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

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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 (Haselbarth 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.
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 x 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 AGA GGA TGG 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
First detection of *Babesia venatorum* in Mongolia

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

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.

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First detection of *Babesia venatorum* in Mongolia


First detection of *Babesia venatorum* in Mongolia


