Molecular detection of vector-borne agents in dogs from ten provinces of China

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Abstract

Background: Although many vector-borne agents are potential zoonoses and cause substantial morbidity and mortality in dogs worldwide, there are limited data on these organisms in dogs of China.

Methods: Quantitative PCRs for vector-borne agents were performed to investigate their prevalences in convenience whole blood samples obtained from 1114 dogs from 21 veterinary clinics and a commercial dog breeding facility in ten provinces of China. In addition, the PCRs were performed on 146 Rhipicephalus sanguineus senso lato and 37 Linognathus setosus collected from dogs in the commercial dog breeding facility.

Results: DNAs of Babesia gibsoni and B. vogeli (1.2 %), Ehrlichia canis (1.3 %), Hepatozoon canis (1.8 %) and Theileria orientalis (0.1 %) or a closely related organism were detected in the bloods of the dogs studied, and B. vogeli (3.4 %) and Ehrlichia canis (4.1 %) in R. sanguineus senso lato. The qPCRs for Anaplasma spp., Dirofilaria immitis and Leishmania spp. were negative for all blood samples, ticks and lice. At least one vector-borne agent was found in dogs from 5 of the 10 provinces investigated in this study. Overall, 4.4 % (49/1117) of the dogs studied were positive for at least one vector-borne agent with the prevalence being highest in the commercial breeding colony (24/97; 24.7 %).

Conclusions: Our study confirms that B. vogeli, B. gibsoni, H. canis, and E. canis occur in China. Also, we present evidence that T. orientalis or a closely related organism can infect dogs.

Keywords: China, Vector-borne agents, FRET-PCR, Dogs
from ticks and lice collected from dogs in one province. Our findings are reported below.

Methods
Collection of whole blood and external parasites
Between November 2012 and February 2014, convenience whole blood samples were collected in EDTA by veterinarians who volunteered to participate (Table 1, Fig. 1). The dogs sampled in Taixing, Jiangsu province, were apparently healthy animals in a commercial dog breeding facility; convenience samples of ticks and lice were also collected from these dogs. The remaining dogs in the study were those attending 21 local veterinary clinics for routine health checks, vaccinations and for various conditions. Veterinarians were asked to comment on the presence or absence of ectoparasites on the dogs sampled.

Blood samples were frozen at −20 °C before being put on ice (over 2 days) and sent to Yangzhou University College of Veterinary Medicine where they were frozen at −80 °C until thawed at room temperature for DNA extraction. The external parasites collected from the dogs were identified following standard morphological criteria [17] and stored in vials with RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics GmbH, Mannheim, Germany) at −80 °C until DNA extraction.

Ethical approval
The study was approved by the Institutional Animal Care and Use Committee of Yangzhou University, China. Written permission for sampling was obtained from the owners of dogs that participated in the study.

Table 1 The distribution of studied samples in ten provinces/municipalities

| Sample type | Source of samples | Province/Municipality | City | Coordinates | Number |
|-------------|-------------------|-----------------------|------|-------------|--------|
| Dog blood   | Beijing           | Beijing               | 39°N, 116°E | 134 |
|             | Gansu             | Lanzhou               | 36°N, 103°E | 96  |
|             | Guangdong         | Guangzhou             | 23°N, 113°E | 35  |
|             | Henan             | Zhengzhou             | 34°N, 113°E | 102 |
|             | Inner Mongolia    | Huhhot                | 40°N, 111°E | 82  |
|             | Jiangsu           | Yangzhou              | 32°N, 119°E | 50  |
|             |                 | Taixing               | 32°N, 120°E | 97  |
|             |                 | Nanjing               | 32°N, 118°E | 130 |
|             | Shanghai          | Shanghai              | 31°N, 121°E | 84  |
|             | Shaanxi           | Yangling              | 34°N, 108°E | 56  |
|             | Xinjiang          | Urumchi               | 43°N, 87°E | 86  |
|             | Yunnan            | Kunming               | 25°N, 102°E | 162 |
| Ticks       | Jiangsu           | Taixing               | 32°N, 120°E | 146 |
| Lice        | Jiangsu           | Taixing               | 32°N, 120°E | 37  |

DNA extraction from whole blood, ticks and lice
Aliquots (200 μL) of whole blood were used for DNA extraction with High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The DNAs were eluted with 200 μL Elution Buffer. Ticks and lice were individually homogenized in a shaker (Bertin Technologies, France) with four 3.0 mm ceramic beads for two periods of 15 s (3160 × g with a 15-s break in between). DNAs were extracted from the homogenates with the QIagen® DNA Mini Kit (Qiagen, Valencia, CA, USA), eluted in 200 μL of 1 × T10E0.1 buffer, and stored at −80 °C until PCR was performed.

Quantitative FRET-PCRs
Eight quantitative PCRs, seven for vector-borne agents and one for the mammalian HMBS gene as an endogenous internal control, were performed on a Roche LightCycler 480-II PCR Instrument. The qPCRs were performed as described previously for *Anaplasma* spp. [5], *Babesia* spp. [18], *Dirofilaria immitis* [19], *Ehrlichia* spp. [5], *Hepatozoon* spp. [20], *Leishmania* spp. [21], *Theileria* spp. [22] and the mammalian HMBS gene [23]. The positive PCR products were verified by gel electrophoresis and sequenced using forward and antisense primers (BGI, Shanghai, China).

Standard PCR
When *T. orientalis* was identified in the *Theileria* qPCR we used a standard PCR amplifying a 591 bp section (including 60-bp primers) of the 18S rRNA gene of *Theileria* spp. [22] to obtain longer sequences for analysis and further verification of identification.

Results
Information provided by veterinarians at the clinics where the study was performed indicated the vast majority of the dogs we sampled were pets that spent most of their time indoors. It was only very infrequently that the veterinarians noted ectoparasites on such dogs as they had very limited exposure to the outside environment and other animals. Ticks and lice were, however, found on the dogs in the Taixing commercial dog breeding facility which were housed more extensively. These were identified as *Rhipicephalus sanguineus* senso lato (*n* = 146) and *Linognathus setosus* (*n* = 37).

The qPCR for the HMBS gene was positive on all samples with 4.5 × 10^6 ± 3.9 × 10^5 copies/ml whole blood. Overall, 4.4 % (49/1117) of dogs were positive for at least one vector-borne agent and we obtained positive qPCR results for 4 of the 7 organisms we studied; there were only negative results for *Anaplasma* spp., *D. immitis* and *Leishmania* spp. At least one vector-borne agent was identified in 5 of the 10 provinces studied (Fig. 1). The
The prevalence of vector-borne agents was highest in the dogs from the commercial breeding facility in Taixing (24/97, 24.7 %) where external parasites were observed and collected. Six of the 146 (4.1 %) *R. sanguineus* sensu lato collected were positive for *E. canis* but none of the *L. setosus* had evidence of a vector-borne agent (Tables 2 and 3).

The most common organism we identified was *Hepatozoon canis* with 1.8 % (20/1114) of the dogs having PCR evidence of infection (Table 2). The prevalences of infection in Jiangsu (1.1 %; 3/277) and Xinjiang provinces (1.2 %; 1/86) were much lower than those found in Shaanxi (8.9 %; 5/56), Henan (4.9 %; 5/102) and Beijing (4.9 %; 6/134). Genomic sequencing and BLASTN demonstrated that the partial 18S rRNA gene sequences for the *H. canis* we identified and deposited in GenBank database (Gene Accession #: KP719091) were identical to one another, and also to that of a *H. canis* found in the spleen of a red fox in Austria (KM115995) (Table 3).

Overall, we found *Ehrlichia canis* DNA in 1.4 % of the dog blood samples (15/1114) and 4.1 % of the ticks (6/146). Ticks and most of the dogs positive for *E. canis* were from the Taixing commercial dog breeding facility (13/97 dogs, 13.4 %; 6/146 ticks, 4.1 %). Only 2 of the positive dogs were from veterinary clinics (Beijing) where their clinical records showed them to be anemic. The sequences of the *E. canis* we found in the dogs and ticks (KP719093, KP719094) were identical but showed 1 nucleotide mismatch with the sequence of the most closely related *E. canis* which was found in a dog in Japan (AF536827) (Table 3).

Thirteen dogs (13/1114, 1.2 %) and 5 ticks (5/146, 3.4 %) were positive for *Babesia* spp. and sequencing showed two *Babesia* spp. occurred, most commonly *B. vogeli* (11 dogs and all ticks) followed by *B. gibsoni* (2 dogs). The sequences of the *B. vogeli* (KP719088, KP719089) from the dogs and ticks were identical but had 1 mismatch with the sequence of a

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**Fig. 1** Prevalences of 4 vector-borne agents in 10 provinces of China. Dog blood samples (1114) were collected from ten provinces (in bisque) of China. The prevalences are shown for *Babesia vogeli* (red), *B. gibsoni* (black), *Hepatozoon canis* (green) and *Ehrlichia canis* (blue) in different colors.
B. vogeli reported in a dog from China (KJ939326). The B. gibsoni sequences (KP719090) were identical to each other and also to a B. gibsoni in a dog from Japan (LC012808) (Table 3).

A 5-year old, female Chihuahua dog with a perineal hernia seen in a veterinary clinic in Henan province was the only dog found to be positive for Theileria spp. The 18S rRNA sequence of the Theileria spp. we identified in our qPCR was identical to that of T. orientalis Thrissur 1 from India (KM609973) and Japan (XR_696404). Similarly, the sequence of the 591 bp amplicon of the 18S rRNA gene we obtained with a subsequent standard PCR [22] was also identical to that of T. orientalis (KM609973, XR_696404).

Multiple infections with vector-borne agents were rare with two dogs and a tick having evidence of infection with H. canis and B. gibsoni, one dog with E. canis and B. vogeli, and one tick with B. vogeli and E. canis.

Table 2 Prevalences of vector-borne agents detected by qPCR in dog bloods, ticks and lice in China

| Sample type | Source of samples | Percent (n) PCR positive |
|-------------|-------------------|-------------------------|
| Dog blood   | Beijing           | 4.5 % (6/134) 1.5 % (2/134) 0 % (0/134) 0 % (0/134) |
|             | Gansu             | 0 % (0/96) 0 % (0/96) 0 % (0/96) 0 % (0/96) |
|             | Guangdong         | 0 % (0/35) 0 % (0/35) 0 % (0/35) 0 % (0/35) |
|             | Henan             | 4.9 % (5/102) 0 % (0/102) 0 % (0/102) 1.0 % (1/102) |
|             | Inner Mongolia    | 0 % (0/82) 0 % (0/82) 0 % (0/82) 0 % (0/82) |
|             | Jiangsu           | 0 % (0/50) 0 % (0/50) 0 % (0/50) 0 % (0/50) |
|             | Taixing           | 0 % (0/97) 13.4 % (13/97) 11.3 % (11/97) 0 % (0/97) |
|             | Nanjing           | 2.3 % (3/130) 0 % (0/130) 1.5 % (2/130) 0 % (0/130) |
|             | Shanghai          | 0 % (0/84) 0 % (0/84) 0 % (0/84) 0 % (0/84) |
|             | Shaanxi           | 8.9 % (5/56) 0 % (0/56) 0 % (0/56) 0 % (0/56) |
|             | Xinjiang          | 1.2 % (1/86) 0 % (0/86) 0 % (0/86) 0 % (0/86) |
|             | Yunnan            | 0 % (0/162) 0 % (0/162) 0 % (0/162) 0 % (0/162) |
|             |                  | 1.8 % (20/1114) 1.3 % (15/1114) 1.2 % (13/1114) 0.1 % (1/1114) |
| Ticks       | Jiangsu           | 0 % (0/146) 4.1 % (6/146) 3.4 % (5/146) 0 % (0/146) |
| Lice        | Jiangsu           | 0 % (0/37) 0 % (0/37) 0 % (0/37) 0 % (0/37) |

Table 3 Comparison of DNA sequences identified in this study with those having similar gene sequences in GenBank

| Isolates identified in this study | Highly similar sequences in GenBank |
|----------------------------------|-----------------------------------|
| Species | GenBank # | Source | GenBank # | Source | Mismatch |
|--------|-----------|--------|-----------|--------|----------|
| E. canis | KP719093 | 13 dogs from Taixing 2 dogs from Beijing 6 ticks (R. sanguineus senso lato) from Taixing | AF536827 | Blood of dog from Kagoshima, Japan | 1/158 |
|        | KP719094 |        |           |        |          |
| B. vogeli | KP719088 | 11 dogs from Taixing 5 ticks (R. sanguineus senso lato) from Taixing | KJ939326 | Blood of Springer Spaniel from Nanning, Guangxi, China | 1/210 |
|        | KP719089 |        |           |        |          |
| B. gibsoni | KP719090 | 2 dogs from Nanjing | LC012808 | Blood of dog from Yamaguchi, Japan | 0/210 |
| H. canis | KP719091 | 3 dogs from Nanjing 6 dogs from Beijing 5 dogs from Zhengzhou 1 dogs from Urumchi 5 dogs from Yangling | KM115995 | Spleen of infected Vulpes vulpes from Lower Austria, Gaenseendorf, Austria | 0/144 |
| T. orientalis | N/A | 1 dog from Zhengzhou | KM609973 | Blood of infected Bubalus bubalis from India | 0/177 |
|        |           |        |           |        | 0/591     |
**Discussion**

To date there have only been a few studies on vector-borne agents in Chinese dogs [7–9, 24, 25] but our study has confirmed that *B. vogeli*, *B. gibsoni*, *E. canis*, *H. canis*, and *T. orientalis* or a closely related organism occur in China. The first three agents are very important pathogens of dogs and veterinarians in China should have an increased awareness of the possibility of infections in their canine patients and appropriate diagnostic tests and treatments should be made available. It is of note that the prevalences of infections were low in the dogs kept as companion animals and which were reported to seldom have ectoparasites. In contrast, ectoparasites were readily found on dogs from commercial dog breeding facilities and vector-borne agents were identified within these parasites. Veterinarians responsible for animals in such facilities should encourage tick control to prevent unnecessary morbidity and mortality. It should also be borne in mind that the prevalence and importance of vectors may vary considerably due to the influence of climatic and other environmental factors. China is a large country that can be divided into seven geographical regions. We studied dogs from each of these areas and found at least one vector-borne agent in five of the seven geographical regions. Future studies with more comprehensive and representative sampling should be performed to investigate the influence of climatic and environmental impact on the distributions of vectors and their agents in the different regions of China.

*Hepatozoon canis* was the most common vector-borne agent we identified and also the most widespread, being identified in dogs from 5 of the 10 provinces we studied. The organism also occurs widely around the world and, although moderate to severe disease can occur [26], most infections are sub-clinical.

The short (177 bp) and long (591 bp) 18S rRNA nucleotide sequences that we obtained for the *Theileria* sp. we identified were identical to that of *T. orientalis* from India (KM609973) and Japan (XR_696404). *Theileria orientalis* is normally found in yaks (Bos grunniens), cattle and buffaloes (Bos bubalis) and is transmitted by *Haemaphysalis* spp. [27, 28]. To the best of our knowledge, ours is also the first report that *T. orientalis* or a closely related organism might occur in dogs. How dogs become exposed to this organism in China and the pathogenicity and significance of infections requires further investigation.

Although Wang and Zhang et al. were unsuccessful in identifying *E. canis* infections in dogs in China using serology and PCR [29, 30], other workers have found serological and molecular evidence of infections in the blood of 2 % of dogs in Shenzhen [7–9] and the organism in *R. sanguineus* senso lato ticks from dogs in China [31]. We have now found PCR evidence that *E. canis* infections are relatively common in Taixing, Jiangsu province. Canine ehrlichiosis is a common disease of dogs around the world that is a cause of considerable morbidity and mortality [32]. There are no vaccines available and treatment can be problematic. The disease is best combated by preventing infections with appropriate tick control strategies.

Our findings of *B. vogeli* and *B. gibsoni* at relatively low levels in dogs from Taixing and Nanjing, Jiangsu province, is consistent with a previous report that levels of infection with *Babesia* spp. are low in domestic dogs in China [24]. The relatively low percentage (3.4 %) of ticks we found with *B. vogeli* in Taixing, Jiangsu province, was similar to that reported in other provinces, mainly Guangdong (3.6 %; 1/28), Hainan (3.3 %; 4/121) and Zhejiang (6.7 %; 1/15) [16]. It was, however, lower than that reported in Chengqing (25.0 %; 4/16) and Guangxi (12.5 %; 11/88), indicating there is considerable regional variation in infection rates in China. While *B. vogeli* infections can cause severe disease in puppies, greyhounds and immune-suppressed dogs, infections usually only result in mild signs or are subclinical [4, 33, 34], *B. gibsoni* on the other hand is generally regarded as being more pathogenic and can cause severe disease which responds poorly to drug therapy [35]. There are no vaccines and prevention depends on adequate tick control and, as *B. gibsoni* can be transmitted in blood and by bites during dog fights [4].

We were unable to identify *Anaplasma* spp., *Dirofilaria* spp. and *Leishmania* spp. in our study. This is in contrast to earlier reports from China where *A. phagocytophilum* was demonstrated by PCR in dog ticks from Suifenhe, Heilongjiang province (5.9 %), and in dog blood samples from nine provinces of China (10.9 %; 11/102) [36, 37]. Further, *D. immitis* infections have been demonstrated by microscopic examination and PCR test in dogs from Dandong, Liaoning province (24.0 %; 147/886), and *Leishmania* spp. demonstrated by real-time PCR test in dogs (24.8 %; 78/314) in Sichuan province [12, 13, 36, 37]. While the different observations might have been due to regional differences in infection rates, it is most likely they were due to demographic differences with the dogs in the above studies being more free-ranging and less well cared for and hence more likely to be exposed to ectoparasites. Larger and more inclusive studies are indicated to more accurately determine the prevalences and distribution of vector-borne agents in dogs in China.

**Conclusions**

In summary, we found the DNAs of *Babesia gibsoni* and *B. vogeli* (1.2 %), *Ehrlichia canis* (1.3 %), *Hepatozoon canis* (1.8 %), and *Theileria orientalis* (0.1 %) in the bloods of the dogs studied. Further, we found *Babesia*...
and Ehrlichia canis (4.1 %) in the R. sanguineus senso lato. Our data from 10 provinces in China show a wide range of important vector-borne pathogens occur in dogs and further larger scale studies are indicated to determine more accurate prevalence data for these agents.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
WC and ZJ participated in the design of the study and performed the statistical analysis. ZJ and XD carried out the experiments. SZ, SC, ZY, HY, DH, WL, El-Mahallawy, HS, XW, WH, LI, ZX KP, and GJ participated in the samples’ collection. WC, KP and ZJ drafted the manuscript. All authors read and approved the final manuscript.

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