# First detection of *Babesia venatorum* (EU1) in *Ixodes persulcatus* ticks in Mongolia

Tuvshintulga, B.[1,2], Battsetseg, B.[2], Battur, B.[2], Myagmarsuren, P.[2], Narantsatsral, S.[2], Sivakumar, T.[1], Takemae, H.[1], Igarashi, I.[1], Inoue, N.[1] and Yokoyama, N[1] *

*Corresponding author: Yokoyama, N., E-mail address: yokoyama@obihiro.ac.jp

[1]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan, [2]Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Mongolian State University of Agriculture, Ulaanbaatar, Mongolia

## ABSTRACT

*Babesia venatorum*, formerly known as *Babesia* sp. EU1, is a zoonotic hemoprotozoan parasites that commonly infects deer. In the present study, we investigated *B. venatorum* infection in *Ixodes persulcatus*, an important tick vector capable of transmitting several tick-borne pathogens that cause babesiosis, encephalitis, tularemia, and Lyme diseases. DNA samples extracted from questing *I. persulcatus* ticks (n=63) that had been collected in Selenge province of Mongolia in 2012 and 2013 were screened for *B. venatorum* using a nested PCR assay. The findings showed that two of 63 DNA samples were positive for *B. venatorum*. The *18S rRNA* sequences amplified from *B. venatorum*-positive DNA samples shared high identity scores (96.1–99.9%) with known *B. venatorum* sequences derived from human and tick isolates. In phylogenetic analysis, the Mongolian *18S rRNA* sequences clustered with the previously characterized *B. venatorum* sequences. In addition to reporting *B. venatorum* in Mongolia for the first time, the present study identifies *I. persulcatus* as a potential vector of this zoonotic *Babesia* in Mongolia. Additional studies to investigate the prevalence of *B. venatorum* in deer, humans, and ticks in different geographical regions are essential to understand the epidemiology of this parasite species in Mongolia.

**Keywords:** *Babesia venatorum*, *18S rRNA*, *Ixodes persulcatus*, Mongolia

## INTRODUCTION

Babesiosis is a tick-borne disease caused by different species of the genus *Babesia*, which belongs to the order Piroplasmida of the phylum Apicomplexa (Homer *et al*., 2000). The *Babesia* parasites are transmitted by ticks and infect a wide range of wild and domestic animals and humans. The classification of *Babesia* species had traditionally been based on their morphology, host specificity, and life cycle (Homer *et al*., 2000). However, these conventional classification criteria were sometimes found to be complicated, as a single tick species may transmit different *Babesia* species to a particular host species and multiple tick

species may transmit a particular *Babesia* species to different host species. By contrast, classification methods based on the parasite's DNA sequences were proven to be effective in identifying more than 100 *Babesia* species (Allsopp *et al*., 1994; Homer *et al*., 2000). In particular, *18S rRNA* sequences are widely used for the genetic identification of different *Babesia* species in vertebrate hosts as well as in tick vectors (Allsopp *et al*., 1994; Armstrong *et al*., 1998; Bonnet *et al*., 2007; Casati *et al*., 2006; Duh *et al*., 2005a,b; Lack *et al*., 2012).

*Babesia* infection can cause a threat to public health, as several *Babesia* species, including *B*. *microti*, *B*. *divergens*, and *B*. *venatorum* (formerly known as *Babesia* sp. EU1) are known to be zoonotic (Hunfeld *et al*., 2008). Microscopic identification of *B*. *venatorum* is difficult, as this parasite species is almost morphologically indistinguishable from *B*. *divergens* and *B*. *odocoilei* (Herwaldt *et al*., 2003; Hunfeld *et al*., 2008; Langton *et al*., 2003). These three *Babesia* species belong to the large *Babesia* (Hunfeld *et al*., 2008; Vannier and Krause, 2012) and commonly infect cervids (Duh *et al*., 2005b; Herwaldt *et al*., 2003; Holman *et al*., 2000; Langton *et al*., 2003). Therefore, *B*. *venatorum* had previously been misidentified as *B*. *divergens* (Herwaldt *et al*., 2003). Recently, Herwaldt *et al*. (2003) demonstrated that *B*. *venatorum* can be differentiated from *B*. *divergens* and *B*. *odocoilei* by comparing the *18S rRNA* sequences of these parasite species. Since then, *B*. *venatorum* infection has been reported in humans in several countries. Two human cases of *B*. *venatorum* infection were reported in Italy and Austria (Herwaldt *et al*., 2003), and a *B*. *venatorum*-like infection in humans was detected in Germany (Häselbarth *et al*., 2007). Recently, several human cases of *B*. *venatorum* infection were described in China (Jiang *et al*., 2015; Sun *et al*., 2014). In particular, Jiang *et al*. (2015) described *B*. *venatorum* infection in 48 humans in northeastern China, where *Ixodes persulcatus* ticks are endemic (Jiang *et al*., 2015). In the same study, the parasite was detected in *I*. *persulcatus*, suggesting that this tick species might be a potential vector of *B*. *venatorum*.

Geographically, Mongolia is a landlocked country, bordered between Russia and China. Several species of *Babesia* parasites, including *Babesia bovis*, *Babesia bigemina, Babesia ovata*, *Babesia caballi*, and *Theileria* (*Babesia*) *equi*, have been reported in livestock in Mongolia (Altangerel *et al*., 2012; Battsetseg *et al*., 2002; Sivakumar *et al*., 2012; Yoshinari *et al*., 2013). In addition, *Babesia microti* infection was recently detected among human and *I*. *persulcatus* ticks in this country (Hong *et al*., 2014; Tuvshintulga *et al*., 2015). As China, a neighboring country of Mongolia, reported *B*. *venatorum* in *I*. *persulcatus* ticks, we hypothesized that the *I*. *persulcatus* ticks in Mongolia might also be infected with this parasite species. In the present study, therefore, we screened DNA samples extracted from *I*. *persulcatus* ticks collected in Mongolia for *B*. *venatorum* and conducted sequence analysis to confirm our findings.

**First detection of *Babesia venatorum* in Mongolia**

**MATERIAL AND METHODS**

**Tick DNA samples**

A total of 63 *I. persulcatus* adult ticks collected from Selenge province in Mongolia during 2012 and 2013 were subjected to DNA extraction (Tuvshintulga *et al.*, 2015). Briefly, individual ticks were digested with lysis buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA pH 7.5, 10 mM NaCl, 1% SDS, and 100 μg/ml proteinase K), and the DNA samples extracted using the phenol/chloroform method were precipitated in ethanol. The DNAs were dissolved in double-distilled water and stored at −30°C until use.

**PCR detection of *B*. *venatorum* in tick DNA samples**

*B. venatorum 18S rRNA* sequences were aligned using the MultAlin online software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). Two sets of primers were designed for a nested PCR assay targeting a 459 bp DNA fragment within the hypervariable region of the *B. venatorum 18S rRNA* sequence (AY046575). For the first round of PCR, a 10-μl reaction mixture was prepared to include 1 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 0.2 mM each of dNTPs (Applied Biosystems), 1 μM of the outer forward (5′-GGA CCA TTC AAG TTT CTG ACC CA-3′) and outer reverse (5′- GCC CCC AAC CGT TCC TAT TA-3′) primers, 1.25 units of Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), 10–25 ng of tick-DNA, and double-distilled water. The PCR reaction mixtures were subjected to an enzyme activation step at 95°C for 5 min and then to 30 cycles comprising a denaturation step at 95°C for 30 sec, an annealing step at 58°C for 30 sec, and an elongation step at 72°C for 40 sec. A final extension step was performed at 72°C for 7 min. For the second round of PCR, 1 μl of PCR product from the first round was used in a similar reaction mixture in which the primers were replaced with the inner forward (5′-ATC AGC TTG ACG GTA GGG TAT TG-3′) and inner reverse (3′- GTC CTA CTC TAT TAT TCC ATG C-5′) primers. For the second round of PCR, the cycle conditions were the same except the cycle number was increased to 40. The nested PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and then visualized under UV light. PCR bands close to the expected size (459 bp) were considered positive for *B. venatorum*.

**Amplification, cloning, and sequencing of longer fragments of *18S rRNA***

Long fragments of protozoan *18S rRNA* were amplified using tick DNA samples that gave a positive result in the nested PCR assay, as described previously (Kawabuchi *et al.*, 2005) with minor modifications. Briefly, a PCR reaction was set up as described above replacing the primers with the forward (5′-GCC AGT AGT CAT ATG CTT GTC TTA-3′) and reverse (5′-CTC CTT CCT TTA AGT GAT AAG GTT CAC-3′) primers described previously (Kawabuchi *et al.*, 2005). Following an initial enzyme activation step at 95°C for 5 min, 40 cycles comprising a denaturation step at 95°C for 60 sec, an annealing step at 61°C for 60 sec, and an elongation step at 72°C for 90 sec were performed, finishing with a final extension step at 72°C for 10 min. The resultant PCR products were gel extracted and cloned, as described previously (Tuvshintulga *et*

31

*al*., 2015). For each PCR product, three clones were sequenced using an ABI PRISM3100 genetic analyzer (Applied Biosystems).

**Phylogenetic analysis**

The newly determined sequences were initially analyzed using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identity scores between the nucleotide sequences were calculated using the MatGAT 2.02 software (Campanella *et al*., 2003). Subsequently, the *18S rRNA* sequences determined in the present study together with those from several *Babesia* species, including *B. venatorum*, which were retrieved from the GenBank database, were used to construct a maximum likelihood phylogenetic tree based on the Tamura-Nei model (Tamura and Nei, 1993) hosted by the MEGA software version 6.06 (Tamura *et al*., 2013).

**RESULTS**

A nested PCR assay was employed to detect *B. venatorum* in DNA samples extracted from *I. persulcatus* ticks collected in Mongolia. Out of 63 DNA samples tested, two (3.2%) were positive for *B. venatorum*. These positive DNA samples were subjected to a previously established PCR assay to amplify a long fragment of *18S rRNA*, which was cloned and three clones per sample were sequenced. The nucleotide sequences of newly amplified *18r RNA* fragments often varied between clones. The sequences generated from the two parasite-positive DNA samples contained five *18S rRNA* variants (GenBank accession numbers: LC005773–LC005777). The identity scores shared between Mongolian sequences were 99.5−99.6%, while these sequences also shared 96.1−96.2% and 96.1−99.9% identity with Italian/Austrian (AY046575) and Chinese (KF724377) human isolates of *B. venatorum*, respectively. These observations suggested that the Mongolian sequences were derived from *B. venatorum*. A phylogenetic tree using the sequences from Mongolia, known *B. venatorum* isolates, and several other *Babesia* species was constructed to confirm our findings. The results showed that the Mongolian sequences clustered with the known *B. venatorum 18S rRNA* sequences from humans, deer, and ticks and formed a *B. venatorum* clade, which was supported by a 99% bootstrap value and clearly separated from the clades formed by the sequences from other parasite species such as *B. divergens*, *B. odocoilei*, *B. capreoli*, *B. gibsoni*, and *B. microti* (Fig. 1). Within the *B. venatorum* clade, the Mongolian sequences were closely related to the sequences derived from a human and an *I. persulcatus* tick isolated in China (KM244044 and JQ993426, respectively).
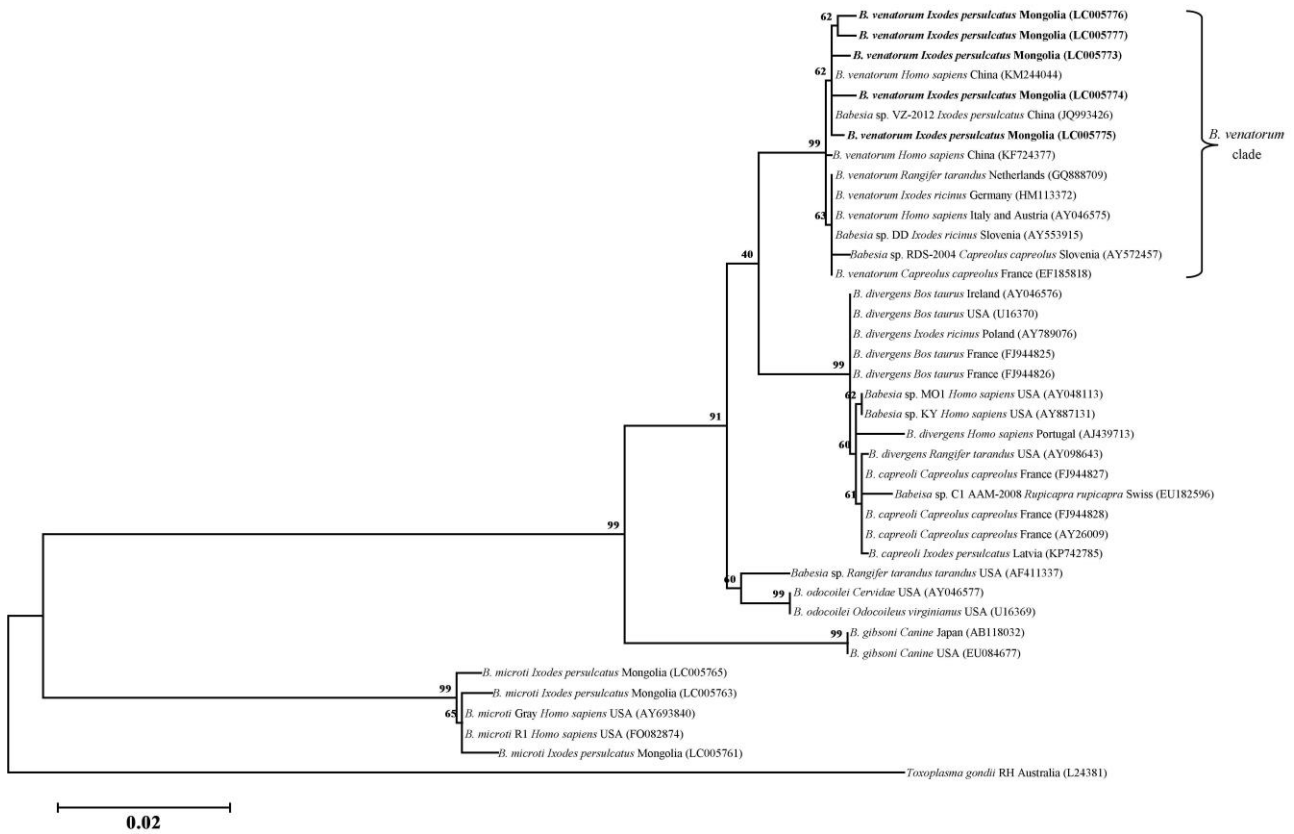
Fig. 1. Phylogenetic tree of the *18S rRNA* sequences. The Mongolian *18S rRNA* sequences together with known *B. venatorum* sequences and sequences from several other *Babesia* species were used to construct a maximum likelihood phylogenetic tree based on the Tamura-Nei model. The Mongolian *B. venatorum* sequences are indicated by boldface letters. Note that the *B. venatorum* sequences formed a separate clade in the phylogeny and that the Mongolian sequences are closely related to the Chinese *B. venatorum* sequences derived from a human and an *I. persulcatus* tick.

## DISCUSSION

Epidemiological studies to investigate *B. venatorum* are important because of its zoonotic potential. Recently, this parasite was reported among humans and deer (Bonnet *et al*., 2007; Kik *et al*., 2011). *B. venatorum* has also been detected in *I. persulcatus* and *Ixodes ricinus* ticks. *I. persulcatus* is an important vector capable of transmitting several pathogens that cause tick-borne diseases, such as babesiosis, encephalitis, tularemia, and Lyme disease (Gray, 1998; Lindquist and Vapalahti, 2008; Zamoto-Niikura *et al*., 2012; Zhang *et al*., 2008). The zoonotic pathogens are mainly transmitted by the adult *I. persulcatus* ticks, as compared with the nymphs (Gray, 1998). Most recently, we demonstrated *B. microti* infection among adult *I. persulcatus* ticks collected in Selenge province of Mongolia (Tuvshintulga *et al*., 2015). In the present study, we analyzed the same *I. persulcatus* DNA samples to detect *B. venatorum* using a nested PCR assay.

Of 63 *I. persulcatus* tick DNA samples, two tested positive for the nested PCR assay employed to detect a *B. venatorum-18S rRNA* fragment. The nucleotide sequences of the long *18S rRNA* fragments amplified from these two positive samples shared high identity scores with known *B. venatorum* sequences, confirming that the Mongolian sequences were indeed derived from *B. venatorum*. *I. persulcatus* is classified

as an important tick species capable of transmitting several zoonotic diseases (Scholz *et al*., 2013; Lindquist and Vapalahti, 2008; Zamoto-Niikura *et al*., 2012). In Mongolia, borreliosis, encephalitis, and human granulocytic anaplasmosis have been serologically demonstrated among humans living in areas where *I. persulcatus* is endemic (Walder *et al*., 2006). Recently, a high prevalence of *Borrelia* sp. in *I. persulcatus* was described in Selenge province of Mongolia (Scholz *et al*., 2013; Masuzawa *et al*., 2014). Furthermore, *B. microti* was recently detected in human blood samples as well as in *I. persulcatus* ticks in Selenge province (Hong *et al*., 2014; Tuvshintulga *et al*., 2015). The findings from the present study suggest that *I. persulcatus* is a potential vector of *B. venatorum* in Mongolia. Therefore, it seems that *I. persulcatus* is an important tick vector for several zoonotic pathogens, including *B. venatorum*, in this country. Therefore, strategies to control *I. persulcatus* should be designed and implemented in Mongolia to reduce the risk of human infection with tick-borne zoonotic pathogens.

On analyzing the phylogeny of the *18S rRNA* sequences, the Mongolian sequences were found to cluster with known *B. venatorum* sequences, confirming the PCR and sequence analysis data. The phylogenetic analysis also demonstrated that the Mongolian *B. venatorum* sequences were closely related to Chinese isolates, which were derived from a human and an *I. persulcatus* tick (Jiang *et al*., 2015). Although these findings suggest that the *B. venatorum*-infected *I. persulcatus* ticks might transmit the infection to humans in Mongolia, additional studies to detect and characterize *B. venatorum* isolated from humans are essential to confirm this assumption.

In conclusion, the present study, which is the first to report *B. venatorum* in Mongolia, found evidence to suggest that *I. persulcatus* as a potential vector of *B. venatorum* in this country. Further studies to determine the prevalence of *B. venatorum* in humans, deer, and ticks in different geographical areas in Mongolia will provide further insight into the epidemiology of this zoonotic pathogen.

**REFERENCES**

Allsopp, M.T.E.P., Cavalier-Smith, T., De Waal, D.T. and Allsopp, B.A. 1994. Phylogeny and evolution of the piroplasms. Parasitology 108: 147−152.

Altangerel, K., Sivakumar, T., Battsetseg, B., Battur, B., Ueno, A., Igarashi, I. and Yokoyama, N. 2012. Phylogenetic relationships of Mongolian *Babesia bovis* isolates based on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes. Vet. Parasitol. 184: 309−316.

Armstrong, P.M., Katavolos, P., Caporale, D.A., Smith, R.P., Spielman, A. and Telford III, S.R. 1998. Diversity of *Babesia* infecting deer ticks (*Ixodes dammini*). Am. J. Trop. Med. Hyg. 58: 739−742.

Battsetseg, B., Lucero, S., Xuan, X., Claveria, F., Byambaa, B., Battur, B., Boldbaatar, D., Batsukh, Z., Khaliunaa, T., Battsetseg, G., Igarashi, I., Nagasawa, H. and Fujisaki, K. 2002. Detection of equine *Babesia* spp. gene fragments in *Dermacentor nuttalli* Olenev 1929 infesting Mongolian horses, and their amplification in egg and larval progenies. J. Vet. Med. Sci. 64: 727−730.

Bonnet, S., Jouglin, M., L'Hostis, M. and Chauvin, A. 2007. *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. Emerg. Infect. Dis. 13: 1208−1210.

Campanella, J.J., Bitincka, L. and Smalley, J. 2003. MatGAT: An application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics 4: 29.

Casati, S., Sager, H., Gern, L. and Piffaretti, J.G. 2006. Presence of potentially pathogenic *Babesia* sp. for human in *Ixodes ricinus* in Switzerland. Ann. Agric. Environ. Med. 13: 65−70.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16: 10881−10890.

Duh, D., Petrovec, M. and Avsic-Zupanc, T. 2005a. Molecular Characterization of human pathogen *Babesia* EU1 in *Ixodes ricinus* ticks from Slovenia. J. Parasitol. 91: 463−465.

Duh, D., Petrovec, M., Bidovec, A. and Avsic-Zupanc, T. 2005b. Cervids as Babesiae hosts, Slovenia. Emerg. Infect. Dis. 11: 1121−1123.

Gray, J.S. 1998. The ecology of ticks transmitting Lyme borreliosis. Exp. Appl. Acarol. 22: 249−258.

Häselbarth, K., Tenter, A.M., Brade, V., Krieger, G. and Hunfeld, K.P. 2007. First case of human babesiosis in Germany − clinical presentation and molecular characterisation of the pathogen. Int. J. Med. Microbiol. 297: 197−204.

Herwaldt, B.L., Cacciò, S., Gherlinzoni, F., Aspöck, H., Slemenda, S.B., Piccaluga, P., Martinelli, G., Edelhofer, R., Hollenstein, U., Poletti, G., Pampiglione, S., Löschenberger, K., Tura, S. and Pieniazek, N.J. 2003. Molecular characterization of a non−*Babesia divergens* organism causing zoonotic babesiosis in Europe. Emerg. Infect. Dis. 9: 942–948.

Holman, P.J., Madeley, J., Craig, T.M., Allsopp, B.A., Allsopp, M.T.E.P., Petrini, K.R., Waghela, S.D. and Wagner, G.G. 2000. Antigenic, phenotypic and molecular characterization confirms *Babesia odocoilei* isolated from three cervids. J. Wildlife Dis. 36: 518-530.

Homer, M.J., Aguilar-Delfin, I., Telford III, S.R., Krause, P.J. and Persing, D.H. 2000. Babesiosis. Clin. Microbiol. Rev. 13: 451.

Hong, S.H., Anu, D., Jeong, Y. II., Abmed, D., Cho, S.H., Lee, W.J. and Lee, S.E. 2014. Molecular detection and seroprevalence of *Babesia microti* among stock farmers in Khutul city, Selenge province, Mongolia. Korean J. Parasitol. 52: 443−447.

Hunfeld, K.P., Hildebrandt, A. and Gray, J.S. 2008. Babesiosis: recent insights into an ancient disease. Int. J. Parasitol. 38: 1219−1237.

Jiang, J.F., Zheng, Y.C., Jiang, R.R., Li, H., Huo, Q.B., Jiang, B.G., Sun, Y., Jia, N., Wang, Y.W., Ma, L., Liu, H.B., Chu, Y.L., Ni, X.B., Liu, K., Song, Y.D., Yao, N.N., Wang, H., Sun, T. and Cao, W.C. 2015. Epidemiological, clinical, and laboratory characteristics of 48 cases of "*Babesia venatorum*" infection in China: a descriptive study. Lancet Infect. Dis. 15: 196-203.

Kawabuchi, T., Tsuji, M., Sado, A., Matoba, Y., Asakawa, M. and Ishihara, C. 2005. *Babesia microti*-like parasites detected in feral raccoons (*Procyon lotor*) captured in Hokkaido, Japan. J. Vet. Med. Sci. 67: 825−827.

Kik, M., Nijhof, A.M., Balk, J.A. and Jongejan, F. 2011. *Babesia* sp. EU1 infection in a forest reindeer, the Netherlands. Emerg. Infect. Dis. 17: 936−938.

Lack, J.B., Reichard, M.V. and Van Den Bussche, R.A. 2012. Phylogeny and evolution of the Piroplasmida as inferred from *18S rRNA* sequences. Int. J. Parasitol. 42: 353−363.

Langton, C., Gray, J.S., Waters, P.F. and Holman, P.J. 2003. Naturally acquired babesiosis in a reindeer (*Rangifer tarandus tarandus*) herd in Great Britain. Parasitol. Res. 89: 194−198.

Lindquist, L. and Vapalahti, O. 2008. Tick-borne encephalitis. Lancet 371: 1861−1871.

Masuzawa, T., Masuda, S., Fukui, T., Okamoto, Y., Bataa, J., Oikawa, Y., Ishiguro, F. and Takada, N. 2014. PCR detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes persulcatus* ticks in Mongolia. Jpn. J. Infect. Dis. 67: 47−49.

Scholz, H.C., Margos, G., Derschum, H., Speck, S., Tserennorov, D., Erdenebat, N., Undraa, B., Enkhtuja, M., Battsetseg, J., Otgonchimeg, C., Otgonsuren, G., Nymadulam, B., Römer, A., Thomas, A., Essbauer, S., Wölfel, R., Kiefer, D., Zöller, L., Otgonbaatar, D. and Fingerle, V. 2013. High prevalence of genetically diverse *Borrelia bavariensis*-like strains in *Ixodes persulcatus* from Selenge Aimag, Mongolia. Ticks Tick Borne Dis. 4: 89−92.

Sivakumar, T., Altangerel, K., Battsetseg, B., Battur, B., AbouLaila, M., Munkhjargal, T., Yoshinari, T., Yokoyama, N. and Igarashi, I. 2012. Genetic detection of *Babesia bigemina* from Mongolian cattle using apical membrane antigen-1 gene-based PCR assay. Vet. Parasitol. 187: 17−22.

Sun, Y., Li, S.G., Jiang, J.F., Wang, X., Zhang, Y., Wang, H. and Cao, W.C. 2014. *Babesia venatorum* infection in child, China. Emerg. Infect. Dis. 20: 896−897.

Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512−526.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725−2729.

Tuvshintulga, B., Sivakumar, T., Battsetseg, B., Narantsatsral, S., Enkhtaivan, B., Battur, B., Hayashida, K., Okubo, K., Ishizaki, T., Inoue, N., Igarashi, I. and Yokoyama, N. 2015. The PCR detection and phylogenetic characterization of *Babesia microti* in questing ticks in Mongolia. Parasitol. Int. 64: 527−532.

Vannier, E. and Krause, P.J. 2012. Human babesiosis. N. Engl. J. Med. 366: 2397−2407.

Walder, G., Lkhamsuren, E., Shagdar, A., Bataa, J., Batmunkh, T., Orth, D., Heinz, F.X., Danichova, G.A., Khasnatinov, M.A., Würzner, R. and Dierich, M.P. 2006. Serological evidence for tick-borne encephalitis, borreliosis, and human granulocytic anaplasmosis in Mongolia. Int. J. Med. Microbiol. 296: S1, 69−75.

Yoshinari, T., Sivakumar, T., Asada, M., Battsetseg, B., Huang, X., Lan, D.T.B., Inpankaew, T., Ybañez, A., Alhassan, A., Thekisoe, O.M.M., De Macedo, A.C.C., Inokuma, H., Igarashi, I. and Yokoyama, N. 2013. A PCR based survey of *Babesia ovata* in cattle from various Asian, African and South American countries. J. Vet. Med. Sci. 75: 211−214.

Zamoto-Niikura, A., Tsuji, M., Qiang, W., Nakao, M., Hirata, H. and Ishihara, C. 2012. Detection of two zoonotic *Babesia microti* lineages, the Hobetsu and U.S. lineages, in two sypatric tick species, *Ixodes ovatus* and *Ixodes persulcatus*, respectively, in Japan. Appl. Environ. Microb. 78: 3424−3430.

Zhang, F., Liu, W., Wu, X.M., Xin, Z.T., Zhao, Q.M., Yang, H. and Cao, W.C. 2008. Detection of *Francisella tularensis* in ticks and identification of their genotypes using multiple−locus variable−number tandem repeat analysis. BMC Microbiol. 8: 152.