

Molecular Identification and Prevalence of *Ehrlichia canis* and *Rhipicephalus sanguineus* (Acari: Ixodidae) Infecting Pet Dogs in Wenzhou, China

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ABSTRACT

Ehrlichia canis is an important zoonotic tick-borne pathogen transmitted by the tick *Rhipicephalus sanguineus* (Latrielle) that causes canine monocytic ehrlichiosis (CME) in dogs. We have investigated the prevalence of *R. sanguineus* and *E. canis* infection in pet dogs in Wenzhou, China. Serum samples were obtained from animal hospitals were examined for antibodies by commercial rapid in-clinic ELISA kits. Ticks were collected from dogs and molecular detection methods were used to identify the tick species and CME by amplification of the cytochrome oxidase subunit I and disulfide oxidoreductase gene, respectively. The results indicated that 1.29% of the serum samples were positive for *E. canis*, and 5.50% of dogs were infested with ticks. The Wenzhou samples of *R. sanguineus* exhibited a high homology (99.7%–99.8%) and these parasites showed a 99.1%–100% homology to previously reported isolates. The *E. canis* derived from *R. sanguineus* in the current study showed a similarity of 98.7%–99.7% to previously published isolates. Our results indicate that precaution should be employed for the potential threat posed by these ticks, especially to pet owners.

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Authors' Contribution

HL, YB and KL contributed in the plan of the study. HQL, YL, PG, WZ, MW, BH and ZZ performed the trial. KL and YB analyzed the data. HL and KL wrote the manuscript.

Key words

Rhipicephalus sanguineus, *Ehrlichia canis*, Pet dog, Prevalence, Molecular detection

INTRODUCTION

The Ixodidae ticks are obligate hematophagous ectoparasites, which are distributed globally (Zhang *et al.*, 2017). These ticks are considered the most important vectors of animal or wild lives' pathogens, and the second most important vector (next to mosquitoes) of human pathogens for the transmission of various kinds of tick-borne diseases (TBDs) caused by these pathogens (bacteria, viruses and parasites) (Zhang *et al.*, 2017; Papa *et al.*, 2017;

Zhao *et al.*, 2020). Emerging and re-emerging TBDs such as spotted fever, Lyme disease, babesiosis, forest encephalitis, Crimean-Congo hemorrhagic fever, anaplasmosis, and severe fever with thrombocytopenia syndrome are ubiquitous and easily disseminated (Malheirosa *et al.*, 2016; Zhang *et al.*, 2017). Also pathogenic and non-pathogenic tick-borne bacteria including *Babesia* spp., *Anaplasma* spp., *Theileria* spp. among others have been observed in animals (Benedicto *et al.*, 2020).

Ehrlichia canis can cause an important severe zoonotic TBD named canine monocytic ehrlichiosis (CME) in dogs (Montenegro *et al.*, 2017; Piantedosi *et al.*, 2017). This gram-negative intracellular bacterium is mainly transmitted by a common tick species named *Rhipicephalus sanguineus* (Piantedosi *et al.*, 2017). The

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symptoms of CME in dogs are high fever, depression, lethargy, myalgia, anorexia, lymphadenomegaly, anemia, splenomegaly, and thrombocytopenia (Mircean *et al.*, 2012; Escribano *et al.*, 2017). Human *E. canis* infection was first isolated from an asymptomatic human in Venezuela in 1996 and detected as clinical ehrlichiosis in Venezuela in 2006 (Perez *et al.*, 1996; Pére *et al.*, 2006). Since then, *E. canis* has been identified as a causative agent of human ehrlichiosis in Brazil, USA and Costa Rica (Diniz *et al.*, 2007; Nicholson *et al.*, 2010; Bouza-Mora *et al.*, 2017).

Previously, 36 tick species from more than 119 tick species were documented and demonstrated to transmit one or more diseases in China (Yu *et al.*, 2015; Zhang *et al.*, 2017; Zhao *et al.*, 2020). The prevalence of *E. canis* infection in dogs was found to be 2.04%–40% in some regions in China (Zhang *et al.*, 2017). However, until now, there is very little information about the tick species and *E. canis* infection in pet dogs in Wenzhou (northern latitude: 27°3′–28°36′; east longitude: 119°37′–121°18′), which is situated to the southeast of the Zhejiang province in China. The present study was undertaken to determine the prevalence and molecular identification of *Ehrlichia canis* and the vector *hipicephalus sanguineus* in pet dogs in Wenzhou, China.

MATERIALS AND METHODS

Ethics statement

Samples were obtained after due permission from the relevant institutions. All procedures were performed under the instructions and approval of the Laboratory Animals Research Centre of Zhejiang province in China and the Ethics Committee of the Wenzhou Vocational College of Science and Technology.

Serum samples and ticks collection

A total of 309 blood samples were collected from pet dogs (male 173, female 136) from the animal hospitals in Wenzhou, China during 2017–2018. The gender and age information were recorded in detail. Each blood sample was centrifuged at 1000×g for 15 min, and serum was separated and stored at -70°C for further analysis. Ticks were obtained from pet dogs without obvious symptoms visiting the University Veterinary Hospital, and ticks were washed by double distilled water. Then these ticks were identified morphologically by using a light microscope (Lu *et al.*, 2017). Ticks from the same dogs were pooled together. All the ticks were stored at -70°C until further testing.

Determination of antibodies against *E. canis*

Serums from pet dogs were examined for antibodies against *E. canis* by commercial rapid in-clinic ELISA (enzyme-linked immunosorbent assay) kits (SNAP®

4Dx®, IDEXX Laboratories, Westbrook, Maine, USA), according to the manufacturer's instructions. Each serum sample (150 µL) was mixed with 200 µL of the testing reagent and loaded into the device detection well.

DNA isolation, amplification and sequencing

The pooled adult ticks were washed thrice using distilled water and then cut into pieces using sterilized scissors, as reported previously (Licari *et al.*, 2017). The genomic DNA (gDNA) of ticks was prepared by using a DNA extraction reagent kit (TIANamp Genomic DNA Kit, Tiangen Biotech CO., LTD, Wuhan, China) according to the manufacturer's instructions. The eluted DNA was kept at -20°C prior to PCR analysis.

The cytochrome oxidase subunit I of the mitochondrial gene of ticks was amplified by utilizing the forward primer pairs LCO1490, 5'-GGTCAACAAATCATAAA-GATATTG G-3' and reverse primer HCO2198, 5'-TAAACT-TCAGGGTGACCAAAAAAT CA-3' (Licari *et al.*, 2017). The disulfide oxidoreductase gene of *E. canis* was amplified by using the forward primer ECAN5, 5'-GATGAT-GTCTGAAGATATGAAACAAAT-3' and reverse primer HE3, 5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3' (Campos-Calderón *et al.*, 2016). The PCR mixture contained 10 µl of autoclaved and distilled water, 8.5 µl of PCR buffer (10×), 2.5 µl of dNTPs (2.5 mM), 1 µl of DNA, 1 µl of Taq, 1 µl of each forward and reverse primer (working concentration: 10 µmol/L), to attain a total reaction volume of 25 µl. Each of the 32 PCR cycles consisted of 94°C for 45 s, 48°C for 30 s, and 72°C for 1 min after an initial hot start at 94°C for 3 min, and ended with 72°C for 5 min. The PCR products were analyzed using electrophoresis on a 1.0% agarose gel, followed by staining with ethidium bromide. The PCR electrophoresis products were purified using a Hi-TIANgel Midi Purification Kit (Tiangen Biotech CO., LTD, Beijing, China) according to the manufacturer's instructions; they were then sent for commercial sequencing by a company (Tsingke, Wuhan, China).

Sequence alignment and phylogenetic analysis

Multiple alignments were performed for the COI and dsb sequences relative to reference sequences available at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) by DNAMAN (5.2.9 Demo version). The phylogenetic analysis was conducted by the NJ (neighbor-joining) algorithm to determine the ticks and rickettsial species, respectively, using the MEGA (version 6.0) software, and the distances were computed using the Tajima-Nei means. The stability of branches was assessed after bootstrapping with 1000 replicates.

Statistical analysis

Statistical analysis was performed by employing the chi-square test with the IBM SPSS (Statistical Analysis System, Version 20.0.) software. The differences were considered significant for $P < 0.05$.

RESULTS

In the current study, 1.29% (4/309) serum samples were found to be positive for *E. canis* (Table I). The seroprevalence was 1.73% and 0.74% in male and female pet dogs, respectively, with no obvious difference in the two genders ($p = 0.441$, i.e. > 0.05) (Fig. 1). The prevalence was 2.80% and 1.41% in ≤ 1 and 1–3-year-old animals, respectively, with no clear difference among the dogs of the different ages ($p = 0.539$, i.e. > 0.05) (Table I).

Table I. Prevalence of *R. sanguineus* and *E. canis* infection in pet dogs in Wenzhou, China.

Variable	Category	Seroprevalence (%)/(No. positive/total samples)	
		<i>R. sanguineus</i>	<i>E. canis</i>
Gender	Male	5.20% (9/173)	1.73% (3/173)
	Female	5.88% (8/136)	0.74% ^a (1/136)
Age (years)	≤ 1	9.35% ^d (10/107)	2.80% ^b (3/107)
	> 1 and ≤ 3	0 (0/71)	1.41% (1/71)
	> 3 and ≤ 5	0 (0/29)	0 (0/29)
	< 5	6.86% ^c (7/102)	0 ^c
Total		5.50% (17/309)	1.29% (4/309)

^aDifference between the prevalence of *R. sanguineus* and *E. canis* infection in female dogs was found to be statistically significant ($p = 0.018$, i.e. < 0.05 , $\chi^2 = 5.631$); ^bA significant difference between the prevalence of *R. sanguineus* and *E. canis* infection in dogs with an age ≤ 1 year was observed ($p = 0.045$, i.e. < 0.05 , $\chi^2 = 4.013$); ^cA significant difference between the prevalence of *R. sanguineus* and *E. canis* infection in dogs with an age > 5 years was observed ($p = 0.007$, i.e. < 0.01 , $\chi^2 = 7.249$); ^dA noteworthy difference was observed between the prevalence of *R. sanguineus* infection in dogs with an age ≤ 1 year and those with an age > 1 year and ≤ 3 years ($p = 0.008$, i.e. < 0.01 , $\chi^2 = 7.030$); ^eA conspicuous difference was observed between the prevalence of *R. sanguineus* infection in dogs with an age > 5 years and those with an age > 1 year and ≤ 3 years ($p = 0.024$, i.e. < 0.05 , $\chi^2 = 5.078$).

A total of 17 out of 309 (5.5%) dogs were found to be infected by ticks (Table I). The prevalence was 5.20% and 5.88% in male and female dogs, respectively. The prevalence was 9.35% and 6.86% in dogs ≤ 1 and ≥ 5 years of age, respectively. The prevalence of tick infection was significantly higher in dogs with an age ≤ 1 year ($p < 0.01$) and < 5 years ($p < 0.05$) than in dogs with an age > 1 year and ≤ 3 years, respectively (Table I). There were noteworthy differences between the prevalence of *R. sanguineus* and

E. canis infection in female dogs ($p < 0.05$). Difference between the prevalence of *R. sanguineus* and *E. canis* infection in dogs with an age ≤ 1 year ($p < 0.05$) and > 5 years ($p < 0.01$) was found to be significant (Table I).

COI genes of 17 pooled tick samples and dsb genes from only one sample were amplified. For testing dogs whose tick pool samples were not analyzed for dsb genes, we utilized the commercial Canine Distemper Virus/Canine parvovirus Antigen Test Kits (Korea); it was found that these dogs were infected with either CDV or CPV. The COI gene and dsb gene sequences were submitted to the GenBank of NCBI with accession numbers of MG969504–MG969508.

The sequence alignment phylogenetic analysis showed that the tick infecting pet dogs in the Wenzhou region was *R. sanguineus* (Fig. 1). The Wenzhou isolates had a high homology, i.e. a 99.7%–99.8% homology to one other, while the homology of the present isolates to previously reported isolates was 99.1%–100% (Fig. 1). The rickettsia derived from *R. sanguineus* in the current study were identified as *E. canis* via phylogenetic analysis (Fig. 2). The Wenzhou *E. canis* isolate presented a high similarity of 98.7%–99.7% to the previously published isolates (Fig. 2).

The available reference sequences of COI are shown in Figure 1. The reference sequences of dsb are shown in Figure 2.

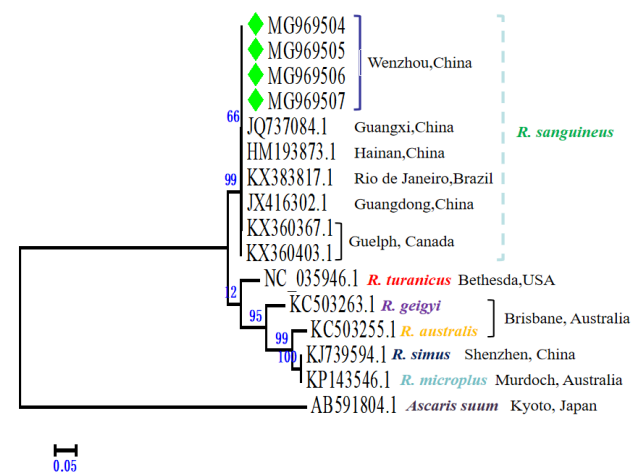


Fig. 1. Phylogenetic tree for *R. sanguineus* based on partial COI gene sequences. Phylogenetic tree based on partial COI gene sequences of *R. sanguineus* was constructed using the neighbor-joining method with Kimura two-parameter analysis and bootstrap analysis of 1000 replicates. The numbers on the branches indicate the percentage of replicates that reproduced the topology for each clad. The green rhombi indicate the sequences acquired from the current study (*Ascaris suum* was used as an outgroup).

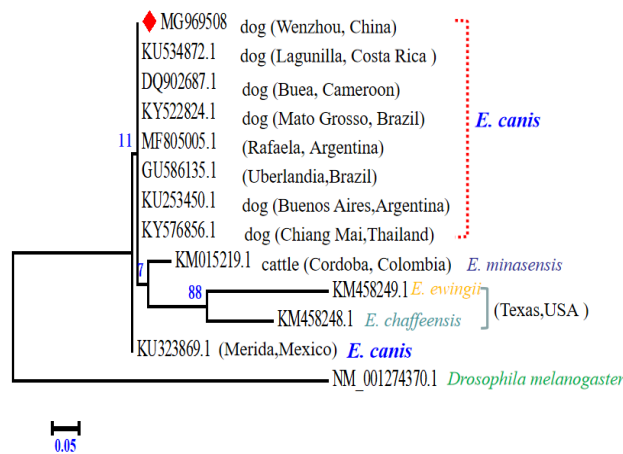


Fig. 2. Phylogenetic tree for *E. canis* based on partial *dsb* gene sequences. Phylogenetic tree based on partial *dsb* gene sequences of *E. canis* was constructed by employing the neighbor-joining method with Kimura two-parameter analysis and bootstrap analysis of 1000 replicates. The numbers on the branches indicate the percentage of replicates that reproduced the topology for each clads. The red rhombi indicate the sequences acquired from the current study (*Drosophila melanogaster* was used as an outgroup).

DISCUSSION

The importance of the potential distribution of the ticks and their species lays a foundation for possible threat prediction and implementation of suitable epidemiologic strategies using DNAMAN (5.2.9 Demo version) (Campos-Calderón *et al.*, 2016). The diversity of ticks is mainly affected by biotic (vegetation, host) and abiotic (climate, temperature) factors (Campos-Calderón *et al.*, 2016). Therefore, it is necessary to monitor tick infections in pet dogs from the Wenzhou region with an average temperature of 17.3–19.4°C and rainfall of 1113–2494 mm. In the current research, it was seen that the prevalence of tick infection in dogs was 5.50%. The molecular characterization of parasites is crucial for understanding the epidemiology of their infections and their control (Li *et al.*, 2016). Previously, genomic regions such as COI, COII, NADH dehydrogenase subunit 1, and the internal transcribed spacers of nuclear ribosomes have been used for phylogenetic studies of different parasitic species (Li *et al.*, 2016, 2017). In the present study, *R. sanguineus*, with a high homology to previously reported isolates with regards to the COI gene was detected (Fig. 1). This is consistent with results from a previous study, where *R. sanguineus* was reported in an imported dog (Wright *et al.*, 2018).

E. canis causes CME, which severely affects domestic dogs (Montenegro *et al.*, 2017). In the present study, the

serum prevalence of *E. canis* infection in dogs was 1.29%, which is in line with its prevalence in dogs (1.3%) in south-eastern China (Zhang *et al.*, 2017). However, the current prevalence of infection was notably lower than the prevalence of *E. canis* infection in dogs from Italy (7.6%), Thailand (9.88%), and Costa Rica (38.2%) (Montenegro *et al.*, 2017; Piantedosi *et al.*, 2017; Kaewmongkol *et al.*, 2017). This difference may be because of the difference in climate, temperature, and host abundance (Campbell-Lendrum *et al.*, 2015). To confirm *E. canis* infection, we amplified the *dsb* genes of *E. canis* from the pooled tick samples; we obtained one *E. canis* sequence, which had a high similarity of 98.7%–99.7% to previously reported isolates, as revealed by the analysis of pair-wise distance of the COI gene from *R. sanguineus* (Fig. 2).

A previous study indicated that dogs in subtropical climates may be at higher risk for infection by *R. sanguineus* (Aktas *et al.*, 2017). The current study found that *R. sanguineus* infects pet dogs in Wenzhou, which has a subtropical climate, contributing to the spread of ticks. From the present results, we can demonstrate that these ticks may pose a threat to public health, as they carry zoonotic TBD pathogens such as *E. canis*. Therefore, efficient precautions should be taken against the potential threat posed by the ticks, especially to pet owners.

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Statement of conflict of interest

The authors have declared no competing interest.

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