis* were excluded.
PCR, polymerase chain reaction.

and pet dogs, respectively ($p < 0.05$). Quantitative PCR test showed that there is a significantly higher *Babesia* DNA copy number in pet versus stray dogs (4.8×10^7 vs. 2.26×10^6 copies/mL) ($p < 0.005$). Sequencing of the 18S rRNA gene showed that most of the *Babesia* positive cases are *B. gibsoni* (accounting for 91.6% [stray] and 93.9% [pet]) rather than *B. canis*, all of the latter are *B. canis* subspecies *vogeli*.

The prevalence of *E. canis* was of 8% and 6% in stray and pet dogs, respectively. There are no significant differences in the prevalence and DNA copy number between the two groups of dogs ($p > 0.5$). *E. canis* antibodies were found in 2 stray and 10 pet dogs; 1 and 3 dogs, respectively, from the two groups were also positive for *Anaplasma* antibodies. In the two *E. canis* antibody-positive stray dogs, the one that was positive for both *Ehrlichia* and *Anaplasma* antibodies was PCR-positive for *E. canis* (1.07×10^5 copies/mL) but PCR-negative for *Anaplasma*. The one that was positive for *E. canis* antibody alone had a very weak color change on SNAP 4Dx, and the sample was PCR-negative for *E. canis*. In the 10 pet dogs with positive *E. canis* antibodies, 5 of them were PCR-negative, suggesting that the antibodies represented a past infection. On the other hand, 1 pet and 2 stray dogs had positive *E. canis* PCR (ranging from 1.13×10^5 to 1.43×10^6 copies/mL) but negative *E. canis* antibodies on SNAP 4Dx. We suspect that these may represent a hyperacute ehrlichial infection before detectable antibodies were developed in the infected dogs.

The prevalence of anaplasmosis was 8% and 0% in stray and pet dogs, respectively. *Anaplasma* antibodies were present in 12 dogs (9 stray and 3 pet), either alone (6 stray) or with heartworm (2 stray) or *E. canis* (1 stray and 3 pet) antibodies. All the pet dogs were PCR-negative for *Anaplasma*, signifying

Table 4. Discrepant Results Between Serology and Polymerase Chain Reaction

Pathogen	*Source of dogs*	*Serology + /PCR–*	*Serology–/PCR +*
E. canis	Strays	1	7
	Pets	5	1
A. platys	Strays	4	3
	Pets	3	0

possible past infections. *Anaplasma* PCR was positive in 8 stray dogs, 6 of which were also PCR positive for *E. canis*. Sequencing of the *groEL* amplicon showed that there was 1 (0.2%) base difference between our samples and that of *A. platys* (AF4781291) but >16 (3.4%) base difference with that of *A. phagocytophilum* (HM7520981), indicating that all the PCR-positive cases were *A. platys* (Fig. 1A).

D. immitis DNAemia was found in 12 dogs (10 stray and 2 pet, $p<0.025$); all of them were positive for circulating heartworm antigen. Sequencing of the ITS showed that all cases have 100% nucleotide identity to those of the reported strains.

Hepatozoonosis is uncommon in our sample with only 2% and 1% prevalence in stray and pet dogs, respectively. Sequencing of the 18S rRNA amplicon showed that all belonged to *H. canis*.

Co-infection by 2 or more pathogens was observed in 19% of stray dogs and 6% of pet dogs ($p<0.01$). The combinations of pathogens in dogs with coinfection are shown in Table 3.

Incidental finding of Wolbachia *DNAemia*

Seventeen and 8 samples from stray and pet dogs, respectively, were initially positive in the *Ehrlichia* PCR reaction. However, on sequencing of their PCR amplicons, 10 from stray and 2 from pet dogs were found to be *Wolbachia* sequences instead, with 100% nucleotide identity to the 16S rRNA sequence of *Wolbachia* endosymbiont of *D. immitis* (AF088187) (Fig. 1B). This can be explained by the significant homology between the *Ehrlichia* and *Wolbachia* 16S rRNA sequences (Fig. 2). Therefore, we designed another *Wolbachia*-specific 16S rRNA primer pair and found that all cases with *D. immitis* DNAemia also had *Wolbachia* DNAemia when tested again by *Wolbachia*-specific PCR test. Sequencing of the 16S rRNA gene of these amplicons showed that they are truly *Wolbachia* endosymbiont of *D. immitis* but not other dog arthropod-related *Wolbachia* species (Fig. 1B). False-positive *Ehrlichia* PCR results were eliminated by the Tagman quantitative PCR for *Ehrlichia* using a specific probe or PCR primers for *E. canis* that will not cross react with *Wolbachia*.

Discussion

Rapid and accurate detection of veterinary pathogens is important for clinical management of sick animals because of the limited sensitivity and specificity of clinical examination and problems associated with empirical treatment. This is the first systematic study to compare SNAP 4Dx against PCR and determine the prevalence of eight canine vector-borne pathogens in Hong Kong. It provides a better understanding of the strengths and limitations of serologic tests and the local prevalence of these pathogens.

The overall concordance between SNAP 4Dx and PCR is 85.2%. Discrepancies between the commercial immunochromatographic test and our PCR tests involved *E. canis* and *A. platys* (Table 4). We suspect that these PCR-positive and antibody-negative cases represented early infections before the development of antibody responses. For example, an *E. canis*-seronegative dog had one of the highest bacterial DNA load in blood (4.03×10^5 copies/mL). It was presumably diagnosed during acute infection before seroconversion, which shows that molecular tests do have an important role in diagnosis at the hyperacute stage of the disease and are not confounded by positive antibody response due to past exposures as in immunochromatographic tests. On the other hand, the antibody-positive but PCR-negative cases may represent past infections that may have been treated, spontaneously resolved, and progressed to the subclinical chronic stage with low levels of bacteremia, or due to cross-reacting antibodies.

Around 10% of dogs in our study had evidence of *Ehrlichia* and/or *Anaplasma* infection by serology or PCR. *E. canis* is the only *Ehrlichia* species found in Hong Kong, presumably due to the absence of *Amblyomma americanum* tick, which is the vector for *Ehrlichia ewingii* and *Ehrlichia chaffeensis*.

All the *Anaplasma* amplicons in our series belonged to *A. platys*, the vector of which is the brown dog tick *Rhipicephalus sanguineus*. *A. phagocytophilum* but not *A. platys* can cause human granulocytic anaplasmosis. The absence of *A. phagocytophilum* in our study suggests that the risk of autochthonous human granulocytic anaplasmosis is low in Hong Kong. In contrast to *A. phagocytophilum*, canine infection due to *A. platys* is often asymptomatic despite the presence of recurrent thrombocytopenia. This may explain the 8% prevalence among apparently healthy stray dogs, which is higher than the prevalence in pet dogs, presumably due to more intense exposure to tick vectors. *E. ewingii*, *E. chaffeensis*, *Neorickettsia risticii*, and *Neorickettsia helminthoeca* are other canine Anaplasmataceae pathogens, whereas *E. chaffeensis* and *A. phagocytophilum* also cause human infections.

Although we initially did not specifically look for *Wolbachia* DNA in our samples, the *E. canis* primers unexpectedly picked up *Wolbachia*, which can only be differentiated from *E. canis* by sequencing the PCR product. This cross reaction could be due to significant homology between their 16S rRNA sequences (Fig. 2). The 16S rRNA primers for *Ehrlichia* and *Anaplasma* are known to amplify *Wolbachia*, and sequencing of the PCR products is essential for differentiating the 3 genera of Anaplasmataceae (Unver et al. 2003, Little 2010). Similar findings have also been previously reported when *H. canis* was unexpectedly detected using *Babesia* primers due to a high degree of homology between the 18S rRNA sequences of the 2 organisms (Spolidorio et al. 2009). *Wolbachia* are endosymbionts of arthropods and filarial nematodes. Humans and animals with filariasis develop immune responses to *Wolbachia* antigens, and the pro-inflammatory antigens from *Wolbachia* participates in the pathogenesis of filariasis and affects the clinical outcome of *Dirofilaria* infections (Bandi et al. 2001, Bazzocchi et al. 2003, Kramer et al. 2008, Dingman et al. 2010). Elimination of *Wolbachia* with tetracyclines is clinically beneficial in the treatment of filariasis and can possibly reduce transmission (Rossi et al. 2010).

The prevalence of *D. immitis* is highly variable, ranging from 0.24% to over 50% in different countries (Lee et al. 2010). The prevalence of canine heartworm infection is 6% in Hong Kong, with a significantly higher prevalence among stray than pet dogs, possibly related to the intensity of exposure to mosquito vectors. Accurate diagnosis is important, because the infection can result in severe cardiopulmonary disease. Diagnosis is commonly made by either blood smear examination by the modified Knott's method or antigen detection. In recent years, molecular diagnosis by using PCR to detect *D. immitis* DNA in canine blood is also possible. We found 100% concordance between SNAP 4Dx and PCR for the diagnosis of *D. immitis* infection, and all dogs with *D. immitis* infection also had positive *Wolbachia* PCR. Unfortunately, since the detection of *Wolbachia* DNAemia was not initially planned as part

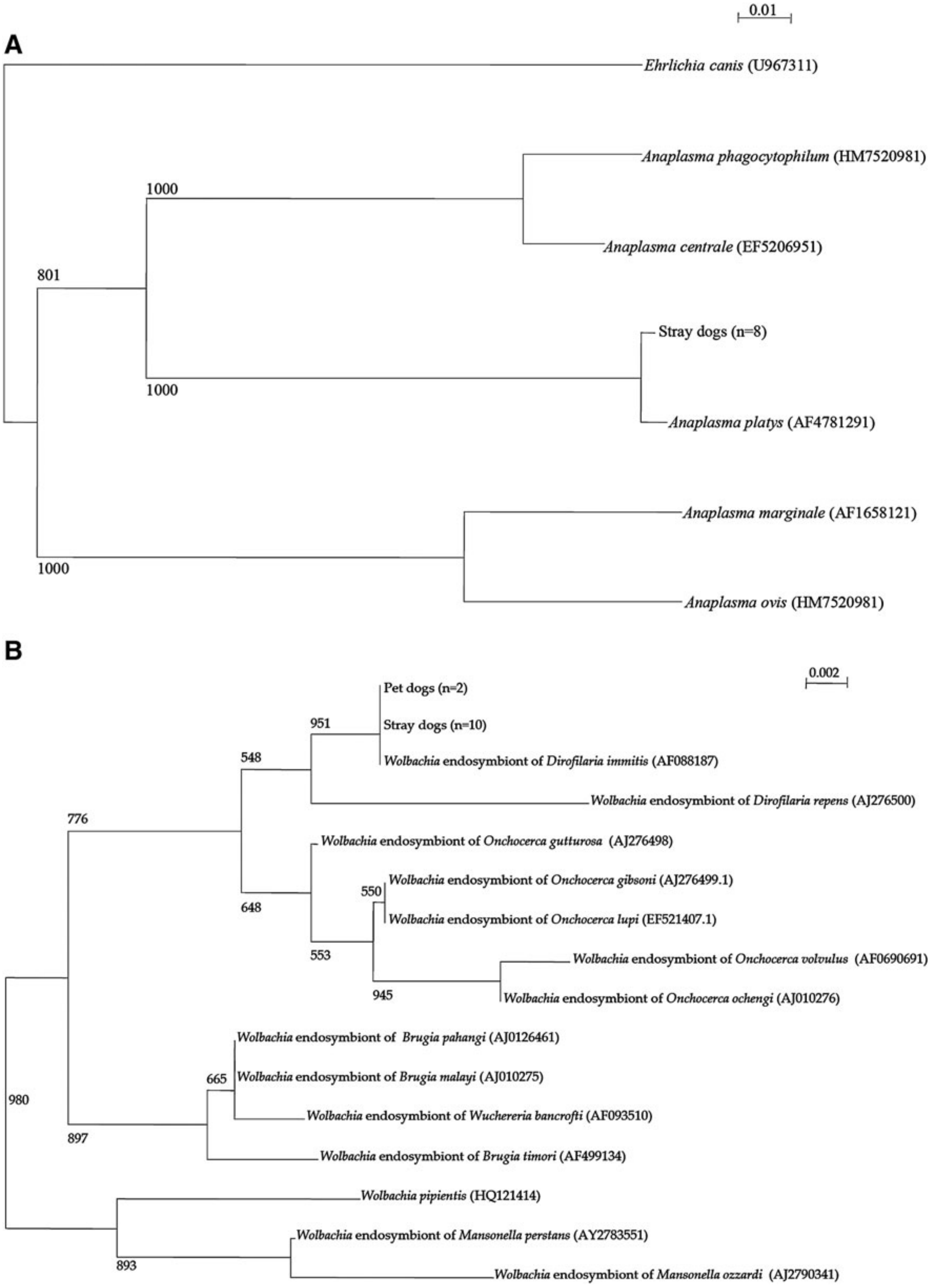
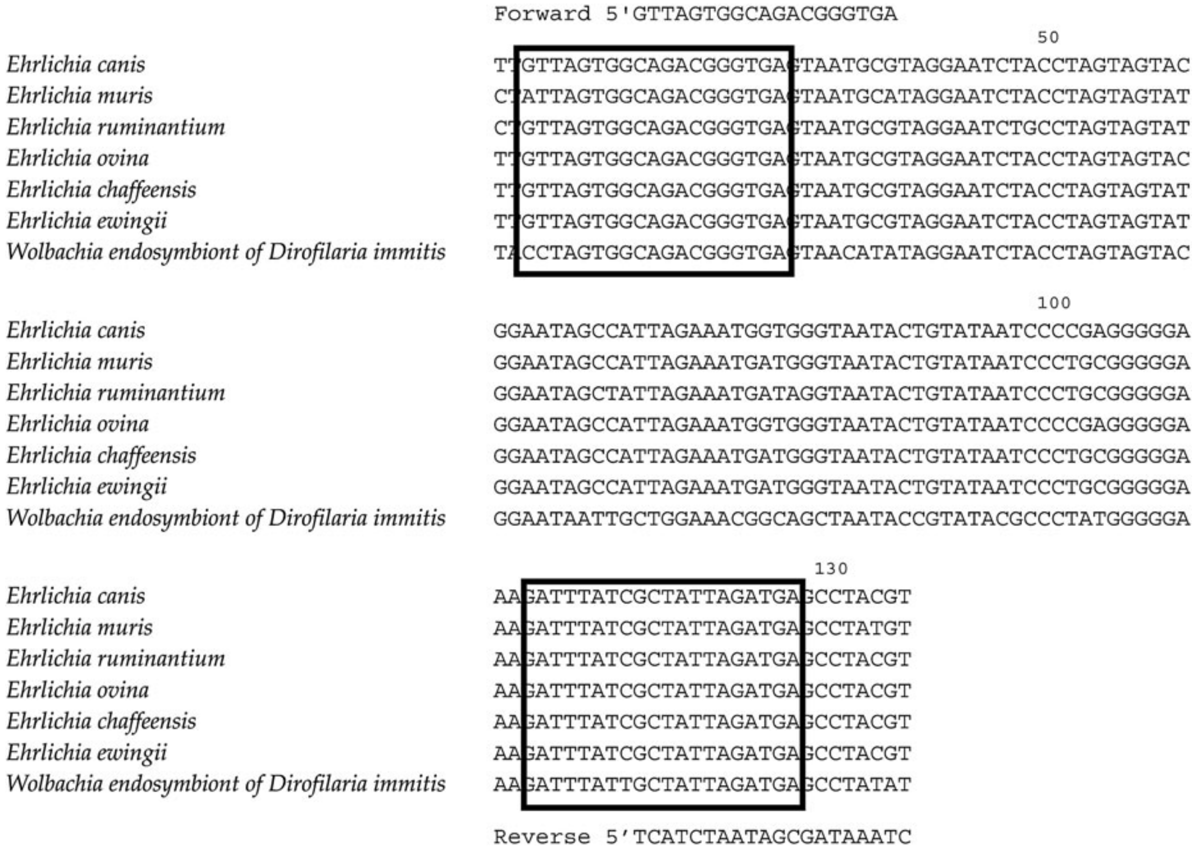


FIG. 1. Phylogenetic relationships based on sequences identified in this study. **(A)** *Anaplasma platys*, *groEL* gene; **(B)** *Wolbachia* endosymbiont of *Dirofilaria immitis*, 16S rRNA gene. The trees were constructed by the neighbor-joining method and bootstrap values calculated from 1000 trees. The scale bar indicates the estimated number of substitutions per 100 and 500 nucleotides as indicated. All names and accession numbers are given as cited in the GenBank database.



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                                                   Forward 5'GTTAGTGGCAGACGGGTGA
                                                                                         50
Ehrlichia canis                                    TTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTAC
Ehrlichia muris                                    CTATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTAT
Ehrlichia ruminantium                              CTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTGCCTAGTAGTAT
Ehrlichia ovina                                    TTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTAC
Ehrlichia chaffeensis                              TTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTAT
Ehrlichia ewingii                                  TTGTTAGTGGCAGACGGGTGAGTAATGCGTAGGAATCTACCTAGTAGTAT
Wolbachia endosymbiont of Dirofilaria immitis      TACCTAGTGGCAGACGGGTGAGTAACATATAGGAATCTACCTAGTAGTAC

                                                                                         100
Ehrlichia canis                                    GGAATAGCCATTAGAAATGGTGGGTAATACTGTATAATCCCCGAGGGGGA
Ehrlichia muris                                    GGAATAGCCATTAGAAATGATGGGTAATACTGTATAATCCCTGCGGGGGA
Ehrlichia ruminantium                              GGAATAGCTATTAGAAATGATAGGTAATACTGTATAATCCCTGCGGGGGA
Ehrlichia ovina                                    GGAATAGCCATTAGAAATGGTGGGTAATACTGTATAATCCCCGAGGGGGA
Ehrlichia chaffeensis                              GGAATAGCCATTAGAAATGATGGGTAATACTGTATAATCCCTGCGGGGGA
Ehrlichia ewingii                                  GGAATAGCCATTAGAAATGATGGGTAATACTGTATAATCCCTGCGGGGGA
Wolbachia endosymbiont of Dirofilaria immitis      GGAATAATTGCTGGAAACGGCAGCTAATACCGTATACGCCCTATGGGGGA

                                                                          130
Ehrlichia canis                                    AAGATTTATCGCTATTAGATGAGCCTACGT
Ehrlichia muris                                    AAGATTTATCGCTATTAGATGAGCCTATGT
Ehrlichia ruminantium                              AAGATTTATCGCTATTAGATGAGCCTACGT
Ehrlichia ovina                                    AAGATTTATCGCTATTAGATGAGCCTACGT
Ehrlichia chaffeensis                              AAGATTTATCGCTATTAGATGAGCCTACGT
Ehrlichia ewingii                                  AAGATTTATCGCTATTAGATGAGCCTACGT
Wolbachia endosymbiont of Dirofilaria immitis      AAGATTTATTGCTATTAGATGAGCCTATAT

                                                   Reverse 5'TCATCTAATAGCGATAAATC
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FIG. 2. Nucleotide sequence alignment of 16S rRNA gene of *Ehrlichia* spp. and *Wolbachia* endosymbiont of *D. immitis*, showing regions containing the 5′ and 3′ primers used for *Ehrlichia* polymerase chain reaction.

of the study, we did not perform blood film examination for microfilaremia and, hence, could not correlate the presence of *Wolbachia* DNAemia with microfilaremia. Although the use of antigen detection kits provides rapid results for immediate management decisions and most of the antigen detection tests are highly sensitive and specific (Nelson et al. 2005), PCR for *D. immitis* DNA is a potentially useful adjunct in cases with low levels of microfilaremia, as the sensitivity of antigen detection tests may be lowered in such cases (Vezzani et al. 2008). However, the added benefits of PCR over serology need to be demonstrated by further studies. On the other hand, in certain parts of the world where other filarial parasites are endemic, these point-of-care tests (primarily targeted against *D. immitis*) may not be clinically useful. With careful selection of primers and/or the use of sequencing, molecular testing could be a better diagnostic test in these areas where non-*D. immitis* filariases are common. *Wolbachia* PCR may provide additional information, because the sensitivity of *D. immitis* antigen detection tests could be limited in dogs with only male or young female worms, as the antigens are derived from the genital organs of adult female worms.

We found no evidence of active canine infection due to *Rickettisa*, *O. tsutsugamushi*, and *B. burgdorferi* in our cohorts. Although the role of dogs as a reservoir host for these bacteria in Hong Kong is probably minor, we cannot completely exclude their existence, because past infections may not be detectable by PCR and serologic tests are required for confirmation. *Rickettsia felis* is an emerging zoonotic pathogen in many parts of the world, with reservoirs in both the cats and dogs. *B. burgdorferi* sensu sticto is primarily found in North America and Europe, and the main vertebrate reservoir hosts are small mammals (especially rodents). However, both *B. burgdorferi* sensu sticto and sensu lato have been isolated from animals in mainland China and Taiwan (Chao et al. 2002, Zhang et al. 1997, Shih et al. 1998a, 1998b). Dogs and humans are accidentally infected by hard tick bites, though neither are important reservoir hosts.

In previous studies from Spain, France, Italy, Czech, the United Kingdom, and the United States, coinfection of vector-borne pathogens in dogs ranged from 0% to over 50%, depending on the location, pathogens, and methods of detection (Shaw et al. 2005, Solano-Gallego et al. 2006, Amusategui et al. 2008, Beall et al. 2008, Kybicová et al. 2009, Pantchev et al. 2009, Couto et al. 2010, Otranto et al. 2010, Tzipory et al. 2010). Evidence of co-infection was found in 19% of stray and 6% pet dogs in our study ($p < 0.01$). The higher prevalence in stray dogs can be explained by the higher risk of exposure to arthropod vectors in the environment.

One of the most important limitations of the point-of-care test is the lack of testing for babesiosis, which is the commonest arthropod-borne infection in both pet and stray dogs in Hong Kong. Most of these are caused by *B. gibsoni* with only 7.3% due to *B. canis*. Although serologic diagnosis of babesiosis using immunofluorescent antibody testing is commercially available, it is rather cumbersome for most veterinarians and requires separate laboratory support. Canine babesiosis is a tick-borne infection with a global distribution caused by *B. canis* (subsp. *canis*, *vogeli*, and *rossi*), *B. gibsoni*, *B. microti*, *B. equi*, and *B. conradae*. *B. gibsoni* generally causes hemolytic anemia, fever, lethargy, hepatosplenomegaly, hemoglobinuria, and icterus. The other less important tick-borne infection is hepatozoonosis. As expected, the Old World species *H. canis* (transmitted by *R. sanguineus*) is the only species found in our study, though the pathogen is not common in our dog population. The absence of American canine hepatozoonosis (*H. americanum*) in Hong Kong is probably related to the absence of its tick vector *Amblyomma maculatum*. The American form of the disease is generally more severe, and infected animals are more debilitated and often fatal.

Compared with conventional blood film examination, PCR offers a highly sensitive means for detecting blood-borne pathogens. A broad range of pathogens can be detected by PCR studies. Sequencing studies also allow differentiation of species or subspecies that may have similar morphological appearances. The accurate speciation of the pathogens carries epidemiologic or prognostic significance. Molecular studies allow the detection of newly described pathogens for which serologic tests are generally not available. However, at the moment, the turnaround time for molecular testing is still longer than blood film examination and point-of-care testing, and the availability of molecular diagnostics for veterinary service is still limited in many countries. A better organization and delivery of such techniques is highly desirable not just for better care of the sick animals but also to allow epidemiologic study of these pathogens and their zoonotic potentials.

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Address correspondence to:
Kwok-Yung Yuen
State Key Laboratory of Emerging Infectious Diseases
Department of Microbiology
The University of Hong Kong
Carol Yu Centre for Infection
University Pathology Building
Queen Mary Hospital
102 Pokfulam Road
Hong Kong

E-mail: kyyuen@hku.hk