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<thead>
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<td>Author(s)</td>
<td>Wong, SSY; Teng, JLL; Poon, RWS; Choi, GKY; Chan, KH; Yeung, ML; Hui, JJY; Yuen, KY</td>
</tr>
<tr>
<td>Citation</td>
<td>Vector-Borne And Zoonotic Diseases, 2011, v. 11 n. 9, p. 1269-1277</td>
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<tr>
<td>Issued Date</td>
<td>2011</td>
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<td><a href="http://hdl.handle.net/10722/157642">http://hdl.handle.net/10722/157642</a></td>
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</tr>
</tbody>
</table>
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 Yuen1,2

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 dirofilarialnosis 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

1State Key Laboratory of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, Hong Kong.
2Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong.
3Agriculture, Fisheries and Conservation Department, Hong Kong Special Administrative Region, Hong Kong.
4PathLab Medical Laboratories Ltd., Hong Kong.
*These two authors contributed equally to this work and are co-first authors.
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlichia spp.</td>
<td>16S rRNA</td>
<td>Forward 5’ GTTAGTGCGCAGACGCCGTGA</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ TCACTATAATACGGAGATAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ehrlichia canis</td>
<td>16S rRNA</td>
<td>Forward 5’ CAATTATTTATAGCCTCTGCATAGGA</td>
<td>400</td>
<td>Murphy et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ TATAGGTACCGTCATTATCTCTCTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babesia spp.</td>
<td>18S rRNA</td>
<td>Forward 5’ GTCTITGTAATGGGATGATGG</td>
<td>560</td>
<td>Beck et al. (2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ CCAAGACTTTGATTTCTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babesia canis</td>
<td>18S rRNA</td>
<td>Forward 5’ GTTTATAGTTGGAACCGG</td>
<td>650</td>
<td>Inokuma et al. (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ GAACCTGAAAAGCCAAAAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babesia gibsoni</td>
<td>18S rRNA</td>
<td>Forward 5’ CTCGGCTACCTGCTTGTGT</td>
<td>650</td>
<td>Inokuma et al. (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ GCCGAAACTGAAAATACGCC</td>
<td></td>
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</tr>
<tr>
<td>Hepatozoon spp.</td>
<td>18S rRNA</td>
<td>Forward 5’ AAGGCAGTTAAAATGTCT</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ AAGCAAGCAGCCGTGTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaplasma</td>
<td>msp2 (outer membrane protein-2)</td>
<td>Forward 5’ CCGGCGCATGTTGTAAGGTGAA</td>
<td>334</td>
<td>Lin et al. (2004), Massung and Slater (2003), M’Ghirbi et al. (2009)</td>
</tr>
<tr>
<td>phagocytophilum</td>
<td>groEL</td>
<td>Forward 5’ AAGCAGCTTTGCGATCTCCAGTTT</td>
<td>516</td>
<td>Beall et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ TCGCGTACAGGAGATGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Borrelia burgdorferi sensu lato</td>
<td>ospA (outer surface protein A)</td>
<td>Forward 5’ AATAGGTCTATAATAGCCCTTTATAGC</td>
<td>308</td>
<td>Demaerschalck et al. (1995), Shaw et al. (2005)</td>
</tr>
<tr>
<td>Spotted fever</td>
<td>groEL</td>
<td>Forward 5’ AATGGTCTATAATAGCCCTTTATAGC</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td>group rickettsiae</td>
<td></td>
<td>Reverse 5’ TCGGCGATCTCCAGTTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>g1A (rickettsial citrate synthase)</td>
<td>Forward 5’ GGGGGCGCTGCTACGGG</td>
<td>382</td>
<td>Regnery et al. (1991), Satoh et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>rompA (SFG rickettsial 190-kDa surface antigen)</td>
<td>Forward 5’ ATIGCAAAAAAGTACAGTAAAA</td>
<td>533</td>
<td>Regnery et al. (1991), Satoh et al. (2002)</td>
</tr>
<tr>
<td>Orientia tsutsugamushi</td>
<td>groEL</td>
<td>Forward 5’ AGATGATTACCGAATTTGAAAAT</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ AAGCCTATATTGGGATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wollbachia spp.</td>
<td>16S rRNA</td>
<td>Forward 5’ AAGCGGCACTATAATGGCATTATA</td>
<td>353</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ ACGCTAGCCCTCTTGTATTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dirofilaria immitis</td>
<td>ITS (internal transcribed spacer)</td>
<td>Forward 5’ ATGATGATTGCTCAATTAATGAGC</td>
<td>290</td>
<td>Thanchomnang et al. (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5’ GATAATCTGATCGATATTGACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 *Orientalia 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 test was performed according to the manufacturer’s instructions by the same investigator. Two groups of 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 within the GenBank sequence database under accession numbers HQ718601 to HQ718730.

**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* endosymbiont 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 and domestic dogs.

**Table 2. Primers and Probes Used for Quantitative Polymerase Chain Reaction of *Ehrlichia*- and *Babesia*-Positive Samples**

<table>
<thead>
<tr>
<th>Pathogen (gene)</th>
<th>Primers/probes</th>
<th>Sequences</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ehrlichia</em> spp. (16S rRNA)</td>
<td>Forward</td>
<td>5’CGGGTGAATGCGTAGGAAT</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’CCCKCRGGGATTACAGTATTACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ [FAM]TACCTAGATAYGGAATACCCAT [MGB]</td>
<td>79</td>
</tr>
<tr>
<td><em>Babesia</em> spp. (18S rRNA)</td>
<td>Forward</td>
<td>5’GACTAGGGATTGAGGTTGTCRT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’TCCCCCAAGAAACCCAAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>5’ [FAM] CCTTCAGSAVCTTGAGAGA [MGB]</td>
<td></td>
</tr>
</tbody>
</table>
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 ($4.8 \times 10^7$ vs. $2.26 \times 10^6$ 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 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* ($1.07 \times 10^5$ 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 $1.13 \times 10^5$ to $1.43 \times 10^6$ 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>
<thead>
<tr>
<th>Pathogen</th>
<th>Source of dogs</th>
<th>Serology+/PCR−</th>
<th>Serology−/PCR+</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. canis</em></td>
<td>Strays</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Pets</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><em>A. platys</em></td>
<td>Strays</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Pets</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>PCR Pathogen</th>
<th>Stray dogs (n=100)</th>
<th>DNA load in blood (mean, range) copies/mL</th>
<th>Pet dogs (n=100)</th>
<th>DNA load in blood (mean, range) copies/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number for tests</td>
<td>Speciation by sequencing</td>
<td>Number</td>
<td>Speciation by sequencing</td>
</tr>
<tr>
<td><strong>Borrelia burgdorferi</strong></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Rickettsia spp.</strong></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>O. tsutsugamushi</strong></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Anaplasma spp.</strong></td>
<td>8</td>
<td>A. platys (8)</td>
<td>6</td>
<td>E. canis (6)</td>
</tr>
<tr>
<td><strong>Ehrlichia spp.</strong></td>
<td>8</td>
<td>E. canis (7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Wolbachia spp.</strong></td>
<td>10</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Babesia spp.</strong></td>
<td>48</td>
<td>B. canis (4)</td>
<td>33</td>
<td>B. gibsoni (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hepatozoon spp.</strong></td>
<td>2</td>
<td>H. canis (2)</td>
<td>1</td>
<td>H. canis (1)</td>
</tr>
<tr>
<td><strong>D. immitis</strong></td>
<td>10</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>SNAP 4Dx</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D. immitis</strong></td>
<td>10</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>E. canis</strong> (+)</td>
<td>1</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>E. canis</strong> only</td>
<td>1</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>A. phagocytophilum</strong></td>
<td>1</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>A. phagocytophilum</strong></td>
<td>6</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>E. canis</strong> (+)</td>
<td>2</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>D. immitis</strong></td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Co-infections</strong></td>
<td>19</td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>Babesia</strong> (+)</td>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Babesia</strong> (+) <strong>D. immitis</strong></td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Babesia</strong> (+) <strong>H. canis</strong></td>
<td>1</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>E. canis</strong> (+) <strong>Anaplasma</strong></td>
<td>7</td>
<td></td>
<td>3</td>
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<tr>
<td><strong>D. immitis</strong> (+) <strong>H. canis</strong></td>
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<td>2</td>
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<td>0</td>
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<td>1</td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Based on both serology and PCR studied. Co-detection of *Wolbachia* and *D. immitis* were excluded.

PCR, polymerase chain reaction.

<table>
<thead>
<tr>
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</tr>
<tr>
<td></td>
<td>Pets</td>
<td>3</td>
<td>0</td>
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</table>
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 (4.05 × 10^4 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.
of the study, we did not perform blood film examination for microfilaremia and, hence, could not correlate the presence of \textit{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 \textit{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 \textit{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-\textit{D. immitis} filariases are common. \textit{Wolbachia} PCR may provide additional information, because the sensitivity of \textit{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 \textit{Rickettsia}, \textit{O. tsutsugamushi}, and \textit{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. \textit{Rickettsia felis} is an emerging zoonotic pathogen in many parts of the world, with reservoirs in both the cats and dogs. \textit{B. burgdorferi} sensu stricto is primarily found in North America and Europe, and the main vertebrate reservoir hosts are small mammals (especially rodents). However, both \textit{B. burgdorferi} sensu stricto 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 \textit{B. gibsoni} with only 7.3% due to \textit{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. conrati. B. gibsoni generally causes hemolytic 
anemia, fever, lethargy, hepatosplenomegaly, hemoglobin- 
uria, and icterus. The other less important tick-borne infection 
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 hepa-

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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References

Amusategui, I, Tesouro, MA, Kakoma, I, Sainz, A. Serological 
reactivity to Ehrlichia canis, Anaplasma phagocytophilum, Neor-
ickettsia risticii, Borrelia burgdorferi and Rickettsia conori in dogs 
from northwestern Spain. Vector Borne Zoonot Dis 2008; 
8:797–803.

Ayoob, AL, Hackner, SG, Prittie, J. Clinical management of ca-

Bazizcchi, C, Genchi, C, Paltrinieri, S, Lecchi, C, et al. Im-
munological role of the endosymbionts of Dirofilaria immitis: 
the Wolbachia surface protein activates canine neutrophils with 

Beall, MJ, Chandrashekar, R, Eberts, MD, Cyr, KE, et al. Ser-
ological and molecular prevalence of Borrelia burgdorferi, 
Anaplasma phagocytophilum, and Ehrlichia species in dogs from 

Babesia and Theileria species in symptomatic and asymptomatic 

Borgo, SN, Sattler, EC, Hogardt, M, Adler, K, et al. PCR for 
Wolbachia in human and canine Demodez mites. Arch 

Chao, LL, Shih, CM. Molecular characterization of Lyme disease 
spirochetes (Borrelia burgdorferi sensu lato) isolated in Taiwan 
by restriction fragment length polymorphism analysis of 

study of selected vector-borne diseases in shelter dogs in 
Central Spain using point-of-care assays. Vector Borne Zoonot 

Demaerschalk, I, Ben Messaoud, A, De Kesel, M, Hoyois, B, 
et al. Simultaneous presence of different Borrelia burgdorferi 
genspecies in biological fluids of Lyme disease patients. 

Dingman, P, Levy, JK, Kramer, LH, Johnson, CM, et al. Associ-
association of Wolbachia with heartworm disease in cats and dogs. 

multiplex PCR for simultaneous detection and discrimination of 
Dirofilaria immitis and Dirofilaria repens in canine peripheral 

Molecular survey of Babesia infection in dogs in Okinawa, 

Irwin, PJ. Canine babesiosis. Vet Clin North Am Small Anim 

influence on the pathology and immunology of Dirofilaria im-

Kybicová, K, Schánilec, P, Hulín, D, Uherková, L, et al. De-
tection of Anaplasma phagocytophilum and Borrelia burgdorferi 
sensu lato in dogs in the Czech Republic. Vector Borne Zoonot 

Lau, SK, Chan, KH, Yip, CC, Ng, TK, et al. Confirmation of the 
first Hong Kong case of human infection by novel swine origin 
influenza A (H1N1) virus diagnosed using ultrarapid, real-
time reverse transcriptase PCR. J Clin Microbiol 2009; 47:2344– 
2346.

Lau, SK, Woo, PC, Li, KS, Huang, Y, et al. Severe acute respira-

tory syndrome coronavirus-like virus in Chinese horseshoe 

Lau, SK, Woo, PC, Tse, H, Fu, CT, et al. Identification of novel 
porcine and bovine parvoviruses closely related to human 

Lau, SK, Woo, PC, Wong, BH, Wong, AY, et al. Identification 
and complete genome analysis of three novel para-
myxoviruses, Tuhoko virus 1, 2 and 3, in fruit bats from China. Virology 2010; 404:106–116.

Address correspondence to: Kaok-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