Epidemiol. Infect. (2017), **145**, 2704–2710. © Cambridge University Press 2017
doi:10.1017/S0950268817001686

Detection of Kobe-type and Otsu-type *Babesia microti* in wild rodents in China's Yunnan province

XIN-RONG CHEN[1,2]†, LI YE[2]†, JUN-WEN FAN[2], CHANG LI[3], FANG TANG[4], WEI LIU[5], LIN-ZHU REN[1] AND JIE-YING BAI[2]*

[1] *Jilin Provincial Key Laboratory of Animal Embryo Engineering, College of Animal Sciences, Jilin University, Changchun, P. R. China*
[2] *Laboratory Animal Center, Academy of Military Medical Sciences, Beijing, China*
[3] *Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary, AMMS, Changchun 130122, People's Republic of China*
[4] *Centers for Disease Control and Prevention of the Armed Police Forces, Beijing, People's Republic of China*
[5] *State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, People's Republic of China*

Received 17 January 2017; Final revision 3 July 2017; Accepted 8 July 2017; first published online 14 August 2017

SUMMARY

Babesiosis is an emerging tick-transmitted zoonosis prevalent in large parts of the world. This study was designed to determine the rates of *Babesia microti* infection among small rodents in Yunnan province, where human cases of babesiosis have been reported. Currently, distribution of *Babesia* in its endemic regions is largely unknown. In this study, we cataloged 1672 small wild rodents, comprising 4 orders, from nine areas in western Yunnan province between 2009 and 2011. *Babesia microti* DNA was detected by polymerase chain reaction in 4·3% (72/1672) of the rodents analyzed. The most frequently infected rodent species included *Apodemus chevrieri* and *Niviventer fulvescens*. Rodents from forests and shrublands had significantly higher *Babesia* infection rates. Genetic comparisons revealed that *Babesia* was most similar to the Kobe- and Otsu-type strains identified in Japan. A variety of rodent species might be involved in the enzootic maintenance and transmission of *B. microti*, supporting the need for further serological investigations in humans.

Key words: *Babesia microti*, China, rodents.

INTRODUCTION

Human babesiosis is a zoonotic, malaria-like illness with variable clinical severity, ranging from asymptomatic disease in healthy adults to life-threatening outcomes in the elderly and asplenic or immunocompromised persons [1]. Over 100 *Babesia* species have been identified; however, only a few can infect humans. The majority of documented cases in the USA are attributed to *B. microti* infection, whereas most of the cases in Europe are caused by *Babesia divergens* infection [2–4]. In recent years, *B. microti* has increasingly been detected in rodents and ticks across Europe, Africa and Asia [5–11]. *Babesia microti* is gaining attention worldwide because of its wide distribution in endemic areas, the increased risk of causing human disease and the potential for transmission through blood transfusion. In China, human babesiosis has largely been overlooked due to the lack of medical awareness and clinical diagnostic methods [12]. To date, only 10 human cases of

* Author for correspondence: J-Y. Bai, Laboratory Animal Center, Academy of Military Medical Sciences, 20 Dong-Da Street, Fengtai District Beijing 100071, People's Republic of China.
(Email: baijieying@126.com)
† These authors contributed equally to this work.

infection by *B. microti*-like organisms have been recorded, mainly from Taiwan [13], Zhejiang province [14] and Yunnan province [15, 16]. In natural foci areas, such as Heilongjiang, Jilin, Henan, Zhejiang, Fujian province, Inner Mongolia, Guangxi Zhuang and Xinjiang Uygur Autonomous Regions, Beijing and Taiwan, *B. microti*-like parasites have been identified in ticks and rodents [12].

Yunnan province is located on the China–Myanmar border in southeastern China, where 83·3% of the national cases of *B. microti* infection have been recorded [15, 16]. Yunnan province is the main area where malaria is endemic in China [12]. Similarities in symptoms and the morphology of the causative agents of babesiosis and malaria can easily result in misdiagnosis [17]. A recent report on the detection of both *B. microti* and plasmodium in this region has further highlighted the necessity of clinical identification and differential diagnosis of babesiosis and malaria by medical care workers. Field investigations are essential for determining the prevalence of *B. microti* in the natural foci of Yunnan province. The current lack of knowledge has hindered the development of informed prevention and control measures by the public health authorities. In this study, we performed field surveys to determine the extent of *B. microti* infection in wild rodents in Yunnan province, where abundant host species exist as potential reservoirs.

MATERIALS AND METHODS

Sample collection

Small wild rodents were collected from the following nine areas of the western Yunnan province: Heqing, Jianchuan, Lanping, Yunlong, Lianghe, Tengchong, Lancang, Menglian and Simao between 2009 and 2011 (Fig. 1 and Table 1). The sampling sites, according to the second national land survey, represent five land cover types: irrigated cropland, rainfed cropland, orchard, forest and shrubland. Altitudes in these regions ranged from 1500 to 3000 meters above sea level. The sampling sites were established on farmlands near the farmhouses where local residents are frequently exposed to wild rodents and ticks. Traps were set nightly at locations where rodent activity was observed and were recovered the next morning. Approximately 250–300 snap traps (baited with peanuts) were placed every night in lines of 20–50 traps at 10 m intervals. Species, age and sex of all captured rodents were identified and the carcasses were subsequently examined for ectoparasites before dissection. Samples were stored in liquid nitrogen until DNA extraction.

DNA extraction

DNA was extracted from spleen tissues using the DNeasy Tissue Kit (QIAGEN, Germantown, MD, USA) following manufacturer's instructions. Negative controls were included throughout the process to exclude the possibility of contamination.

Polymerase chain reaction (PCR) amplification

PCR targeting a specific fragment of *Babesia* 18S rRNA gene was performed using the outer primers Bab 1 and Bab 4 and the inner primers Bab 2 and Bab 3, which amplified a 238 base pairs (bp) and a 154 bp fragments, respectively [18]. Both primary and nested PCR amplifications were performed in a 20 µl volume containing 2 µl of 10 × PCR buffer, 0·2 µl Taq DNA polymerase (5 U/µl), 0·2 µl dNTP mix (10 mM) (all from TaKaRa, Shuzo Co. Ltd, Kyoto, Japan), 15·8 µl deionised water, 1 µl DNA template and 0·4 µl of each primer (12·5 mM). DNA amplification was carried out under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s; followed by a final extension step at 72°C for 7 min. One microliter of the first-round PCR product was used in the second amplification. Conditions and systems used for the second amplification were identical to those used for the first round. For further confirmation, nested PCR was performed; a 1198 bp sequence of 18S rRNA gene was amplified using the specific primer pair *B. microti* 155F 5′– CTAGGGCTAATACATG CTCG –3′ and *B microti* 1606R 5′– ACTAGGCA TTCCTCGTTCA –3′ for the initial amplification and the inner primer pair *B. microti* 255F 5′– AAAT TAGCGAATCGCATGG –3′ and *B. microti* 1453R 5′– ACAGACCTGTTATTGCCTTAC –3′ for the nested PCR reaction. The conditions for both rounds of amplification were 94°C for 5 min; 40 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min 30 s; followed by a final extension step at 72°C for 7 min.

Sequence analysis

Amplicons were sequenced in both directions by automated dideoxynucleotide cycle sequencing (ABI

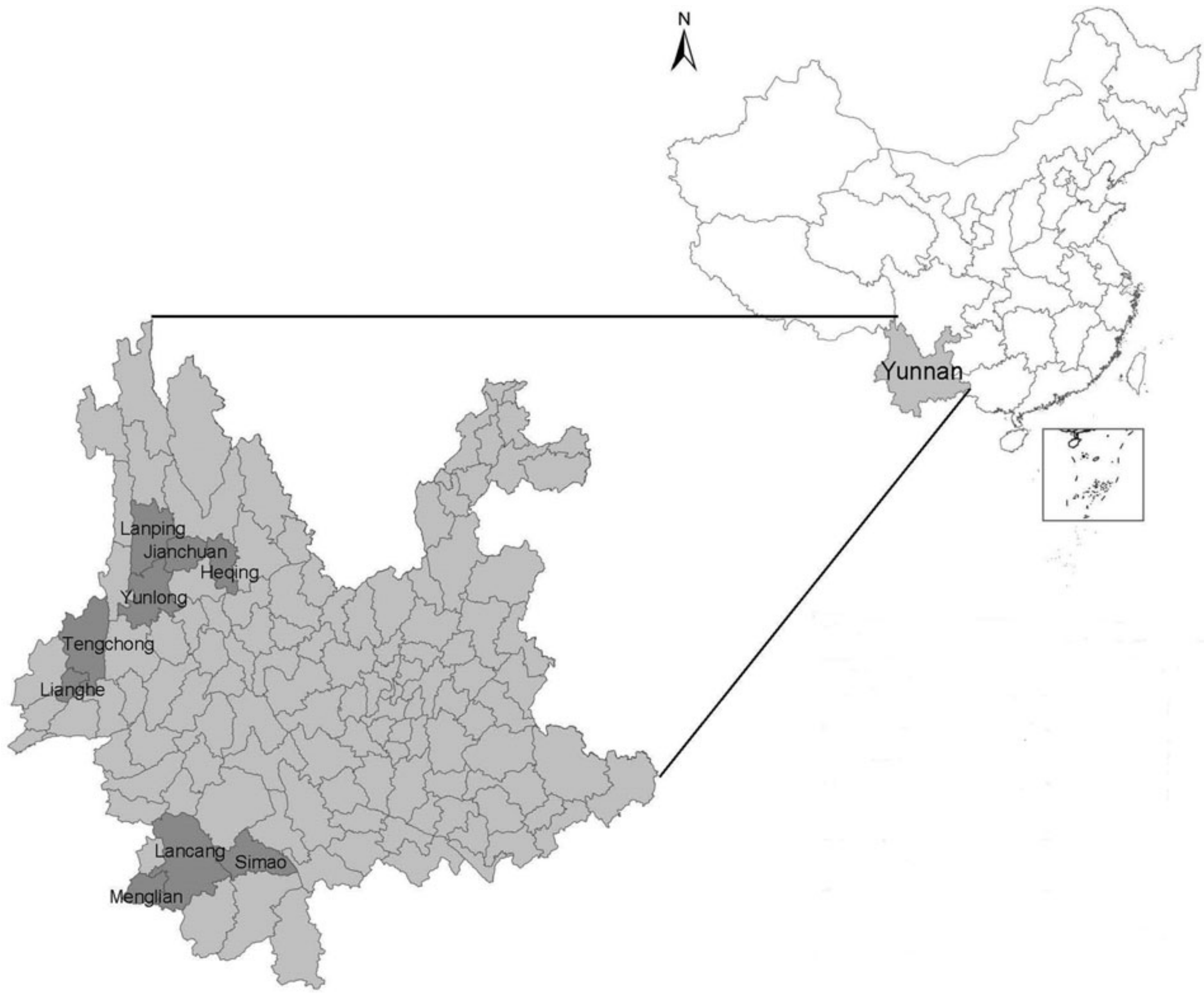

Fig. 1. Geographic locations of captured wild rodents in Yunnan province.

Table 1. *The infection rates of Babesia microti in rodents captured from nine sampling sites in Yunnan province*

Sampling sites	No. rodents tested	No. positive	Detection rate (%)
Tengchong	286	18	6·3
Menglian	271	6	2·2
Lancang	206	8	3·9
Yunlong	203	2	1
Heqing	184	5	2·7
Simao	159	1	0·6
Lianghe	141	11	7·8
Lanping	115	20	17·4
Jianchuan	107	1	0·9
Total	1672	72	4·3

PRISM 377, PerkinElmer, Inc.). The 18S rRNA nucleotide sequences were used for phylogenetic analysis by Mega 5·1 software (http://mega.software.informer.com/5.1b/) based on the available GenBank nucleotide sequences (925 bp) from the USA, Japan and China [11, 16, 19]. *Babesia leo* was used as an outgroup. Phylogenetic trees were constructed using the neighbor-joining algorithm method with the Kimura two-parameter model [20]. Maximum parsimony analyses were conducted to examine the effect of the method used for analysis on the resulting phylogeny. Reliability of the phylogenetic analysis was estimated by bootstrap analysis with 1000 replications.

Statistical analysis

The prevalence of *B. microti* among rodent species and within various geographic locations was compared by χ^2 or the Fisher exact test. For each sampling site, the environmental factors, such as elevation and land use type, were determined using ArcGIS 9·3

https://doi.org/10.1017/S0950268817001686 Published online by Cambridge University Press

software (ESRI Inc.). Poisson regression analysis was done to identify the risk factors for *B. microti* infection using STATA 10·0 software (StataCorp LP, College Station TX, USA). Significance level for all tests was set at $P < 0{\cdot}05$.

RESULTS

A total of 1672 rodents comprising 4 orders were captured at nine locations during a 3-year survey. *B. microti* was detected in 72 (4·3%) rodents (Table 1) and the species *Apodemus chevrieri* exhibited the highest number of animals with *B. microti* infection (273, 16·3%) (Table 2). The 72 infected animals comprised 20 rodent species with *B. microti* infection rates ranging from 1·6% to 50% (Table 2). The infection rates were significantly higher in *Apodemus chevrieri* and *Niviventer fulvescens* than in any other species (χ^2 or the Fisher exact test, $P = 0{\cdot}018$ and 0·023, respectively).

Rodents from all nine survey sites harbored *B. microti* at rates ranging from 0·6% to 17·4% (Table 1). The Lanping (20/115, 17·4%), Lianghe (11/141, 7·8%) and Tengchong regions (18/286, 6·3%) exhibited the highest rates, which increased significantly when Simao was used as a control site (odds ratios 33·26, 13·37 and 10·61, respectively; $P < 0{\cdot}05$ for all) (Table 1). The infection rate was highest in shrub areas, followed by forests and rainfed cropland areas (Table 3). Using the Poisson regression model, we demonstrated that forests ($P = 0{\cdot}024$) and shrublands ($P = 0{\cdot}003$) had significantly higher rates of *B. microti* infection (Table 3). Variation in elevation had no discernible influence on the rates of detection (Table 4). Three *B. microti* variants were identified, including Yunnan-1 (GenBank accession number KC147722) in 49 rodents, Yunnan-2 (GenBank accession number KC147723) in 13 rodents and Yunnan-3 (GenBank accession number KC147724) in 10 rodents. Prevalence of the three *B. microti* variants differed significantly among the various rodent species, land use types and the sampling sites (Supplementary Tables 1, 2 and 3). Detection rates of Yunnan-1, Yunnan-2 and Yunnan-3 strains were highest in *Rattus* (11/49, 22·5%), *Apodemus* (10/13, 76·9%) and *Crocidura* (5/10, 50·0%), respectively. Yunnan-1 was more prevalent in forest areas (25/49, 51·0%), whereas the incidence of Yunnan-2 (4/13, 30·8%) and Yunnan-3 (4/10, 40·0%) infections were higher in rainfed croplands.

Table 2. *Detection rates of Babesia microti among wild rodent species in Yunnan Province*

Animal species	No. tested rodents	No. positive	Infection rate (%)
Apodemus chevrieri	273	19	7·0
Rattus tanezumi	259	8	3·1
Rattus rattus	251	6	2·4
Mus pahari	115	4	3·5
Anourosorex squamipes	99	3	3·0
Eothenomys miletus	79	2	2·5
Crocidura fuliqinosa	71	4	5·7
Mus caroli	64	1	1·6
Crocidura attenuata	53	2	3·8
Suncus murinus	50	3	6·0
Rattus yunnanensis	49	2	4·1
Niviventer fulvescens	45	5	11·1
Hylomys suillus	42	3	7·1
Eothenomys eleusis	40	2	5·0
Niviventer niviventer	30	1	3·3
Apodemus draco	30	2	6·7
Micromys minutus	24	1	4·2
Tupaia belangeri	22	2	9·1
Rattus nitidus	22	0	0
Crocidura horsfieldii	17	0	0
Niviventer coxinqi	7	0	0
Apodemus latronum	6	0	0
Crocidura russula	6	0	0
Berylmys bowersi	4	1	25·0
Eothenomys custos	3	0	0
Crocidura lasiura	2	1	50·0
Rattus norveqicus	2	0	0
Eothenomys miletus	2	0	0
Ochotona thibetana	1	0	0
Bandicota indica	1	0	0
Vernaya fulva	1	0	0
Mus musculus Linnaeus	1	0	0
Soriculus leucops	1	0	0
Total	1672	72	4·3

Phylogenetic analysis revealed that *B microti* sequences obtained in this study clustered with sequences from Japan and southeast China (Fig. 2). The Yunnan-1 sequence varied only by 1 bp from the Kobe-type sequences obtained from samples of a Japanese patient (AB 032434) and rodents in southeastern China (AB 241632). Using random basic local alignment search tool analysis of sequences in GenBank (http://blast.ncbi.nlm.nih.gov), Yunnan-2 and Yunnan-3 sequences were found to be 99% and 98% identical to the Otsu-type strain from Japan (AB 119446·1), respectively.

Table 3. *Comparison of Babesia microti infection rates in different land use types in Yunnan Province*

Land use type	No. rodent tested	No. positive	Infection rate (%)	*P* value
Irrigated cropland	277	7	2·53	
Rainfed cropland	291	11	3·78	0·476
Orchard	360	3	0·83	0·108
Forest	508	31	6·10	0·024
Shrub	238	20	8·40	0·003

The comparison among different land use types was made by Poisson analysis.

Table 4. *Poisson regression analysis of the environmental factors associated with detection of Babesia microti in wild rodents in Yunnan Province*

Influencing variables	*P* value	OR	95% CI
Elevation (30 m)*	0·30	0·99	0·98–1·06
Land use types†	0·03	1·67	1·06–2·31
NDVI‡	0·73	2·54	0·32–5·17

* A continuous variable with the minimum interval of 30 m.
† Five kinds of land use types are rainfed cropland, irrigated cropland, orchard, shrubland and forest.
‡ Normalized difference vegetation index.

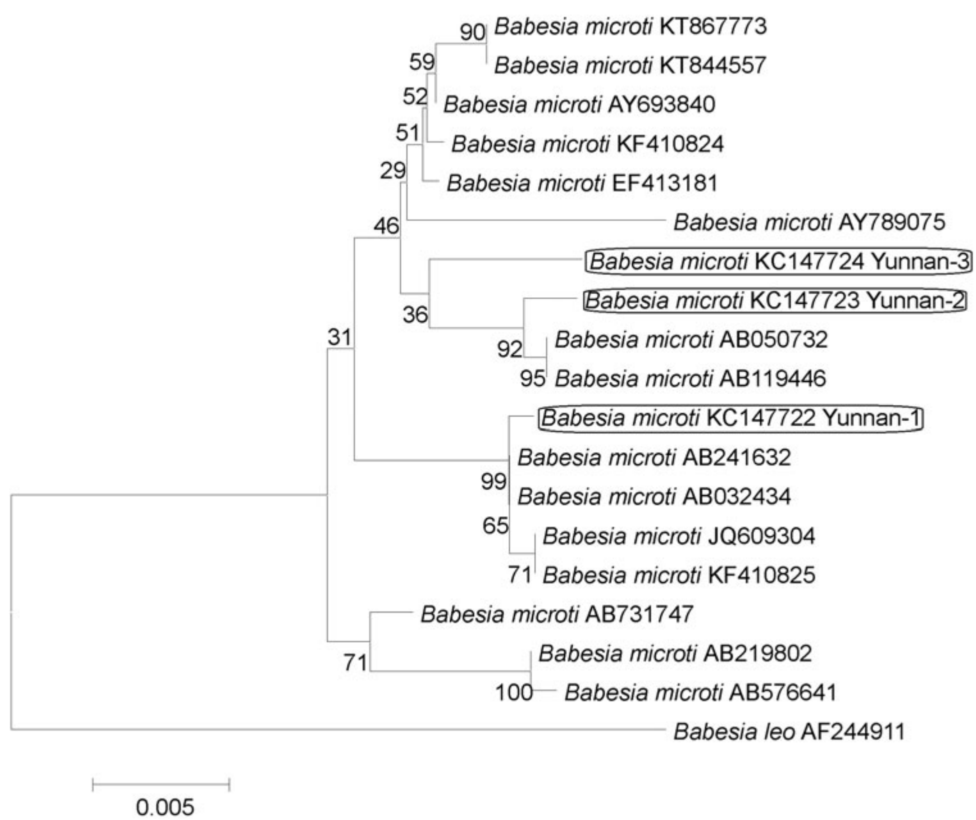

Fig. 2. Neighbor-joining tree of *Babesia microti* inferred from the 1078 bp sequence of 18S rRNA gene using the Neighbor-joining algorithm method with the Kimura two-parameter model. The number on each branch denotes the percent occurrence in 1000 bootstrap replicates. Three genetically different *B. microti* variants, Yunnan-1, Yunnan-2 and Yunnan-3, were detected. Yunnan-1, Yunnan-2 and Yunnan-3 were associated with *Rattus* (11/49, 22·5%), *Apodemus* (10/13, 76·9%) and *Crocidura* (5/10, 50·0%) species, respectively.

DISCUSSION

Babesia spp. are important parasitic protozoans carried by several mammalian hosts and known to cause diseases in humans. High tick infestation rates coupled with the prevalence of the pathogen in cats and dogs was demonstrated in the Wrocław Agglomeration of southwest Poland [21]. *B. microti* and *B. venatorum* were found in 9·0% of *Ixodes ricinus*. Evidence suggests that dogs and cats may contribute substantially to the circulation of the pathogen

https://doi.org/10.1017/S0950268817001686 Published online by Cambridge University Press

among ticks. Some small mammals, such as voles [22] and other rodents [23], are also involved in the enzootic maintenance and transmission of *B. microti*.

In this study, we detected *B. microti* in 4·3% of the rodent species in Yunnan. It is postulated that there might be more rodent species harboring *B. microti* than have been previously reported [12], suggesting that a variety of mammalian species could be involved in the enzootic maintenance and transmission of *B. microti*. According to Zhou *et al.*, *Macaca* species, *Rattus* species, *Niviventer* species and *Citellus* species are also capable of harboring *B. microti* [12]. Our results indicate that a far greater number of rodents are potentially important for maintaining and transmitting *B. microti*. Prevalence of infected rodents is much higher in Lanping (20/115, 17·4%) than in other areas indicating that the residents of Lanping might be at a higher risk for *B. microti* infection. Similarly, people in Lianghe (11/141, 7·8%) and Tengchong (18/286, 6·3%) could also be at a higher risk of infection with *B. microti,* especially because 10 human cases of *Babesia* infection have already been reported in Tengchong [16]. The highest risk of *B. microti* infection was observed in rodents captured from forests or shrublands, which may be related to the geographic distribution of ticks in these natural environments.

Three genetically different *B. microti* variants were detected (Fig. 2). Majority of the identified *Babesia* variants were similar to the Kobe-type genetic variant found in Japan with the capacity for infecting humans [24]. This strain of *B. microti* is different from the strains reported from Tengchong in Yunnan so far [16]. The Kobe-type *Babesia* strain has also been detected in Zhejiang and Fujian provinces [11]. A wide distribution of *Babesia* strains implies a higher risk of human infections in other regions of China as well.

In conclusion, a variety of rodent species were found to be involved in the enzootic maintenance and transmission of *B. microti*. Future investigations are warranted to determine the potential risk of infection in local residents. The most common mode of infection was through tick bites, supporting the need for further investigation into ticks as the important carriers of *B. microti*.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0950268817001686.

ACKNOWLEDGEMENTS

This work was supported by the Natural Science Foundation of Beijing (grant no. 5152023), the Natural Science Foundation of China (grant numbers 31272385 and 30860250), and the State Key Laboratory of Biological Safety of Pathogenic Microorganisms (grant no. SKLPBS1435). The sponsors of the study had no role in the study design, data collection, data analysis, interpretation or writing the report.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All studies and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of State Key Laboratory of Pathogen and Biosecurity (IACUC-2009-036).

AVAILABILITY OF DATA AND MATERIAL

All data generated and analyzed during this study are included in this published article and its supplementary information files.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Bai JY, Ren LZ and Liu W conceived and designed; acquisition, analysis and interpretation of data were done by Chen XR, Fan JW, Ye L, Li C, Tang F and Liu W; Ren LZ and Bai JY drafted the manuscript or revising; final approval of the version to be published were done by Chen XR, Ye L, Fan JW, Li C, Tang F, Liu W, Ren LZ and Bai JY. Accountable for all aspects of the work and for ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved by Chen XR, Ye L, Fan JW, Li C, Tang F, Liu W, Ren LZ and Bai JY. All authors read and approved the final version of the manuscript.

REFERENCES

1. **Homer MJ, *et al.*** Babesiosis. *Clinical Microbiology Reviews* 2000; **13**: 451–469.

2. **Vannier E, Krause PJ.** Human babesiosis. *New England Journal of Medicine* 2012; **366**: 2397–2407.
3. **Vannier E, Krause PJ.** Babesiosis in China, an emerging threat. *Lancet Infectious Diseases* 2015; **15**: 137–139.
4. **Leiby DA.** Transfusion-transmitted *Babesia* spp.: bulls-eye on *Babesia microti*. *Clinical Microbiology Reviews* 2011; **24**: 14–28.
5. **Hildebrandt A, *et al.*** First confirmed autochthonous case of human *Babesia microti* infection in Europe. *European Journal of Clinical Microbiology & Infectious Diseases* 2007; **26**: 595–601.
6. **Sinski E, Welc-Faleciak R.** *Babesia* spp. in Poland: the identity and epidemic reality. *Postepy Mikrobiologii* 2008; **47**: 299–305.
7. **Rar VA, *et al.*** Genetic diversity of Babesia in Ixodes persulcatus and small mammals from North Ural and West Siberia, Russia. *Parasitology* 2011; **138**: 175–182.
8. **Maamun JM, *et al.*** Prevalence of *Babesia microti* in free-ranging baboons and African green monkeys. *Journal of Parasitology* 2011; **97**: 63–67.
9. **Mazyad SA, Shoukry NM, El-Alfy NM.** Efficacy of Ixodes ricinus as a vector of zoonotic babesiosis in Sinai Peninsula, Egypt. *Journal of the Egyptian Society of Parasitology* 2010; **40**: 499–514.
10. **Saito-Ito A, *et al.*** Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. *Journal of Clinical Microbiology* 2000; **38**: 4511–4516.
11. **Saito-Ito A, *et al.*** Detection of Kobe-type *Babesla microti* associated with Japanese human babesiosis in field rodents in central Taiwan and southeastern mainland China. *Parasitology* 2008; **135**(6): 691–699.
12. **Zhou X, *et al.*** Human babesiosis, an emerging tick-borne disease in the People's Republic of China. *Parasites & Vectors* 2014; **7**(18): 509.
13. **Shih CM, *et al.*** Human babesiosis in Taiwan: asymptomatic infection with a *Babesia microti*-like organism in a Taiwanese woman. *Journal of Clinical Microbiology* 1997; **35**(2): 450–454.
14. **Yao LN, *et al.*** Pathogen identification and clinical diagnosis for one case infected with Babesia. *Chinese Journal of Parasitology and Parasitic Diseases* 2012; **30**(2): 118–121 (in Chinese).
15. **Zhou X, *et al.*** Emergence of human babesiosis along the border of China with Myanmar: detection by PCR and confirmation by sequencing. *Emerging Microbes & Infections* 2014; **3**: e55.
16. **Zhou X, *et al.*** Co-infections with *Babesia microti* and plasmodium parasites along the China–Myanmar border. *Infectious Diseases of Poverty* 2013; **2**: 24.
17. **Zhou X, *et al.*** Emergence of babesiosis in China–Myanmar border areas. *Parasites & Vectors* 2015; **8**: 390.
18. **Persing DH, *et al.*** Detection of *Babesia microti* by polymerase chain reaction. *Journal of Clinical Microbiology* 1992; **30**: 2097–2103.
19. **Sun Y, *et al.*** *Babesia microti*-like rodent parasites isolated from Ixodes persulcatus (Acari: Ixodidae) in Heilongjiang Province, China. *Veterinary Parasitology* 2008; **156**: 333–339.
20. **Kimura M.** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 1980; **16**: 111–120.
21. **Król N, *et al.*** Detection of selected pathogens in ticks collected from cats and dogs in the Wrocław Agglomeration, South-West Poland. *Parasites & Vectors* 2016; **9**: 351.
22. **Rar V, *et al.*** High prevalence of *Babesia microti* 'Munich' type in small mammals from an Ixodes persulcatus/Ixodes trianguliceps sympatric area in the Omsk region, Russia. *Parasitology Research* 2016; **115**(9): 3619–3629.
23. **Hamšíková Z, *et al.*** Babesia spp. in ticks and wildlife in different habitat types of Slovakia. *Parasites & Vectors* 2016; **9**(1): 292. doi: 10.1186/s13071-016-1560-z.
24. **Tsuji M, *et al.*** Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasite. *Journal of Clinical Microbiology* 2001; **39**: 4316–4322.