First Molecular Detection of Piroplasm Infection in Pet Dogs from Gansu, China

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Babesiosis, the hemolytic disease caused by Babesia, which is a tick-transmitted obligate intraerythrocytic protozoan parasite. This disease is responsible for significant mortality and morbidity rates and enormous economic losses to the livestock industry in tropical and subtropical regions worldwide. In this study, blood samples were collected from 141 pet dogs from Gansu, China, and analyzed for Babesia or Theileria spp. infection using specific PCR and sequencing based on 18S rRNA gene fragments. The results indicated that 18S rRNA gene sequences from 11 samples were similar to the 18S rRNA gene sequences in Babesia canis vogeli (2) and Theileria sinensis (9). The total infected rates of B. canis vogeli and T. sinensis were 1.4% (2/141) and 6.4% (9/141), respectively. This represents the first molecular report of T. sinensis in dogs worldwide and of B. canis vogeli in dogs from Gansu province of China. Furthermore, the finding of T. sinensis in dogs may represent the common infection of this parasite occurring in Gansu.

Keywords: pet dog, piroplasm, detection, Gansu, infection

INTRODUCTION

Babesiosis is a haemoparasitic disease, caused by the intraerythrocytic multiplication of protozoa of the genus Babesia and transmitted by ticks. It is a frequent infection of domestic and wild animals worldwide, including humans (Telford et al., 1993; Bush et al., 2001). The clinical symptoms of babesiosis are due to the repeated asexual rounds of multiplication of parasites inside of host erythrocytes, and are usually characterized by fever, depression, hemolytic anemia, hemoglobinuria, icterus, finally resulting in death in severe cases if not treated. The prevalence of babesiosis correlates with the geographic distribution and activity of vector ticks. Furthermore, environmental conditions changing, including global warming, favors tick survival and reproduction, which correlate with a significant increase in the abundance of ticks (Slenning, 2010). This disease can be responsible of great direct and indirect economic losses, due to the death of the animals, a reduction in the production or restriction in animal movements (McCosker, 1981).

Canine babesiosis is a common and clinically significant emerging hemoprotozoan infection of domestic dogs and wild canids geographically widespread by tick borne pathogen Babesia (Vichová et al., 2016). The clinical characteristics include fever, haemolytic anemia, thrombocytopenia, and vomiting. The earliest descriptions of intraerythrocytic parasites in dogs were in Africa in 1896,
and the first documented case of canine babesiosis was in the United States in 1934 (Adaszek and Winiarczyk, 2008). Recently, six main Babesia species, responsible for canine babesiosis, have been reported in the world, including the large form of B. canis (three distinct subspecies B. canis canis, B. canis rossi, and B. canis vogeli) and the small form of B. gibsoni, B. conradae, and B. vulpes (Zähler et al., 2000; Boozer and Macintire, 2003; Irwin, 2009, 2010; Matijatko et al., 2012; Berzina et al., 2013; Kamani et al., 2013; Baneth et al., 2015). Moreover, two large Babesia spp. have been reported, one is distributed across North Carolina, New Jersey, New York, and Texas (Birkenheuer et al., 2004; Holman et al., 2009; Sikorski et al., 2010), and another one was only reported in a dog from Great Britain (Holm et al., 2006). Arthropod vectors of these two Babesia species have not been identified.

Little information is available on the prevalence of piroplasmosis in dogs in Gansu province, China. Therefore, the aim of the present study was to investigate the occurrence of Babesia and Theileria spp. infections in pet dogs from Gansu province of China.

**MATERIALS AND METHODS**

**Animals and Sample Collection**

A total of 141 blood samples were collected from randomly selected pet dogs (84 males and 57 females) of different breeds in a pet clinic. Venous blood samples were collected into EDTA tubes between March 2015 and March 2016. The ages of the dogs were between 2 months and 18 years. One hundred of the pet dogs were without clinical symptom, and 41 animals presented with clinical signs: fever, pale mucous, vomiting, cough, and thrombocytopenia, and thus were suspected to have haemoparasite infection.

**Genomic DNA Extraction, PCR Amplification and Sequencing**

Genomic DNA was isolated from 300 µl of each blood sample using a QIAamp DNA Blood Mini Kit (Gentra, United States) according to the manufacturer’s instructions. The DNA samples were stored at −20°C until further use. Genomic DNA from Babesia sp. Lintan (Babesia cf. motais) and T. ovis was used as the positive control and distilled water was used as the blank control.

For the molecular detection and identification of piroplasm parasites at the molecular level, PCR amplification of the 18S rRNA gene was performed with the genomic DNA of all samples. A set of forward and reverse primers (Piro1-S: 5′-CTTGACGGTAGGTATTGCC-3′, Piro3-AS: 5′-CCITCCCTTAATGTAAGGTTCAC-3′) was used to amplify a gene fragment of 1400 bp (Yang et al., 2014), after which a nested-PCR was performed on primary PCR products with internal primers (PIRO-A: 5′-ATACCGTTGTAATTGTAGG-3′ and PIRO-B: 5′-TGTTATCTTTGTACATTACC-3′) to amplify a gene fragment of 406–421 bp (Olmeda et al., 1997). The PCR amplified conditions of the 18S rRNA gene were previously described by Yang et al. (2014). All 114 samples of secondary PCR products were subjected to electrophoresis on 1.5% agarose gels treated with GoldView I nucleotide stain (Solarbio) and visualized under UV illumination for the expected size of amplified fragments by comparison to molecular weight marker.

The positive PCR products with 18S rRNA gene fragments of 11 isolates were purified using a MiniBEST DNA Fragment Purification Kit (TaKaRa), cloned into a pGEM-T Easy Vector System (Promega), and then transformed into Escherichia coli JM109 Chemically Competent Cells (TaKaRa) according to the manufacturers’ instructions. Colonies were selected by direct colony PCR using vector primers. Ten sub-colonies from each sample were selected for sequencing.

The 11 partial 18S rRNA sequences obtained were subjected to a blast search on the NCBI website¹ using the BLASTn program and deposited in GenBank under accession nos. KY608898-KY608908. Multiple sequence alignments were analyzed using Clustal W 2.0.12 software. A phylogenetic tree was constructed with the sequences obtained in this study and sequences of the 18S rRNA genes of the main Babesia and Theileria species available in GenBank using Neighbor-Joining in MEGA 7 software (Kumar et al., 2016).

**Ethics Statement**

This study was approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (No. LVRIAE2013-010). All pet dogs were handled in accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China.

**RESULTS AND DISCUSSION**

Vector-borne infections in dogs have been increasing worldwide. However, few studies have been performed on piroplasm in dogs in Gansu. In 2015, there was a report investigating prevalence of tick-borne pathogens in 10 provinces of China, including Gansu province, but no samples indicated Babesia or Theileria species infections in dogs from Gansu (Xu et al., 2015).

In our study, out of the 141 dogs sampled, 11 positive samples for piroplasm infections were found, including 2 (1.4%) infected with B. canis vogeli and 9 (6.4%) infected with T. sinensis. Two pet dogs (one male, Shiba Inu and one female, mixed-breed) infected with B. canis vogeli were both 2 months old and showed the clinical symptom of fever. Studies have revealed that young animals are susceptible to B. canis vogeli infection and their clinical presentations are more severe (Greene, 2011). At present, B. canis vogeli and B. gibsoni have been reported to infect dogs in Jiangxi, Fujian, Anhui, Jiangsu, Chongqing, Guangdong, Guangxi, Hainan, Zhejiang, Shanghai, and Shandong provinces (Shen et al., 1997; Wei et al., 2012; Cao et al., 2015). The transmission vector for B. canis vogeli is Rhipicephalus sanguineus, a tick that is distributed in 15 provinces of China, including Gansu (Teng and Jiang, 1991). No B. gibsoni infections were found in pet dogs in our study. T. sinensis, which normally infects cattle, was found to infect two pet dogs (male, 6 years old, asymptomatic) belonging to the

¹http://blast.ncbi.nlm.nih.gov/Blast.cgi
FIGURE 1 | Phylogenetic tree of the nucleotidic sequences of *Babesia* and *Theileria* spp. 18S rRNA obtained from pet dogs in this study and deposited in GenBank from different piroplasm species, the isolate, host, countries and accession numbers are shown after species name. The 18S rRNA sequences obtained in this study were indicated with bold triangle. The tree was inferred using the neighbor joining method of MEGA7, bootstrap values are shown at each branch point. Numbers above the branch demonstrate bootstrap support from 1000 replications. All sites of the alignment containing insertions–deletions, missing data were eliminated from the analysis (option "complete deletion").
Bichon Frise breed; 2 Nisos dogs (male, one was 2 months old and one was 5 months old) with the clinical feature of fever; 
one Husky dog (male, 7 months old) with a cough; and four 
mixed-breed dogs (three males, two of them are 3 months old and 
another one was 8 years old; one female, 4 months old) with 
the clinical features of fever, vomiting and cough. Several studies 
based on molecular methods have revealed that dogs could be 
sporadically infected by the genus Theileria, including Theileria 
equi (Beck et al., 2009), T. ovis (Takeet et al., 2017), T. annulata 
(Criado et al., 2006; Aktaş et al., 2015) and T. orientalis (Xu 
et al., 2015), from Croatia, Spain, Turkey, Iran, and China. These 
parasites are usually found in horses, sheep and cattle. T. sinensis 
is distributed only in Gansu Province China and is transmitted by 
Haemaphysalis qinghaiensis (Zhao et al., 2017). In our study, 
records from dogs infected with B. canis vogeli and T. sinensis 
provided by veterinarians from clinics indicated that these pet 
dogs spent most of their time indoors and they had limited 
exposure to the outside environment with active R. sanguineus and 
H. qinghaiensis ticks. Thus, how these dogs were exposed to 
B. canis vogeli and T. sinensis requires further study. Possibilities 
of blood transfusion and vertical transmission might be existed. 

A phylogenetic tree, based on 18S rRNA sequences (n = 11) 
determined in the present study and sequences of this gene 
deposited in GenBank from different species, was constructed by 
Neighbor-Joining method using the software MEGA 7 (Figure 1). 
In general, the tree indicated that the 18S rRNA sequences 
formed two clades, including Theileria spp. clade and Babesia 
sp. clade. Nine Gansu isolates formed two sub-branches in the 
first clade, forming a sister clade with all the Theileria spp. 18S 
rRNA sequences from our study, and formed on the same sub-
clade with T. sinensis, which normally infected cattle. Two Gansu 
isolates formed a sub-branch with B. canis vogeli isolates from 
Brazil and Italy. Other canine Babesia species formed separated 
sub-clusters on Babesia spp. clade, including B. canis canis, 
B. gibsoni, B. canis rossi clusters, while B. vulpesm, B. conradi 
and Babesia sp. Coco formed single clusters, respectively. Taken 
together, based on the sequences and phylogenetic analysis, the 
isolates from pet dogs might be infected two piroplasm species, 
T. sinensis and B. canis vogeli.

To the best of our knowledge, this is the first report of 
B. canis vogeli in dogs from Gansu province and the first report of 
T. sinensis in dogs in the world. The results of this study 
provide evidence for the presence of two distinct piroplasm 
parasites among the canine population from Gansu that had 
previously not been molecularly documented. It is suggested 
that increasing piroplasm parasite infections in pet dogs might 
pose an increased health threat for pet dogs. Dog owners and 
veterinarians should be better informed on the possibility of 
infections of canine piroplasm in pet dogs so that appropriate 
prevention and treatment measures can be adopted.

AUTHOR CONTRIBUTIONS

QN and YP carried out the experiments, including PCR, cloning, 
sequencing and data analysis. QN drafted the manuscript. JY and 
YC collected samples, ZL, SG, GG, GL, JL, and HY supervised 
all work. All authors read and approved the final version of the 
manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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