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Short Communication

Molecular detection and genetic diversity of *Babesia canis canis* in pet dogs in Henan Province, China

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ABSTRACT

Canine babesiosis is an important tick-borne disease caused by *Babesia* spp. A total of 130 blood samples were randomly collected from pet dogs in Zhengzhou city, Henan Province, China, and screened for the presence of piroplasms with nested PCR and gene sequencing targeting the 18S rRNA gene. On the basis of blast analysis of the 18S rRNA gene sequences, our results revealed that seven dogs (5.4%) were infected with *Babesia canis canis*. The sequences were compared with those in GenBank, and alignments showed that all *B. canis canis* isolates belonged to genotype B. This is the first report of *B. canis canis* infection in dogs in China.

1. Introduction

Canine babesiosis is caused by obligate intraerythrocytic protozoa of the genus *Babesia*, and is transmitted by tick vectors. The most common clinical signs of infected dogs are fever, anemia, icterus, hemoglobinuria/bilirubinuria, anorexia, weight loss, and in some cases death [4]. The severity of the disease depends on several factors, including the species of the parasite and its genotype, and the host's immune status [1,2,12].

Based on morphology, *Babesia* spp. are classified as large or small [15]. Three large *Babesia* species have been identified in domestic or stray dogs: *Babesia canis canis*, *Babesia canis rossi*, and *Babesia canis vogeli* [5,14]. In addition, an unnamed *Babesia* species that is closely related to *Babesia bigemina* has been described in North Carolina in the United States [4]. Three small *Babesia* species are known to infect canines, *Babesia gibsoni*, *Babesia conradae*, and *Babesia microti*-like spp. [3,19].

In China, the first case of canine babesiosis caused by *Babesia gibsoni* was reported in Henan Province in 1985 [16]. Thereafter, cases of canine babesiosis had been reported in several provinces across China [9]. Molecular epidemiological surveys of canine babesiosis have been conducted in Gansu and Hubei Provinces. The available studies have revealed that two *Babesia* spp. were responsible for canine babesiosis in those regions, namely *Babesia canis vogeli* and *Babesia gibsoni* [6,17]. Limited information is available about canine babesiosis in Henan Province, located in the central area of China, where the average temperature ranges from 0 °C to 15 °C during the winter. In this study, to evaluate the status of *Babesia* species infection in winter, blood samples were randomly collected from pet dogs in this area, between November 2017 and February 2018.

2. Materials and methods

2.1. Blood samples and DNA isolation

From November 2017 to February 2018, a total of 130 blood samples were randomly collected from pet dogs (61 males and 69 females, 3 months to 14 years old) and taken to a veterinary hospital located in Zhengzhou city, Henan Province, China. Blood samples were collected into EDTA-coated vacutainer tubes, and transported in iceboxes to the laboratory of Vectors and Vector-Borne Diseases (VVBD), Lanzhou Veterinary Research Institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS). Genomic DNA was extracted from 200 µl of each blood sample using a commercial DNA extraction kit, according to the manufacturer's instructions (Qiagen DNA Blood Mini-Kit, Germany). DNA samples were stored at −20 °C until use.

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2.2. Detection of piroplasms

A nested PCR (nPCR) was conducted to detect the presence of piroplasms in the blood samples, according to a report by Yang et al. [18]. In the first round of PCR, a set of primers reported previously (Piro1-S: 5′-CTTGACGGTAGGGTATTGGC-3′, Piro3-AS: 5′-CCTTCCTTTAAGTGA TAAGGTTCAC-3′) was used to amplify a 1400 bp gene fragment of 18S rRNA. Genomic DNA extracted from *Babesia bovis* and distilled water were used as the positive and negative controls, respectively. In the second round of reactions, 1 μl of the products of first-round reactions was used as template. Minor modifications were made to the primers, according to 18S rRNA gene sequences of canine piroplasms available in GenBank, to amplify an 407 bp fragment, Piro-A: 5′-ATTACCCAAT-MCBGACACVGKG-3′ and Piro-B: 5′-TTAAATACGAATGCCCCCAAC-3′ [10]. The PCR parameters and composition of the PCR mixture have been described previously [18]. Briefly, reactions were conducted in a total of 25 μl, including 2.5 μl of 10 × PCR buffer, 2.0 μl of dNTP (2.5 mM each), 1.25 U of *Taq* DNA polymerase (TaKaRa, Dalian, China), 1.0 μl of template DNA, 1.0 μl of each primer (10 pmol), and 16.25 μl of double distilled water. The PCR reaction was started with a one-step initial denaturation at 95 °C for 3 min, which was followed by 35 cycles of denaturation at 95 °C for 30 s, 55 °C for 30 s and extension at 72 °C for 90 s, with a final extension step at 72 °C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel containing gold view dye (SolarBio, Beijing, China) in Tris-acetate-EDTA buffer at 120 V for 30 min, and then examined under UV light. Positive amplicons were purified using a gel DNA purification kit (ZYMO, USA), cloned into pGEM-T Easy vector (Promega, USA), and then transformed into *Escherichia coli* JM109 competent cells. For each amplicon (407 bp), three positive clones, identified by PCR amplification with the pair of primers SP6 and T7, were sequenced using BigDye Terminator Mix (Genscript, Nanjing, China).

2.3. PCR amplification of the large fragments of the 18S rRNA gene

A long fragment of 18S rRNA was amplified with an nPCR assay to identify the species of *Babesia* that infects pet dogs. A primer pair (PIRO-F; 5′-GAAAYTGCGA ATGGCTCATTAM-3′ and PIRO-R; 5′-CACC GGATCACTCGATCGGTAGG-3′) was designed according to the sequences alignment of the 18S rRNA gene of *Babesia* species that infect companion animals, and was used in the first round PCR. After that, in the second round of PCR 1, 418 bp of DNA fragments were generated using another pair of primers, PIRO-nest F (5′-GGATAACCGTGSTAAT-TSTAGGGC-3′) and PIRO-nest R (5′-GTGTGTACAAAG GGCAGGG ACG-3′). The PCR conditions, gene cloning, and sequencing were the same as described in Section 2.2.

2.4. Sequences analysis

The 18S rRNA gene sequences were subjected to blast analysis on the NCBI website using the BLASTn program. The representative sequence was deposited in the GenBank database with accession number MK256974, MK571830- MK571835.

To assess *B. canis canis* genotypes [1,7], the sequences obtained in this study were compared with those of group A (AY703072) and group B (AY649326). Furthermore, the sequences obtained in the present study, which were previously submitted to GenBank, were aligned using Clustal W in MegAlign 7.0 software (DNAStar, USA). The consensus sequences were manually trimmed to 1251 bp in the final data set and a phylogenetic tree was created using the maximum likelihood method with MEGA 7.0 software.

3. Results

The results of nPCR revealed that the piroplasm prevalence was 5.4% (7/130) in the domestic dogs, basic information provided in Table 1. To confirm the classification of the *Babesia* species, large fragments of the 18S rRNA gene were amplified from the each sample that was positive for infection with *Babesia*. The results of sequence analysis were the same as with the short sequences. The seven nucleotide sequences from canine samples were identical to each other and had 99.6–99.9% identity with *B. canis canis* derived from dogs in Croatia (AY072926) and Estonia (KT008057). Comparison of the *B. canis canis* sequences obtained in this study with genotypes A and B revealed that all of our isolates were classified as genotype B (Fig. 1). The phylogenetic tree that we constructed also indicated that the 18S rRNA gene sequences obtained in this study were grouped as *B. canis canis* (Fig. 2).

4. Discussion

Early in 1985, the first documented case of canine babesiosis in China was reported in Henan Province. Subsequently, sporadic cases of this disease have been reported in Shandong, Jiangsu, Anhui, Zhejiang, Jiangsu, Jiangxi, and Guangxi Provinces. These reports indicated that *B. gibsoni* and *B. canis vogeli* were the causative agents of canine babesiosis in those areas. The prevalence of *B. gibsoni* and *B. canis vogeli* was 1.5–11.86% and 1.2–11.3%, respectively [6,17]. The epidemiology of several tick-borne pathogens in specific areas, such as *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia burgdorferi* and *B. canis canis*, is greatly affected by the spatial and temporal distribution of tick vectors [7,8]. In China, most of the previous studies of canine piroplasmosis were conducted in spring and autumn. Therefore, our study evaluated the infection status and classification of the piroplasm species in dogs during winter. Our results revealed that *B. canis canis* was prevalent in this area; however, *B. gibsoni*, which has a prevalence of 6.67% according to Zhang et al. [20], was not detected in this study. A similar situation was described in Southern France, where *B. canis canis* infection was only reported in winter or very early spring [11]. However, further studies are needed to elucidate the impact of temporal and seasonal changes on parasite distribution.

On the basis of 18S rRNA gene sequence analysis, genetic heterogeneity of *B. canis canis* has been documented in Poland, Lithuania, and Hungary. Two genotypes of *B. canis canis*, genotypes A and B, have been identified so far, and have shown to have differing virulence [2]. Based on clinical signs and mortality rates, *B. canis canis* genotype B is considered more virulent than *B. canis canis* genotype A [1,2,13]. The clinical manifestations of *B. canis canis* infection are moderate to severe, and include petechiae, epistaxis, vomiting, anemia. The severity of disease depends on the species of *Babesia* causing infection and other factors such as immune status, age. In the present study, the clinical signs of dogs infected with *B. canis canis* were fever, vomiting, cough, and lethargy. It has not been reported whether dogs infected with *B. canis canis* genotype A present a similar clinical manifestation. Therefore, we cannot draw the conclusion that dogs in this area are exposed to pathogenic *B. canis canis* genotype B. More data are needed to explore the clinical significance of finding this genotype of *B. canis canis* in dogs in this area.

Genetic variability is a vital mechanism for piroplasm survival in hosts. Molecular epidemiological surveys and studies of genetic diversity are essential to provide important information for the development of vaccines and for taking appropriate preventive measures against canine babesiosis. Our study is the first to describe *B. canis canis* infection in pet dogs in China. It indicates that increasing numbers of piroplasm species infecting dogs may pose a severe health threat to pet dogs in China. Our data provide valuable insight into the distribution of canine *Babesia* species in China.

5. Conclusion

This is the first report to describe the prevalence of *B. canis canis* in China. Two canine *Babesia* species that have been previously reported

Table 1
Basic information on the animals sampled and the *Babesia* species identified in the present study.

Samples ID				Clones	Pathogen	Clinical symptom	Accession number(s)	Blood work[a]	Diagnostic investigation
Breed	ID of animal	Age	Sex						
Welsh Corgi	Hen171001	1 year	Male	2A	*B. canis canis*	Fever,	MK571830	RBC: 4.3 HGB: 90 HCT: 27.6%	PCR: positive Blood smear: Not tested
Bichon frise	Hen171103	9 year	Female	3A	*B. canis canis*	Fever and vomiting	MK571831	Not tested	PCR: positive Blood smear: Not tested
Poodle	Hen171012	8 month	Male	12A	*B. canis canis*	Cough	MK571832	RBC: 3.9 HGB: 86 HCT: 26%	PCR: positive Blood smear: Not tested
Husky dog	Hen171014	6 month	Male	15A	*B. canis canis*	Loss of appetite	MK571833	Not tested	PCR: positive Blood smear: Not tested
Poodle	Hen171033A	2 month	Female	33-AA	*B. canis canis*	lethargy	MK571834	RBC: 4.1 HGB: 76 HCT: 25.7%	PCR: positive Blood smear: Not tested
Miniature schnauzer	Hen171033B	8 month	Female	33-BA	*B. canis canis*	Fever and vomiting	MK571835	RBC: 4.1 HGB: 85 HCT: 23.3%	PCR: positive Blood smear: Not tested
Japanese Spitz	Hen171026-1	2 year	Male	26-1A	*B. canis canis*	Fever and cough	MK256974	RBC: 3.95 HGB: 92 HCT: 26.3%	PCR: positive Blood smear: Not tested

[a] Reference ranges: red blood cell (RBC) count, 4.6–10 × 10^9/L; Hemoglobin concentration (HGB), 93–153 g/L; Haematocrit (HCT), 28–49%.

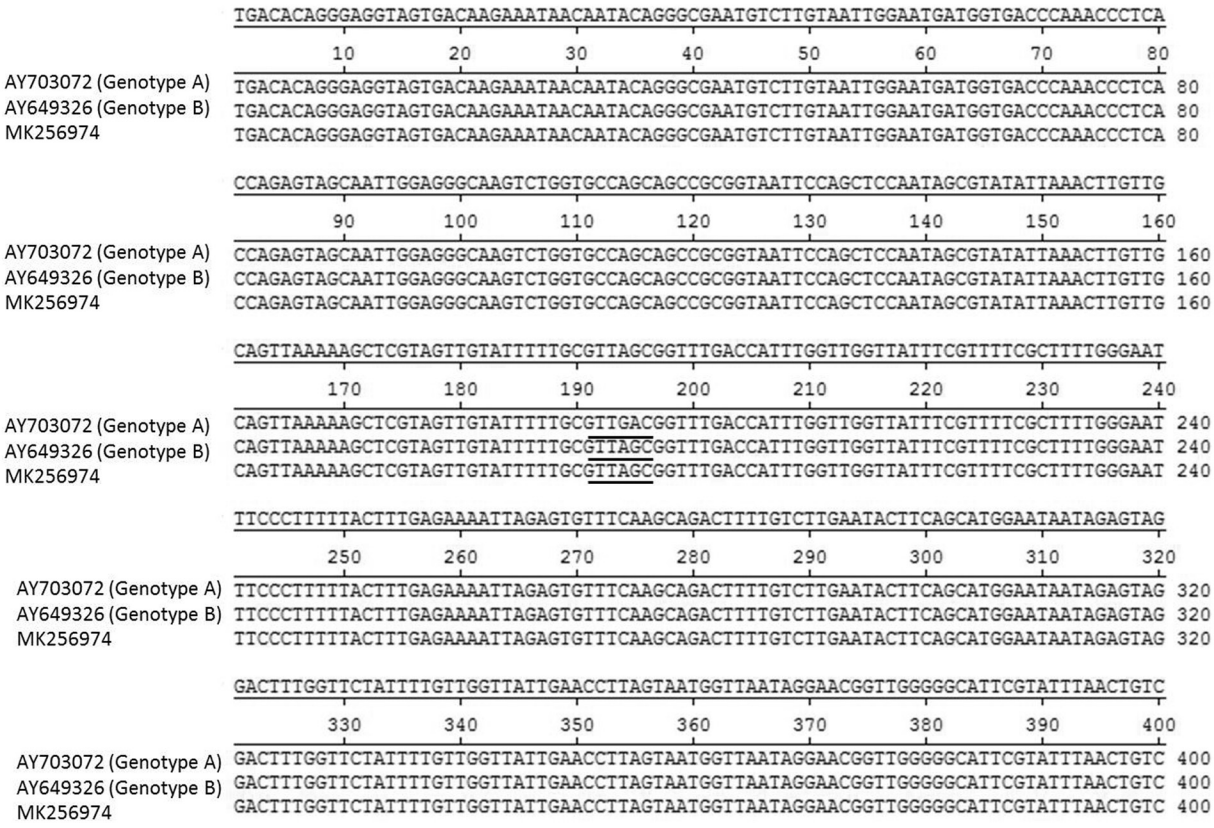

Fig. 1. Comparison of nucleotide sequences of *B. canis canis* isolates with those of genotypes A and B. *Hinc* II restriction sites are underlined. According to the restriction pattern of *Hinc* II, isolates of *B. canis canis* can be defined as genotype A or B.

in China (*B. canis vogeli* and *B. gibsoni*) were not detected in Henan Province. All isolates of *B. canis canis* were classified as virulent genotype B. The finding of this study provides essential data for the control and prevention of the canine babesiosis in China.

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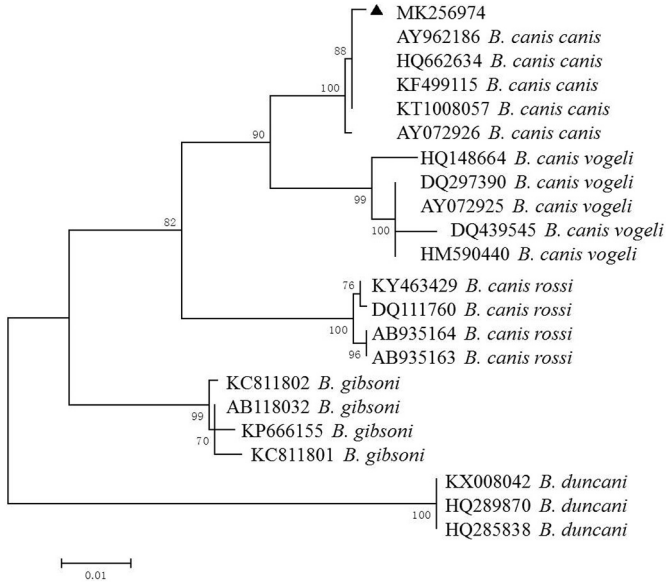

Fig. 2. Phylogenetic tree inferred from the sequences of the *Babesia* spp. 18S rRNA gene (around 1200 bp). The sequence newly generated in this study is indicated with a bold triangle. Bootstrap values obtained from 1000 replications are provided on each branch.

Conflicts of interest

The authors declare no competing financial interests.

Ethics statement

Our study was approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, CAAS (Permit No. LVRIAEC-2018-001). All the procedures were conducted according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Informed consent was obtained from the pet owners, who agreed to participate in this study.

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