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Babesia vesperuginis in insectivorous bats from China

Hui-Ju Han^{1†}, Jian-Wei Liu^{1†}, Hong-Ling Wen², Xiang-Rong Qin¹, Min Zhao¹, Li-Jun Wang¹, Chuan-Min Zhou¹, Rui Qi¹, Hao Yu³ and Xue-Jie Yu^{1*}

Abstract

Background: To increase understanding of human bacterial and parasitic pathogens in bats, we investigated the prevalence of *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *Coxiella burnetii* in bats from China.

Methods: Bats were captured from Mengyin County, Shandong Province of China using nets. DNA was extracted from the blood and spleen of bats for molecular detection of *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *Coxiella burnetii* with specific primers for each species.

Results: A total of 146 spleen samples and 107 blood samples of insectivorous bats, which belonged to 6 species within two families, were collected from Mengyin County, Shandong Province of China. We found that two *Eptesicus serotinus* (2/15, 13.3%) were positive for *Babesia vesperuginis*. We were unable to detect genomic sequences for *Rickettsia* spp., *Anaplasma* spp. and *Coxiella burnetii*.

Conclusions: To our knowledge, our study showed for the first time the presence of *Babesia vesperuginis* in *Eptesicus serotinus* collected from China, suggesting that *Babesia vesperuginis* has a broad host species and geographical distribution.

Keywords: Bat, China, *Babesia vesperuginis*

Background

Bats have been studied in recent years due to their association with several serious emerging viruses, such as SARS-Coronavirus, Hendra virus, Nipha virus, Ebola virus and Marburg virus [1]. Most studies have focused on emerging viruses; however, bacterial and parasitic agents in bats have been largely neglected. We previously showed that bats from northern China carried several novel *Bartonella* spp. [2] as well as a diversity of pathogenic *Leptospira* spp. [3]. To have a better understanding of bacterial and parasitic pathogens in bats, we expanded our study to several tick-borne bacterial and parasitic pathogens, including *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *Coxiella burnetii*.

Babesia spp. are tick-transmitted protozoan hemoparasites associated with a wide range of vertebrate hosts worldwide [4]. So far, *Babesia* spp. detected in bats have

been almost exclusively *Babesia vesperuginis* [5–9], with the exception of a study reporting *Babesia canis*, the causative agent of canine babesiosis, in the feces of bats from Hungary [10]. In addition, a recent study reported the detection of *B. vesperuginis*, *Babesia crassa* and *B. canis* in ixodid ticks on bats [11], which indicated that bats could harbor a greater diversity of *Babesia* species and hard ticks could also play a role in *Babesia* transmission among bats. The role of bats in the ecology of *Babesia* spp. as well as the vectors involved in transmission of *Babesia* spp. among bats deserves further investigation.

Rickettsia spp. are intracellular bacteria that are responsible for life-threatening spotted and typhus fevers in humans [12]. So far, *Rickettsia* spp. infections in bats were limited to several serological and molecular surveys in America, Africa and Europe. Antibodies against several spotted fever group (SFG) *Rickettsia* spp. were reported in bats from Brazil and USA [13, 14]. DNA of *Rickettsia* spp. was also detected in the blood samples of bats from Swaziland, South Africa and Saint Kitts Island [15, 16]. A recent study conducted in Europe showed that *Rickettsia*

* Correspondence: yuxuejie@whu.edu.cn

†Hui-Ju Han and Jian-Wei Liu contributed equally to this work.

¹Wuhan University School of Health Sciences, Wuhan, Hubei, China
Full list of author information is available at the end of the article



spp. DNA was detected in bat feces [17]. Moreover, bat ectoparasites, including soft ticks (Argasidae), hard ticks (Ixodidae) and flies (Nycteribiidae), were found to carry a diversity of *Rickettsia* spp. that were identical to those found in bats, indicating the vector-borne transmission of *Rickettsia* spp. [18–23]. So far, there is a lack of knowledge on *Rickettsia* spp. in bats in Asia.

Anaplasma spp. belong to the order Rickettsiales, causing tick-borne anaplasmosis in animals and humans [24]. So far, there is no report of *Anaplasma* spp. in bats.

Coxiella burnetii is an obligate intracellular gram-negative bacterium, and is the agent of Q fever [25]. So far, there are no reports of *C. burnetii* in bats. However, its existence in ticks from bats has been reported in Algeria [26].

Therefore, the aim of the study was to investigate the prevalence of *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *C. burnetii* in bats from China.

Methods

Bat sampling

Bats were captured with nets from Mengyin County, Shandong Province of China (117°45' to 118°15'N, 35°27' to 36°02'S) as part of an ongoing program of detecting novel microorganisms (viruses, bacteria and parasites) in bats. Identification of bat species was performed by DNA sequencing the PCR amplified cytochrome *b* (*cytb*) gene as described previously [27]. Details on the collection of bat specimens are as described previously [2].

Briefly, bats were anesthetized for collecting blood samples, and were then killed with overdosed anesthetic to collect organs.

Molecular detection for *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *C. burnetii*

Bat blood DNA extraction was performed with the Qiagen DNA Kit (Qiagen, Hilden, Germany) and the spleen was extracted with the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Blood DNA samples were screened for *Babesia* spp., *Rickettsia* spp. and *Anaplasma* spp. Spleen DNA samples were screened for *C. burnetii*. Primers used in this study are shown in Table 1.

For *Babesia* spp., an initial screening PCR targeting 18S rDNA was conducted in a 50 µl mixture containing 25 µl DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA), 0.8 µl 25 µmol/l of each forward and reverse primer (Sangon Biotech, Shanghai, China), 16.4 µl nuclease-free water, and 7 µl blood DNA of each sample. Nuclease-free water was used as negative controls. PCR was performed under the following conditions: 1 denaturing cycle at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, 55°C for 30 s, and 72 °C for 1 min and an additional final cycle at 72 °C for 10 min.

For 18S rDNA positive samples, an additional nested PCR targeting *cox1* was performed. The first round PCR

Table 1 PCR primers used for *Babesia* spp., *Rickettsia* spp. and *Anaplasma* spp. and *C. burnetii* screening

Target agent	PCR method	Primer	Primer sequences (5'→3')	Target gene	Amplicon size (bp)	Tissue tested	Reference
<i>Babesia</i> spp.	PCR	BJ1	GTCTTGAATTGGAATGATGG	18S rDNA	~500	Blood	[10]
		BN2	TAGTTTATGGTTAGGACTACG				
	Nested PCR	Bab_For1	ATWGGATTYATATGAGTAT	<i>cox1</i>	924	[7]	
		Bab_Rev1	ATAATCWGGWATYCTCCTTGG				
		Bab_For2	TCTCTWCATGGWTTAATTATGATAT				
Bab_Rev2	TAGCTCCAATTGAHARWACAAAGTG						
<i>Rickettsia</i> spp.	qPCR	gltA-F	GTGAATGAAAGATTACACTATTTAT	<i>gltA</i>	–	Blood	[30]
		gltA-R	GTATCTTAGCAATCATTCTAATAGC				
	qPCR	338-F	GAMAAATGAATTATATACGCCGCAAA	RC0338 gene	–		
		338-R	ATTATTKCCAAATATTCGTCTGTAC				
<i>Anaplasma</i> spp.	Nested PCR	AE1-F	AAGCTTAACACATGCAAGTCGAA	16S rRNA	926	Blood	[31]
		AE1-R	AGTCACTGACCCAACCTTAAATG				
		EE3	GTCGAACGGATTATTCTTTATAGCTTGC				
		EE4	CCCTTCCGTTAAGAAGGATCTAATCTCC				
<i>Coxiella burnetii</i>	Nested PCR	omp1	AGTAGAAGCATCCAAGCATTG	<i>com1</i>	438	Spleen	[32]
		omp2	TGCCTGCTAGCTGTAACGATTG				
		omp3	GAAGCGCAACAAGAAGAACA				
		omp4	TGGAAGTTATCACGCAGTTG				

was conducted in a 25 μ l mixture containing 0.125 μ l 5 U/ μ l TaKaRa Ex Taq (TaKaRa, Shiga, Japan), 2.5 μ l 10 \times ExTaqbuffer (Mg²⁺ free), 2 μ l 25 mM MgCl₂, 2 μ l dNTP mixture (2.5 mM for each), 0.4 μ l 25 μ mol/l of each forward and reverse primer, 12.6 μ l nuclease-free water and 5 μ l blood DNA of each sample. The second round PCR was the same as described above for 18S rDNA except that 3 μ l of first round PCR product was used as a template. The PCR condition was the same as described for 18S rDNA, but the annealing temperature for the first and second rounds of PCR were 45 °C and 49 °C, respectively.

For *Anaplasma* spp. and *C. burnetii*, a nested PCR was conducted as described for *cox1* of *Babesia* spp. Blood DNA and spleen DNA were used for the detection of *Anaplasma* spp. and *C. burnetii*, respectively. The PCR conditions were the same as described for 18S rDNA of *Babesia* spp.

PCR products were analyzed by 1.2% agarose gel electrophoresis and detected using ethidium bromide under UV light. PCR products with expected sizes were excised from gels and extracted using a Gel Extraction Kit (Promega, Madison, WI, USA), which were then cloned into the pMD19-T vector (TaKaRa) for sequencing.

Quantitative real-time PCR (qPCR) was used for the detection of *Rickettsia* spp. The reaction was conducted in a 50 μ l mixture containing 25 μ l FastStart Universal SYBR Green Master (ROX), 0.8 μ l 25 μ mol/l of each forward and reverse primer, 16.4 μ l nuclease-free water, and 7 μ l blood DNA of each sample. The tests were performed using a Light Cycler 480 II (Roche, Mannheim, Germany) with the following conditions: an initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and at 58 °C for 30 s. Nuclease-free water was used as negative controls in each run. Results were considered positive if the cycle threshold (Ct) value was lower than 36.

Phylogenetic analysis

Chromatograms were checked with Chromas 2.5.1 (Technelysium, Tewantin, QLD, Australia) to exclude double peaks, and sequences were analyzed with the BLAST programme (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

After alignment by ClustalW with MEGA 7.0 [28], phylogenetic trees were constructed using the Maximum Likelihood method with the Tamura-Nei model by using MEGA7.0, and bootstrap values were calculated with 1000 replicates.

Results

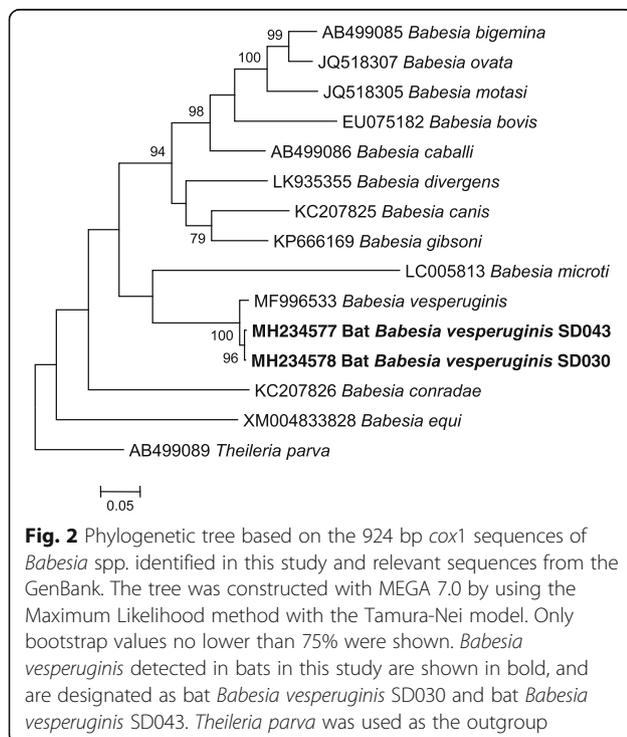
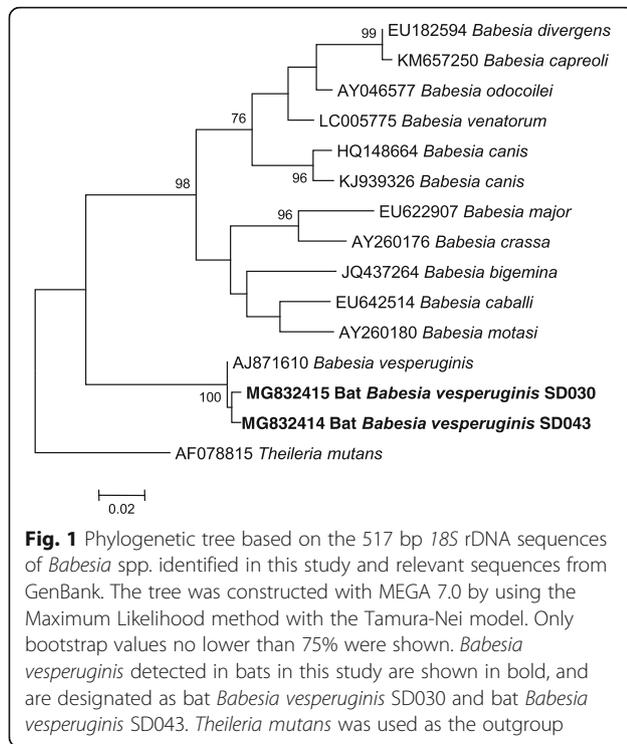
A total of 146 bats belonging to 6 species within two families were sampled. Bats of the family Rhinolophidae included 4 *Rhinolophus ferrumequinum* and 14 *Rhinolophus pusillus* captured from a karst cave; bats of the family of Vespertilionidae included 26 *Eptesicus serotinus* from two farmers' houses, 34 *Myotis fimbriatus* and 10 *Myotis ricketti* from a city sewer and 58 *Myotis pequinius* from a cave (Table 2). Finally, 146 spleen DNA samples were screened for *C. burnetii*, and 107 blood DNA samples were screened for *Babesia* spp., *Rickettsia* spp. and *Anaplasma* spp.

In this study, we found that 2 out of 15 blood samples of *E. serotinus* (2/15, 13.3%) were positive for *Babesia* spp., while blood samples of the other 5 bat species (*Rh. ferrumequinum*, *Rh. pusillus*, *My. fimbriatus*, *My. ricketti* and *My. pequinius*) were all negative. BLAST analysis of the 517 bp 18S rDNA sequences showed that the two *Babesia* spp. detected in *E. serotinus* in this study (designated as bat *Babesia vesperuginis* SD030 and bat *Babesia vesperuginis* SD043), which differed by 4 nucleotides, shared 99.4% similarity with *B. vesperuginis* (GenBank: AJ871610). BLAST analysis of the 924 bp *cox1* sequences showed that the bat *Babesia vesperuginis* SD030 and bat *Babesia vesperuginis* SD043 differed by 3 nucleotides, and shared 98.2% and 98.1% similarity with *B. vesperuginis* (GenBank: MF996533), respectively. Phylogenetic analysis of 18S rDNA and *cox1* genes also showed that *Babesia* spp. detected in bats in this study clustered together with *B. vesperuginis* (Figs. 1 and 2). The 18S rDNA and *cox1* sequences of *B. vesperuginis* of this study were deposited in the GenBank with accession numbers: MG832414-MG832415 and MH234577-MH234578.

We were unable to detect genomic sequences for *Rickettsia* spp., *Anaplasma* spp. and *C. burnetii*.

Table 2 Information of bats sampled from Mengyin County, Shandong Province of China

Family	Sampling site	Species	Common name	Spleen samples	Blood samples
Rhinolophidae	Karst Cave	<i>Rhinolophus ferrumequinum</i>	Greater horseshoe bat	4	3
		<i>Rhinolophus pusillus</i>	Least horseshoe bat	14	10
Vespertilionidae	Farmers' houses	<i>Eptesicus serotinus</i>	Common serotine	26	15
	City sewer	<i>Myotis fimbriatus</i>	Fringed long-footed myotis	34	16
		<i>Myotis ricketti</i>	Rickett's big-footed myotis	10	5
	Cave	<i>Myotis pequinius</i>	Peking myotis	58	58
Total				146	107



Discussion

Babesia vesperuginis in bats was first described in bats from Italy, and later also found in bats from other parts of Europe (UK, Austria, Czech Republic, Romania) and South America (Colombia) [5, 7–9]. So far, *B. vesperuginis* has been detected in *Nyctalus noctula* and *Pipistrellus* sp. from Italy; *My. mystacinus* and *Pipistrellus* sp. from the UK; *Mormoops megalophylla* from Colombia; *My. alcaethoe*, *My. bechsteinii*, *My. myotis* and *Vespertilio murinus* from Romania; *Ny. noctula*, *Pi. nathusii* and *Pi. pipistrellus* from the Czech Republic; *Pi. pipistrellus* and *Ve. murinus* from Austria; and *Pi. pipistrellus* from China [5–9]. The prevalence of *B. vesperuginis* in *Pipistrellus* spp. in Europe has been reported as 8.45% (6/71), 9.22% (19/206), 16.7% (6/36) and 10% (5/48) [6, 7, 9, 29]. The prevalence of *B. vesperuginis* in *Mo. megalophylla* in South America and in *N. noctula* in Europe was reported to be 1.19% (2/168) and 1.63% (4/246), respectively [5, 7]. However, the prevalence of *B. vesperuginis* in other bat species might be biased due to the limited sample size [7, 8]. In this study, the prevalence of *B. vesperuginis* in *E. serotinus* from China was 13.3% (2/15), which might also be biased by the limited sample size. To our knowledge, this is the first report of *B. vesperuginis* in *E. serotinus*, suggesting that *B. vesperuginis* has a broad host species and geographical distribution.

Natural and experimental infection showed that *B. vesperuginis* was pathogenic to bats, which could result in symptoms such as lowered blood haemoglobin, raised white blood cell counts and enlarged spleen in bats [8]. Soft ticks (*Argas vespertilionis*) were suspected to play a role in the transmission of *B. vesperuginis* among bats [8]. Although no ticks were found on bats in this study, a recent study reported that soft ticks (*Argas vespertilionis*) collected from *B. vesperuginis*-positive bats (*Pi. pipistrellus*) were also positive for *B. vesperuginis* in northwestern China [6], indicating that soft ticks might be the vector for *B. vesperuginis* transmission among bats.

Conclusions

We detected *B. vesperuginis* in *E. serotinus* collected from China, suggesting that *B. vesperuginis* has a broad host species and geographical distribution. Since *B. vesperuginis* is pathogenic to bats, the finding of this species in China has some implications for the conservation of bats in China.

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Availability of data and materials

The *B. vesperuginis* sequences of this study are available in the GenBank under the accession numbers MG832414-MG832415 and MH234577-MH234578.

Authors' contributions

HJH designed the study. HJH, JWL, HLW, XRQ, MZ, LJW, CMZ and YLZ participated in bat sampling and performed the experiments. HJH and RQ helped in data analysis. HJH, XYJ and HY wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval

The collection of bats for microbiological studies was approved by the Ethics Committee of Prevention Medicine of Shandong University (No.20150501).

Competing interests

The authors declare that they have no competing interests.

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Author details

¹Wuhan University School of Health Sciences, Wuhan, Hubei, China.

²Shandong University School of Public Health, Jinan, Shandong, China.

³Fudan University School of Medicine, Shanghai, China.

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