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RESEARCH ARTICLE

First molecular report of *Babesia gibsoni* infection in pet dogs in Hunan province, subtropical China

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ABSTRACT

Canine babesiosis caused by *Babesia* spp. is a noteworthy tick-borne zoonotic disease of domestic dogs and wild canids. In present study, a total of 556 blood samples were randomly collected from pet dogs in eight cities of Hunan province, subtropical China. Genomic DNA was extracted and *Babesia* DNA was detected by amplification of partial 18S rRNA gene sequences. A total of 56 (10.1%) blood samples were tested positive for *Babesia* species. Sequence analysis showed that 29 dogs (5.2%) were positive for *B. gibsoni*, and other 27 dogs for *B. vogeli* (4.9%). The age and health status were considered as important risk factors for *B. gibsoni* and *B. vogeli* infections in pet dogs in this study ($P<0.05$). Phylogenetic analysis showed that the examined positive samples were highly clustered in the same branch with *B. gibsoni* and *B. vogeli*, respectively. This is the first molecular report of *B. gibsoni* infection in pet dogs in Hunan province, subtropical China. Our finding has provided a guide for the control of dog babesiosis in China and elsewhere.

Keywords: Canine babesiosis; *Babesia gibsoni*; *Babesia vogeli*; pet dogs; subtropical China.

INTRODUCTION

Babesiosis caused by *Babesia* spp. is an emerging tick-borne disease. The *Babesia* spp. can infect humans and animals, causing health problem and economic losses (Gray *et al*., 2010; Schnittger *et al*., 2012). Canine babesiosis is a significant tick-borne disease in dogs with worldwide distribution. The first record of *Babesia* spp. infection in dogs was occurred in Italy (1895) (Roncalli Amici, 2001). Nowadays, this disease has increased rapidly and is prevalent around the world. The clinical symptoms include fever, lethargy, weakness, anemia, vomiting, jaundice, splenomegaly and partial organs dysfunction, sometimes even death in severe condition (Beck *et al*., 2009; Bajer *et al*., 2014).

Babesia spp. are intraerythrocytic protozoan parasites, belonging to phylum Apicomplexa, order Piroplasmorida. Traditionally, they were divided into two major categories according to morphology, the small *Babesia* (1.0–2.5 µm), usually called *B. gibsoni* and the large *Babesia* (2.6–5.0 µm), generally named *B. canis*. However, with the application of molecular detection and DNA sequencing in parasite classification and identification, many *Babesia* species infecting dogs have been deem to morphologically similar, but genetically distinguishing (Panti-May & Rodríguez-Vivas, 2020). Up to now, the large types of *Babesia* infecting dogs comprise *B. canis*, *B. vogeli*, and *B. rossi*, while the small forms are consisted of *B. gibsoni*, *B. vulpes* and *B. conradae* (Panti-May & Rodríguez-Vivas, 2020). Thereinto, *B. gibsoni* is the most rampant worldwide as the pathogen of canine babesiosis (Trapp *et al*., 2006; Hartelt *et al*., 2007; Cao *et al*., 2015).

In the last decades, there has been rapid socio-economic development in China. With the increase of the living standards and changes in life styles, more and more dogs are raised as family pets. Based on the statistical data from previous study, the number of dogs in China was approximately between 150 and 200 million (Ma *et al*., 2012). The first case of canine babesiosis caused by *B. gibsoni* in China was reported 37 years ago, from Henan province. Then, cases of canine babesiosis were gradually reported across China. Currently, canine babesiosis has been reported from 11 provinces and one municipality of China (Jin *et al*., 2005; Xu *et al*., 2015; He *et al*., 2017; Niu *et al*., 2017; Zhang *et al*., 2017; Zheng *et al*., 2017; Li *et al*., 2020; Wang *et al*., 2020), which revealed the existence of *Babesia* spp. in pet dogs. These results have showed that *B. gibsoni* and *B. vogeli* were common in China. In spite of the prevalence of *Babesia* spp. reported in pet dogs in many provinces of China, limited information is available for *Babesia* spp. infection in Hunan province, subtropical China. Importantly, a recent study has indicated that *B. canis* is the most widespread species in Hunan province (Wang *et al*., 2020). Therefore, the objective of the present study was to investigate the prevalence of *Babesia* spp. infection in pet dogs from Hunan province, subtropical China. Our results should provide important epidemiological information for the control of canine babesiosis in China and elsewhere.

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MATERIALS AND METHODS

Ethics statement

This study was approved by the Animal Ethics Committee of Hunan Biological and Electromechanical Polytechnic (No. 43121603).

Blood sample collection

During April 2021 to July 2021, a total of 556 blood samples were randomly collected from pet dogs in different pet hospitals located in Hunan province, subtropical China (Table 1). EDTA coated vacutainers were used for collecting. One blood sample was collected from each pet dog. All samples were labelled individually and these samples were cooled in an ice box during transport to the laboratory at Hunan Biological and Electromechanical Polytechnic, then stored at -20°C until used. The necessary information (pet age, breed and gender) was recorded in detail from pet hospitals.

DNA extraction, PCR detection and sequencing

Total genomic DNA was extracted from 200 ul of whole blood sample using the TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) strictly according to the manufacturer's instructions. The extracted DNA was then stored at -20°C until used. The primers used to amplify 18S rRNA gene were as follow: BF: 5'-3' AATACCCAATCCTGACACAGGG and BR: 5'-3' TTAAATACGAATGCCCCCAAC (Xia *et al*., 2020). PCR amplification was initial denaturation at 94°C for 4 min, then 94°C for 45 s (denaturation), 55°C for 40 s (annealing), 72°C for 45 s (extension) for 35 cycles, followed by 72°C for 5 min (final extension). The expected size of the target fragment was 317 bp. Positive control and negative control (without DNA template) was included in each amplification run. In order to avoid mutual contamination between the experiment operation, all procedures were strictly operated in the SW-CJ-2FD clean workstation [Cleanliness level: 100@ $\geq$ 0.5um (Federal 209E)]. The PCR products were validated by electrophoresis using 1% agarose gels containing GoldView^{TM} (Beijing, China). The positive PCR products were sequenced in both directions by Sangon Biotech (Shanghai, China).

Sequence and phylogenetic analysis

The obtained forward and reverse nucleotide sequences were checked using Chromas v.2.4 (https://technelysium.com.au/chromas.html), respectively, and then assembled by the DNAMAN v.7.0 program after quality inspection. All the assembled sequences in this study were matched through the Basic Local Alignment Search Tool (BLAST) from NCBI website. The software Clustal X 2.0 (Larkin *et al*., 2007) was used to align the sequences assembled in this study and the reference sequences published in NCBI, then MegAlign software (DNAstar Lasergene 12 Core Suite) was used to calculate the sequences identities.

A total of 24 representative 18S rRNA gene sequences of the genus *Babesia* infecting dogs from GenBank were selected to do phylogenetic analysis, along with *Theileria ovis* as the outgroup (GenBank accession number: MW131360). The selected nucleotide sequences were aligned together by Clustal X 2.0 (Larkin *et al*., 2007), then edited to a new format through the software BioEdit version 7.1.3.0 (Alzohairy, 2011). Phylogenetic tree was constructed by the program MEGA 11.0 (Tamura *et al*., 2021) with Neighbor-joining (NJ) method. The best substitution model was Kimura 2-parameter model. Tree was performed with 1000 bootstrap replicates, and gap / missing data treatment was complete deletion.

Statistical analysis

Statistical analysis was performed using SPSS V26.0 (IBM, Chicago, USA). Each variable was included in a Binary Logit Model (BLM) and as an independent variable in a multivariate regression analysis. Odds ratios (OR) and their 95% confidence intervals (95% CI) based on likelihood ratio statistics were also analyzed in this study. When $P<0.05$, the results were considered statistically significant.

RESULTS

Prevalence of *Babesia* spp. based on PCR detection

In this study, 56 of 556 (10.1%, 56/556; 95% CI: 7.6-12.6) examined pet dogs were positive for *Babesia* spp. (Table 1). The prevalence of *Babesia* spp. in adults (15.4%) was higher than that in Juvenile (3.0%), and in sick dogs (16.5%) was six times higher than that in healthy dogs (2.7%) (Table 1). The prevalence of *Babesia* spp. in male (10.7%) and female (9.3%) was nearly identical (Table 1). Sequencing results showed that there were two *Babesia* spp. in the pet dogs. *B. gibsoni* (5.2%) was identified from 29 samples and *B. vogeli* (4.9%) was detected in 27 samples (Tables 2 and 3).

The prevalence of *B. gibsoni* was significantly different in health status and age. The risk of infecting *B. gibsoni* in sick pet dogs (8.4%) was nearly six times higher than that in apparently healthy pet dogs (1.5%) (Table 2). The risk in adult pet dogs (7.8%) was nearly five times higher than that in juvenile pet dogs (1.7%) (Table 2). Across the whole breed of pet dogs in the study, the prevalence of *B. gibsoni* was highest in Poodle (11.5%), and no *B. gibsoni* was detected in Schnauzer, Springer spaniel and Yorkshire (Table 2) ($P > 0.05$). The prevalence of *B. gibsoni* in Chenzhou city was 10.3%, higher than that in other seven cities and towns ($P > 0.05$) (Table 2). the prevalence of *B. gibsoni* in female (4.9%) pet dogs was slightly lower than that in male (5.5%) (Table 2).

The prevalence of *B. vogeli* in healthy status (1.2%) was evidently lower than that in sick pet dogs (8.1%) (Table 3), and the risk in adult (7.5%) was 6.3 times higher than that in juvenile (1.3%) ($P < 0.05$). Moreover, the highest prevalence of *B. vogeli* in the diverse breed was also in Poodle (13.1%). In addition, the prevalence of *B. vogeli* in eight regions was similar, from 2.5% to 5.1% (Table 3).

Table 1. Prevalence of *Babesia* spp. in pet dogs in Hunan province, subtropical China

Factors	Parameters	Positive/ examined	Prevalence (95%CI)
Age	Adult ($\geq$ 1 year)	49/319	15.4 (11.4-19.3)
	Juvenile (< 1 year)	7/237	3.0 (0.8-5.1)
Breed	Bichon Frise	1/35	2.9 (0-8.4)
	Chinese Rural Dog	8/86	9.3 (3.2-15.4)
	Corgi	6/63	9.5 (2.3-16.8)
	Golden	5/85	5.9 (0.9-10.9)
	Labrador	4/52	7.7 (0.4-14.9)
	Pomeranian	1/39	2.6 (0-7.5)
	Poodle	30/122	24.6 (16.9-32.2)
	Schnauzer	0	
	Shiba Inu	1/48	2.1 (0-6.1)
	Springer spaniel	0	
	Yorkshire	0	
Gender	Female	23/247	9.3 (5.7-12.9)
	Male	33/309	10.7 (7.2-14.1)
Health status	Apparently healthy	7/259	2.7 (0.7-4.7)
	Sick	49/297	16.5 (12.3-20.7)
Region	Changde	3/31	9.7 (0-20.1)
	Changsha	25/175	14.3 (9.1-19.5)
	Chenzhou	4/29	13.8 (1.2-26.3)
	Hengyang	5/68	7.4 (1.1-13.6)
	Loudi	1/25	4.0 (0-11.7)
	Yueyang	4/62	6.5 (0.3-12.6)
	Xiangtan	5/80	6.3 (0.9-11.6)
	Zhuzhou	9/86	10.5 (4.0-16.9)
Total		56/556	10.1 (7.6-12.6)

Table 2. Prevalence and risk factors of *Babesia gibsoni* in pet dogs in Hunan province, subtropical China

Factors	Parameters	Prevalence (Positive/examined)	OR (95%CI)	*P*-value
Age	Adult (≥ 1 year)	7.8% (25/319)	4.953 (1.700-14.432)	0.001
	Juvenile (< 1 year)	1.7% (4/237)	Reference	
Breed	Bichon Frise	2.9% (1/35)	1.382 (0.083-22.885)	1.000
	Chinese Rural Dog	3.5% (3/86)	1.699 (0.172-16.797)	1.000
	Corgi	6.3% (4/63)	3.186 (0.345-29.473)	0.387
	Golden	2.4% (2/85)	1.133 (0.100-12.825)	1.000
	Labrador	5.8% (3/52)	2.878 (0.289-28.652)	0.619
	Pomeranian	2.6% (1/39)	1.237 (0.075-20.433)	1.000
	Poodle	11.5% (14/122)	6.093 (0.778-47.681)	0.070
	Schnauzer	0		
	Shiba Inu	2.1% (1/48)	Reference	
	Springer spaniel	0		
	Yorkshire	0		
Gender	Female	4.9% (12/247)	Reference	
	Male	5.5% (17/309)	1.140 (0.534-2.435)	0.848
Health status	Apparently healthy	1.5% (4/259)	Reference	
	Sick	8.4% (25/297)	5.859 (2.011-17.069)	0.000
Region	Changde	6.5% (2/31)	2.069 (0.277-15.433)	0.598
	Changsha	5.1% (11/175)	2.012 (0.433-9.343)	0.523
	Chenzhou	10.3% (3/29)	3.462 (0.546-21.958)	0.322
	Hengyang	4.4% (3/68)	1.385 (0.224-8.574)	1.000
	Loudi	0		
	Yueyang	3.2% (2/62)	Reference	
	Xiangtan	3.8% (3/80)	1.169 (0.189-7.219)	1.000
	Zhuzhou	5.8% (5/86)	1.852 (0.347-9.871)	0.699
Total		5.2% (29/556)		

Table 3. Prevalence and risk factors of *Babesia vogeli* in pet dogs in Hunan province, subtropical China

Factors	Parameters	Prevalence (Positive/examined)	OR (95%CI)	*P*-value
Age	Adult (≥ 1 year)	7.5% (24/319)	6.346 (1.888-21.332)	0.000
	Juvenile (< 1 year)	1.3% (3/237)	Reference	
Breed	Bichon Frise	0		
	Chinese Rural Dog	5.8% (5/86)	3.148 (0.358-27.723)	0.409
	Corgi	3.2% (2/63)	1.672 (0.147-18.976)	1.000
	Golden	3.5% (3/85)	1.866 (0.189-18.425)	1.000
	Labrador	1.9% (1/52)	Reference	
	Pomeranian	0		
	Poodle	13.1% (16/122)	7.698 (0.993-59.661)	0.024
	Schnauzer	0		
	Shiba Inu	0		
	Springer spaniel	0		
	Yorkshire	0		
Gender	Female	4.5% (11/247)	Reference	
	Male	5.2% (16/309)		0.843
Health status	Apparently healthy	1.2% (3/259)	Reference	
	Sick	8.1% (24/297)	7.502 (2.232-25.215)	0.000
Region	Changde	3.2% (1/31)	1.300 (0.114-14.872)	1.000
	Changsha	5.1% (14/175)	3.971 (0.752-15.291)	0.104
	Chenzhou	3.4% (1/29)	1.393 (0.130-14.646)	1.000
	Hengyang	2.9% (2/68)	1.182 (0.162-8.621)	1.000
	Loudi	4.0% (1/25)	1.625 (0.141-18.712)	0.562
	Yueyang	3.2% (2/62)	1.300 (0.178-9.497)	1.000
	Xiangtan	2.5% (2/80)	Reference	
	Zhuzhou	4.7% (4/86)	1.902 (0.339-10.682)	0.683
Total		4.9% (27/556)		

Sequence analysis

The sizes of PCR positivity amplification product were both ~ 300 bp (Figure 1), the partial 18S rRNA gene sequences obtained in this study had 98.9-100% identity to previously published corresponding *B. gibsoni* sequences, and shared 99.7-100% identity with *B. vogeli* which published in GenBank. Multiple nucleotide sequence variations within the 18S rRNA gene sequences of *B. gibsoni* ranged from 98.9 to 100%, while the sequence identities of *B. vogeli* were 99.7-100%. The representative nucleotide sequences of *B. gibsoni* and *B. vogeli* have been deposited in GenBank under the accession numbers OP168996-169000, OP169007-10, respectively.

Phylogenetic analysis

Our results showed that *B. gibsoni* sequences obtained in the present study are in the same branch with other *B. gibsoni* isolates, with a strong support (Bpp = 100) (Figure 2), while *B. vogeli* sequences in the study were clustered with other *B. vogeli* sequences from different geographical regions (Bpp = 97) (Figure 2). This results further demonstrated there were *B. gibsoni* and *B. vogeli* infections from pet dogs in Hunan province. It is evidently that three large canine species (*B. vogeli*, *B. canis* and *B. rossi*) formed a large clade, which were sister taxa with clade of *B. gibsoni* (Bpp = 100). Meanwhile, other two small *Babesia* species *B. vulpes* and *B. conradae* were in the different clades, while the latter was in the outermost branch (Figure 2). Phylogenetic analyses also indicated that the genus *Babesia* is monophyletic

DISCUSSION

In the last decade, an increasing number of canine babesiosis have been reported across the world (Matijatko *et al*., 2012). This may be due to the increased awareness of pet health among veterinarians and owners, and the reduction in molecular sequencing cost. Nowadays, *Babesia* spp. infecting dogs has been found in 11 provinces and one municipality of China, with a highest frequency of *B. gibsoni*, followed by *B. vogeli* (Jin *et al*., 2005; Xu *et al*., 2015; He *et al*., 2017; Niu *et al*., 2017; Zhang *et al*., 2017; Zheng *et al*., 2017; Li *et al*., 2020; Wang *et al*., 2020). A recent study showed that four dogs (3.5%) were positive for *B. canis*, and one for *B. vogeli* (0.87%) in Hunan province of China (Wang *et al*., 2020). However, in the present study, *B. gibsoni* and *B. vogeli* have been detected, but no *B. canis*. Compared with previous studies, the prevalence of *B. gibsoni* (5.2%) in the present study was higher than that in east China (3.5%) (including six province and one municipality) and Kunming, Yunnan province (1.2%) (Cao *et al*., 2015; Xu *et al*., 2015), and significantly lower than that in Shenzhen, Guangdong province (7.0%) and Wuhan, Hubei province (11.9%) (He *et al*., 2017; Zhang *et al*., 2017). Meanwhile, the prevalence of *B. vogeli* (4.9%) in the

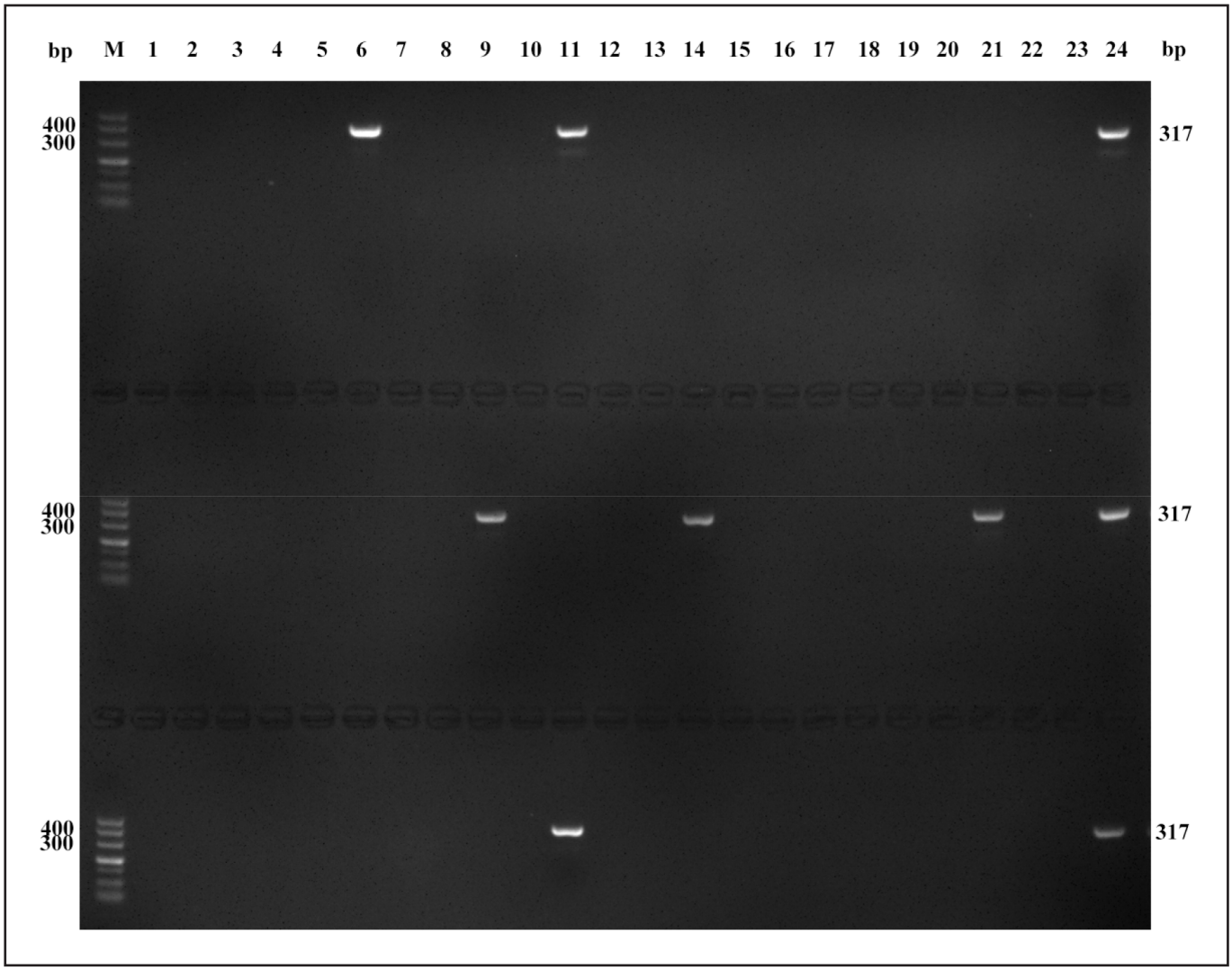

Figure 1. Electrophoretogram of partial PCR products of *Babesia* spp.. Lane M: DL500 DNA marker. Lane 1–22: samples. Lane 23: negative control. Lane 24: positive control.

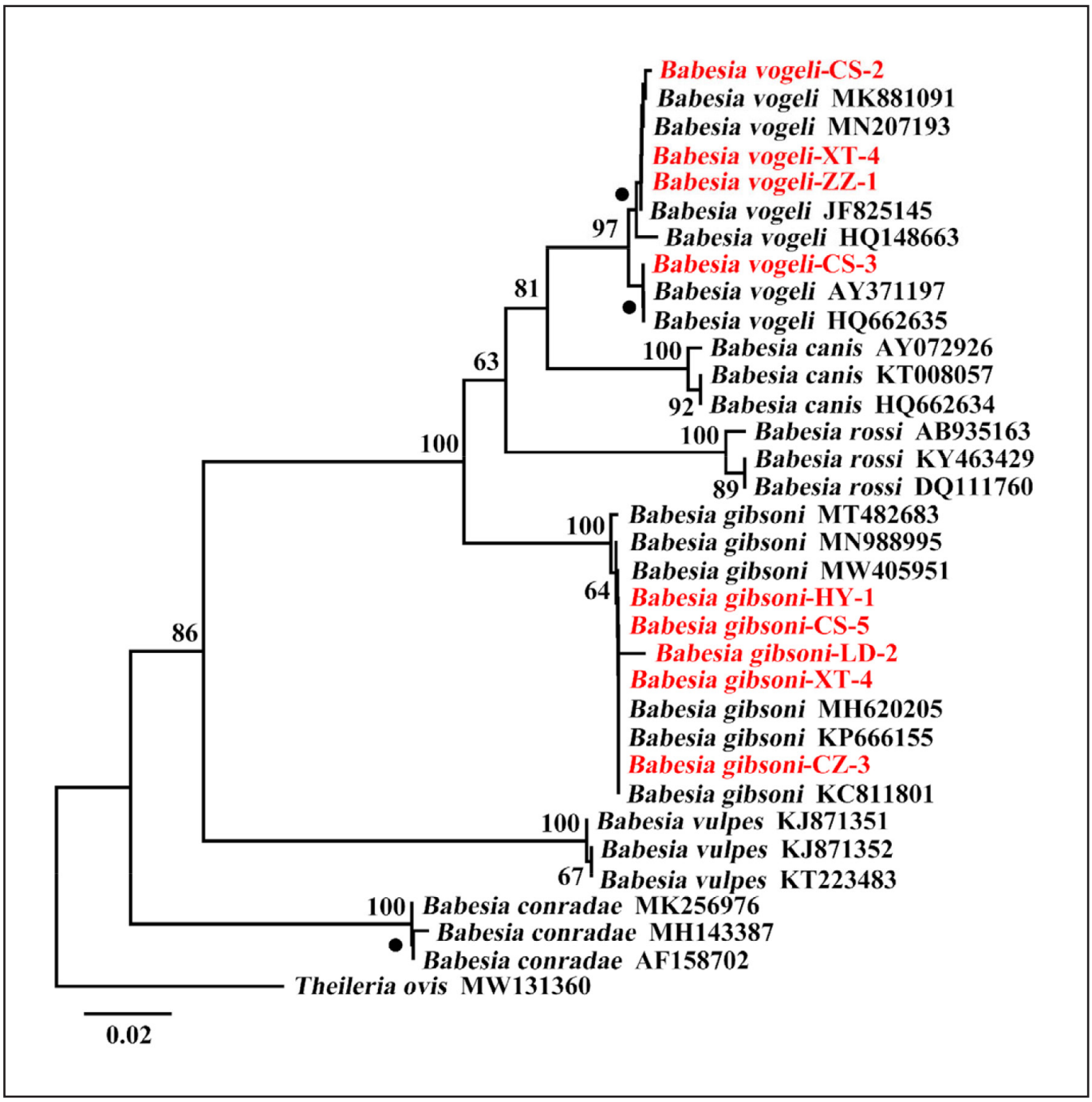

Figure 2. Phylogenetic trees based on the 18S rRNA of *Babesia* species. Color in red indicates representative sequences determined in this study. Circle in black indicates bootstrap values are lower than 60.

present study was consistent with that in Jiangxi province (4.9%) (Zheng *et al*., 2017), however, it was obviously lower than that in Shenzhen, Guangdong province (11.0%) and Taixing, Jiangsu province (11.3%), and higher than that in Gansu province (1.4%) (Xu *et al*., 2015; Niu *et al*., 2017; Li *et al*., 2020). These differences in prevalence may be due to different detection methods, geographical origins and animal welfares.

Babesia gibsoni is mainly transmitted by the hard tick *Haemaphysalis longicornis*, *H. bispinosa* and *Rhinpicephalus sanguineus* (Schoeman, 2009; Iwakami *et al*., 2014). Especially, *B. gibsoni* can be transmitted via direct contact from dog to dog through infected blood by bite (Yeagley *et al*., 2009; Birkenheuer *et al*., 2018). Therefore, it is easier infected in fighting dogs than others. The only vector of *B. vogeli* is *R. sanguineus*. In Hunan province, *H. longicornis* is a little bit more active than *R. sanguineus* (Ding *et al*., 1997), this may be the reason that the prevalent of *B. gibsoni* (5.2%) is higher than *B. vogeli* (4.9%). Moreover, the prevalent of two *Babesia* in juvenile group were both lower than that in adult group, which is consist with previous study *(*Mritunjay *et al*., 2008). Co-infections with *Babesia* spp. are not detected in the present study, which rarely reported in dogs in the past, too, only *B. microti*-like sp. with *B. canis* and *B. gibsoni* were found. However, phylogenetic analyses suggest that the diversity of *Babesia* species that co-infect dogs may be more than previously thought (Zahler *et al*., 2000; Beck *et al*., 2009). Some recent studies which reported mix infection with *B. gibsoni* and *B. vogeli* in dogs (Padmaja *et al*., 2022; Thomas *et al*., 2022) have proved the view above.

Babesiosis as an emerging tick-borne zoonosis is more universally noticeable, which is harmful for livestock and companion animal welfare to the inclusion of human health (Guo *et al*., 2022).

The previous (Wang *et al*., 2000) and present studies provide strong evidence that *B. gibsoni*, *B. vogeli* and *B. canis* infections are common in pet dogs in Hunan province, and can cause considerable health problem in these animals. Four *Babesia* species (*B. microti*, *B. divergens*, *B. duncani*, and *B. venatorum*) can infect human (Al-Nazal *et al*., 2022). Although these *Babesia* species (*B. microti*, *B. divergens*, *B. duncani*, and *B. venatorum*) were not detected in present study, but *B. microti* was also reported in pet dogs (Checa *et al*., 2019), posing significant public health threats to human health.

The present survey follows the principle of random sampling; however, the samples incompletely cover pet hospitals. Secondly, the number of samples in each group is not evenly distributed, which may have impact on the results. Moreover, although PCR method used in this study is a common method for molecular detection and characterization, multiple real time PCR method is more specific and accurate than the former (Troskie *et al*., 2019). Therefore, further work is required to confirm whether other *Babesia* species (including *B. microti*) infect pet dogs in Hunan province using larger numbers of specimens from more pet hospitals based on multiple real time PCR method.

CONCLUSION

Our results indicated that *B. gibsoni* and *B. vogeli* infections are prevalent in pet dogs in Hunan province, subtropical China, which provide a foundation for the prevention and control of canine babesiosis in Hunan province, subtropical China. This is the first report of *B. gibsoni* infection in pet dogs in Hunan province, subtropical China.

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Conflict of Interests

The authors declare that there is no conflict of interests in this paper.

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