

Detection and characterization of an emerging type of *Babesia* sp. similar to *Babesia motasi* for the first case of human babesiosis and ticks in Korea

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ABSTRACT

Babesiosis is a tick-transmitted intraerythrocytic zoonosis. In Korea, the first mortalities were reported in 2005 due to *Babesia* sp. detection in sheep; herein we report epidemiological and genetic characteristics of a second case of babesiosis. Microscopic analysis of patient blood revealed polymorphic merozoites. To detect *Babesia* spp., PCR was performed using *Babesia* specific primers for β -tubulin, 18S rDNA, COB, and COX3 gene fragments. 18S rDNA analysis for *Babesia* sp., showed 98% homology with ovine *Babesia* sp. and with *Babesia* infections in Korea in 2005. Moreover, phylogenetic analysis of 18S rDNA, COB, and COX3 revealed close associations with *B. motasi*. For identifying the infectious agent, *Haemaphysalis longicornis* (296) and *Haemaphysalis flava* (301) were collected around the previous residence of the babesiosis patient. *Babesia* genes were identified in three *H. longicornis*: one sample was identified as *B. microti* and two samples were 98% homologous to *B. motasi*. Our study is the first direct confirmation of the infectious agent for human babesiosis. This case most likely resulted from tick bites from ticks near the patient house of the babesiosis patient. *H. longicornis* has been implicated as a vector of *B. microti* and other *Babesia* sp. infections.

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Introduction



Babesiosis, caused by intraerythrocytic protozoans of the Genus *Babesia*, is a zoonotic tick-borne disease, but also can be transmitted through blood transfusion [1,2]. Babesiosis is an emerging health concern and has been progressively diagnosed beyond known endemic areas [3–5]. More than 100 *Babesia* species cause infections in many wild and domestic animals, with varying degrees of virulence among different species of *Babesia* in humans and animals. *B. microti*, *B. divergens*, and *B. venatorum* are the three most predominant species known to infect humans, while other species, *B. ovis*, *B. major*, *B. bovis*, *B. bigemina*, *B. ovata*, *B. orientalis*, *B. motasi*, and *B. caballi* are causal agents for animal infections [6].

Four Genera, *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, and *Hyalomma* within the *Ixodidae* have been reported as vectors of *Babesia* spp. Geographically, *Ixodes scapularis* in the USA, *I. Ricinus* in Europe, and *I. persulcatus* in Asia transmit *Babesia* parasites to natural hosts [7–9]. *B. ovis*, *B. motasi*, and *B. crassa* are primarily transmitted via *Haemaphysalis qinghaiensis* and *H. longicornis* [10]. In addition, *B. bovis* and *B. bigemina* are also

transmitted primarily via *Rhipicephalus microplus* and *R. annulatus*. Recently, *B. divergens*, *B. microti*, and *B. venatorum* were detected in *I. persulcatus* and *B. divergens* in *H. longicornis* was detected in China.

Human cases of *B. microti*, *B. duncani*, and *B. divergens*-like infections have been reported in several regions in the USA [1,11–13]. Sporadic cases of infection by *B. microti*-like or uncharacterized *Babesia* species were reported in Africa, South America, and Asia [1,14]. In East Asia, cases of human babesiosis have been reported in Japan [15] and Taiwan [16], caused by *B. microti*-like parasites; however, the patients were asymptomatic. In Korea, the first case of human babesiosis with a suspected *B. ovis*-like infection was reported in 2005; however, there were no epidemiological investigations conducted to determine the accepted vectors at that time [17].

The purpose of this study was to: (i) identify the *Babesia* sp., reported as a novel large *Babesia* parasite infecting humans; (ii) investigate the distribution and diversity of *Babesia* sp. in ticks collected around the patient's previous residence; and (iii) analyse the phylogenetic association of the *Babesia* sp. detected in the patient with that detected in associated ticks.

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Materials and methods

Blood staining

A blood sample was obtained from the jugular vein in the patient that presented with dizziness and general weakness. Thin blood films were made on dry and clean slides, air-dried, and fixed with methyl alcohol for 5 min, followed by staining with 5% Giemsa solution [18]. The slide was stained for 30 min and then dried for 30 min. Thereafter, the stained films were examined via light microscopy at $\times 100$ magnification with an oil-immersion lens for blood parasites (Leica Microsystems, Heeburg, Singapore). Percentage parasitemia was determined by counting the number of parasitized erythrocytes and dividing that by the total number of red blood cells (RBCs): % parasitemia = (infected RBCs/RBCs) \times 100. A minimum of 500 RBCs were counted and RBCs infected with multiple parasites were counted as a single infected cell.

DNA isolation from blood

Genomic DNA was extracted from blood samples, using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The extracted DNA was eluted in 100 μ L of elution buffer. The quantity and quality of the isolated DNA were assessed with a spectrophotometer Nanodrop 2000c (Thermo Fisher Scientific, Massachusetts, USA) and stored at -20°C until use.

Tick collection and DNA extraction

Questing ticks were collected by flagging and a dry ice bait trap in Hoengseong-gun, Gangwon-do, Korea as described by H.S. Ginsberg and C.P. Ewing [19]. The collection area was divided into each three parts, adjusting the differential collecting methods depending on short and long grasses for efficient tick sampling

regardless of tick spp. The ticks with short grasses were collected by flagging and those with long grasses were collected by dry ice trap. A total of 597 ticks were collected in the survey area. Genomic DNA was isolated from individual ticks, using the DNeasy tissue kit (Qiagen) in accordance with the manufacturer's instructions. Quantity and quality of the isolated DNA were assessed with a spectrophotometer Nanodrop 2000c (Thermo Fisher Scientific) and stored at -20°C until use.

Polymerase chain reaction (PCR) assays

Babesia spp. were detected via PCR for 18S rDNA, Cytochrome b (COB) and Cytochrome c oxidase subunit III (COX3) in accordance with a previously described method [20–23] (Table 1). *B. microti* and *B. divergens* were detected via PCR, as described previously [24–26]. Reactions were carried out in 20- μ L reaction mixtures by using AccuPower PCR master mix (Bioneer, Daejeon, Korea) containing 1 μ M each of the 1st forward and reverse primers, sterile water, and 500 ng of DNA template. Amplification products were electrophoresed using an auto-electrophoresis device (QIAxcel, Hilden, Germany) [27]. The PCR products were then purified using an agarose gel DNA purification kit (Qiagen) [27]. TA cloning was performed using the TOPO TA cloning kit with isolated PCR products for sequencing (Invitrogen, Carlsbad, CA, USA). These samples were sequenced using an ABI PRISM 3730xl Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis

PCR products were purified and sequenced in both directions, using the specific primers. Nucleotide sequences were analysed using BLAST-N and aligned with ClustalW. The pairwise distance was analysed using the Kimura's 2-parameter model. Phylogenetic

Table 1. Forward and reverse primers used for the detection of *Babesia* spp.

Gene	Primers	Amplification protocol	Size	Ref
18S rRNA (<i>Babesia</i> sp.)	1st PCR BTH 1F 5'-CCTGAGAAACGGCTACCACATCT-3'	94°C: 10 min, 45 cycles: (95°C: 30 s, 68°C: 1 min, 72°C: 1 min) 72°C: 10 min	561 bp	[20]
	BTH 1R 5'-TTGCGACCATACTCCCCCA-3'			
18S rRNA (<i>Babesia</i> sp.)	2nd PCR GF2F 5'-GTCTTGTAATTGGAATGATG-3'	94°C: 10 min, 40 cycles: (95°C: 30 s, 60°C: 1 min, 72°C: 1 min) 72°C: 10 min	~1.7 kb	[21]
	GR2R 5'-CCAAAGACTTTGATTTCTCTC-3'			
18S rRNA (<i>Babesia</i> sp.)	EUK F 5'-ACCTGGTTGATCCTGCCAGT-3'	94°C: 5 min, 35 cycles: (94°C: 1 min, 55°C: 1 min, 72°C: 1 min 30s) 72°C: 10 min		
	EUK R 5'-TGATCCTTCTGCAGGTTCCACTAC-3'			
Cytochrome b (COB) (<i>Babesia</i> sp.)	COB F 5'-CCATAGCAATTAATCCAGCTA-3'	94°C: 10 min, 35 cycles: (94°C: 40 s, 54°C: 30 s, 72°C: 1 min) 72°C: 10 min	550 bp	[22]
	COB R 5'-ACCTTGGTCATGGTATTCTGG-3'			
Cytochrome c oxidase subunit III (COX-3) (<i>Babesia</i> sp.)	COX3 F 5'-TCAACAAAATGCCAATATGT-3'	94°C: 10 min, 35 cycles: (94°C: 40 s, 54°C: 30 s, 72°C: 1 min) 72°C: 10 min	552bp	[23]
	COX3 R 5'-AAGTGCATCTTTGGGAGAAG-3'			
β -tubulin (<i>B. microti</i>)	1st PCR Tubu93 F 5'-GAYAGYCCCTTRCAACTAGAAAGAGC-3'	95°C: 10 min, 35 cycles: (95°C: 30 s, 58°C: 1 min, 72°C: 1 min 30 s) 72°C: 10 min	551 bp	[24]
	Tubu897 R 5'-CGRTCGAACGAACATTTGTHGTCARTTC-3'			
18S rRNA (<i>B. divergens</i>)	2nd PCR Tubu192 F 5'-ACHATGGATTCTGTTAGATCYGGC-3'	94°C: 10 min, 45 cycles: (94°C: 30 s, 61°C: 30 s, 72°C: 1 min) 72°C: 10 min	353 bp	[25]
	Tubu782 R 5'-GGGAADGGDTRAGATTCACAGC-3'			
	B.diver F 5'-GTTTCTGMCCCATCAGCTTAC-3'			
	B.diver R 5'-CAATATTAACACCACGCAAAAATC-3'			

analyses were conducted using the software MEGA 6 [28]. The neighbor-joining method was employed to construct a phylogenetic tree. The reliability of the branches in the tree was evaluated via bootstrapping analysis with 1000 replicates [29], and a bootstrap value more than 60% was considered significant.

Results

Clinical signs

A 70-year-old man was hospitalized with a history of dizziness and general weakness for 2 d. The patient received medication for diabetes mellitus, hypertension, and hyperlipidaemia, and underwent total pancreatectomy for intraductal papillary mucinous neoplasm 19 months ago. The patient resided in a suburban area in Hoengseong-gun, Gangwon-do, Korea. Initial analyses of vital signs were: a blood pressure 86/30 mmHg; pulse rate, 138 beats/min; respiratory rate, 20 breaths/min; body temperature, 39.5°C. Initial laboratory analysis: leukocyte count, 8840/ μ L with 93.5% neutrophils; haemoglobin, 14.5 g/dL; platelet count, 90,000/ μ L; blood urea nitrogen, 38.1 mg/dL; creatinine, 3.03 mg/dL; total bilirubin, 2.49 mg/dL; aspartate transaminase (AST), 336 IU/L; alanine transaminase (ALT), 86 IU/L; C-reactive protein, 8.2 mg/dL. Abdominopelvic computed tomography revealed no suspicious focal infection or local and distant metastasis. Piperacillin/tazobactam were administered as an initial empirical antimicrobial therapy. Blood analysis on the second day, 22 h after hospitalization changed as follows: leukocyte count, 6590/ μ L with 90.7% neutrophils; haemoglobin, 11.9 g/dL; platelet count, 21,000/ μ L; blood urea nitrogen, 33.0 mg/dL; creatinine, 2.21 mg/dL; total bilirubin, 7.98 mg/dL; AST, 1,010 IU/L; ALT, 191 IU. The patient's consciousness was drowned and the oxygen saturation decreased to 82–83%. Chest X-ray revealed no abnormal findings. The patient was transferred to the intensive care unit and subjected to intubation and continuous renal replacement therapy; however, the patient died 36 h after hospitalization. No microorganisms were isolated from the blood culture.

Microscopic findings

Upon light microscopic examination, variable intraerythrocytic parasites as ring forms, pear-shaped forms, paired pyriforms, pleomorphic ring forms, and multiple-infected parasites and clusters of extracellular rings were detected in Giemsa-stained blood smears. The percentage of parasitaemia was 1.8% (Figure 1). Maltese cross forms comprising four masses in an erythrocyte that are often described as a characteristic of *B. microti* infection were not detected in most blood smears (Figure 1).

Molecular characteristics of *Babesia* sp. in human

Genomic DNA was extracted from the patient's blood sample and subjected to 18S rDNA analysis for *Babesia* species, Beta-tubulin for *B. microti*, and 18S rDNA for *B. divergens*. Only the 18S rDNA, *Babesia* sp. KCDC-1 (MK930513) was positive for *Babesia* species. Consequently, the samples were subjected to full-length (~1.7 kb) amplification of 18S rDNA for *Babesia* spp. Samples with positive results upon PCR sequenced sequences then subjected to phylogenetic analysis with related *Babesia* species for 18S rRNA gene sequences in GenBank. The analysis demonstrated that the positive sequences were closely related to *Babesia* sp. KO1, ovine *Babesia* sp. Liaoning 2005, and ovine *Babesia* sp. Hebei-2005 (DQ346955, DQ159075, and DQ159074) (Figure 2). To further evaluate the classification of each sample, the *Babesia* species was subjected to genomic DNA analysis using *COB* and *COX3* gene fragments of *Babesia* species. Positive sequences from the analysis of both genes were identified and sequenced. The sequences were then phylogenetically analysed with *COB*, *Babesia* sp. KCDC-1 (MK918505) and *COX3*, *Babesia* sp. KCDC-1 (MK918507) sequences of related *Babesia* species in GenBank. This analysis demonstrated that the positive sequences of *COB* and *COX3* were closely related with ovine *Babesia motasi* Ningxian (JX440507) (Figure 3 (A)) and ovine *B. motasi* Ningxian (JX866781) (Figure 3(B)), respectively. As these sequences were clustered with *B. motasi* in the phylogenetic tree, the parasite detected from the patient's blood belonged to ovine *B. motasi*.

Identification and molecular detection of *Babesia* sp. in questing ticks

Tick collections were performed by dividing the area around the patient's residence into six parts (1: front yard of patient's residence, 2: patient's residence hill III, 3: back yard of patient's residence, a: Fields surrounding the patient's residence, b: patient's residence hill I, c: patient's residence hill II) (Figure 4). A total of 597 ticks were collected around the patient's residence, including 296 *H. longicornis* (186 adult, 41 nymphs, and 68 larvae) and 301 *Haemaphysalis flava* (1 adult and 300 larvae) (Table 2). Among these, 94% of the ticks were collected in both the front yard of patient's residence (442 ticks) and associated hill III (124 ticks). Based on the results of the amplification of *Babesia* genes in each tick, 2 (0.3%) were positive for 18S rDNA of *Babesia* species, 1 (0.2%) for *COB* and *COX3*, and 1 (0.2%) for β -tubulin gene of *B. microti*. While the nymph of *H. longicornis* yielded a positive result for only 18S rDNA, one female tick of *H. longicornis* yielded positive results for 18S rDNA,

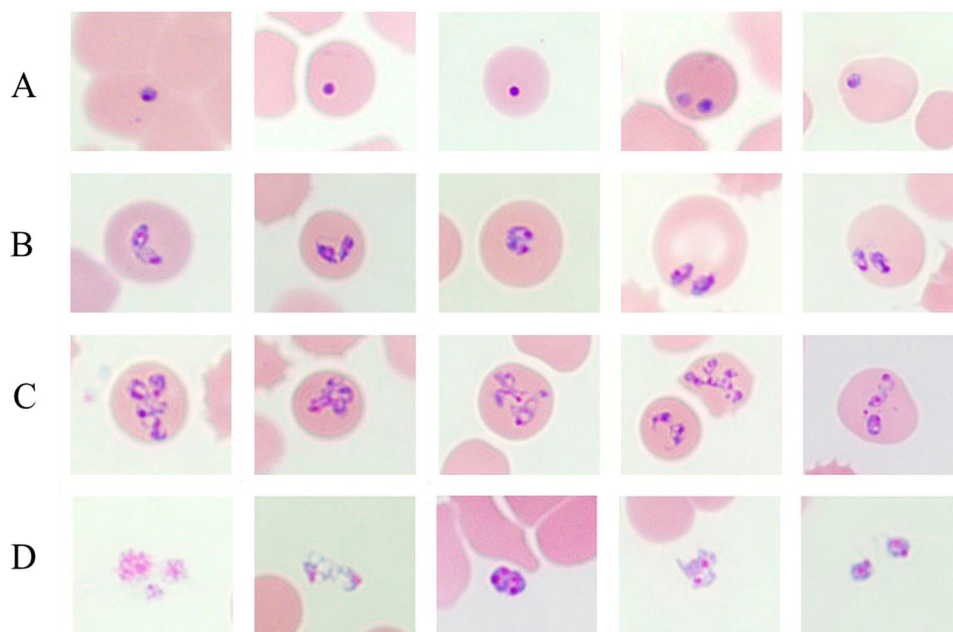


Figure 1. *Babesia* spp in a thin blood smear stained with 5% Giemsa on May 30, 2017, from a patient in Hoengseong-gun, Gangwon-do, Korea, showing pleomorphic and pyriform rings and multiple-infected RBCs. Pigment not present in any of the parasites. (A) Ring-form parasites; (B) Paired-pyriform parasites; (C) Pleomorphic ring forms and multiply infected parasites; (D) Cluster of extracellular rings.

COB, and COX3 gene fragments. Also, one female tick of *H. longicornis* only yielded positive results for β -tubulin gene of *B. microti* (Table 3).

Molecular characteristics of *Babesia* sp. in questing ticks

Sequencing of the PCR product allowed for the determination of *Babesia* species. The sequences are then phylogenetically analysed with related *Babesia* species for 18S rDNA, COB, COX3, and β -tubulin sequences in GenBank. Sequences of the 18S rRNA gene, *Babesia* sp. KCDC-GT-270 and KCDC-GT-272 (MK918504 and MK918503) in 2 *H. longicornis* were closely related with that of ovine *Babesia* sp. Hebei-2005 (DQ159074) (Figure 2). Also, the sequence of COB, *Babesia* sp. KCDC-GT-270 (MK918506) and COX3, *Babesia* sp. KCDC-GT-270 (MK918508) in *H. longicornis* was very closely related with ovine *Babesia* sp. Ningxian (JX866781) (Figure 3(A,B)). Since these sequences clustered with *B. motasi*, the *Babesia* sp. detected from *H. longicornis* was identified as ovine *B. motasi*. The sequence of the β -tubulin gene, *B. microti* KCDC-GT-6 (MK918509) in a female *H. longicornis* was very similar to that of the US-type *B. microti* (Figure 5). Furthermore, the β -tubulin sequence in the positive samples in the present study was closely related to those reported previously in Korea and Russia in East Asia.

Discussion

The severity of *Babesia* infections in humans is rapidly becoming apparent, irrespective of whether the disease

is transmitted via a tick bite or secondarily transmitted via transfusion of infected blood [30–32]. Previously, seven different *Babesia* spp., *B. microti*, *B. divergens*, *B. bovis*, *B. canis*, *B. duncani*, *B. venatorium*, and a novel *Babesia* sp. similar to ovine babesias were reported to cause human babesiosis [5,17,26,32,33]. Regarding the geographical distribution of human babesiosis, *B. microti* is the primary species responsible for disease in the USA [34] while *B. divergens* is the causative agent in Europe [35,36]. In Asia, human cases of a *B. microti* infections have been reported in Taiwan, Japan, China, and Mongolia [16,37,38]. In Korea, the first case of human babesiosis (KO1) was reported in 2007 and KO1 was highly related to Chinese ovine *Babesia* sp. [17]. Unfortunately, there was no epidemiological surveys conducted involving patients to determine the causative infects. Human babesiosis (KCDC-1) in 2017 was the second case identified in Korea and the sequence of *Babesia* sp. was very closely related to that of KO1 and Liaoning, China. These large *Babesia* are clearly distinct from other agents of human babesiosis based on their shape and phylogeny. These results suggest that the causative agent in their case of babesiosis is a novel large *Babesia* parasite infecting humans and may be highly fatal.

Human babesiosis infections and virulence depend on a number of factors, such as parasite species, patient, age, and host immune competency [39]. In general, old age and reduced cellular immunity are associated with a higher risk of symptomatic infection and more severe illness. In particular, most cases of severe babesiosis were reported among individuals lacking a spleen [14,40]. When *Babesia* sp. sporozoites

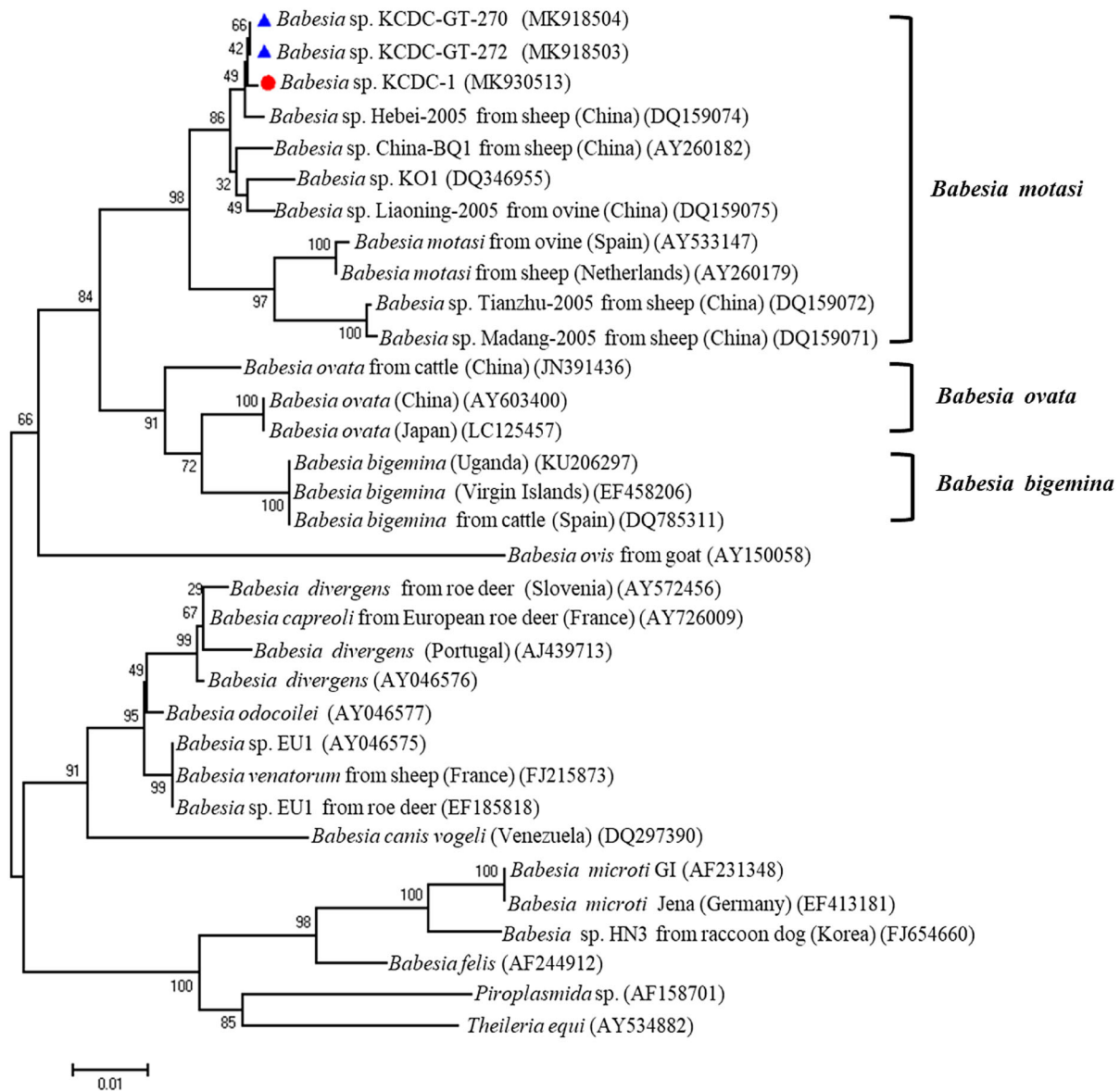


Figure 2. Phylogenetic relationships based on the 18S rRNA gene sequence of *Babesia* species in a human babesiosis sample and in ticks, *Babesia* sp. KCDC-GT-270, *Babesia* sp. KCDC-GR-272 and *Babesia* sp. KCDC-1, in accordance with the polymerase chain reaction-amplified sequence. The evolutionary history was inferred via the Neighbor-Joining method. The percentage of replicate trees wherein the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted using MEGA6 (● *Babesia* positive in this study) (▲ *Babesia* positives in ticks).

first infect humans, they immediately target the host RBCs. Further, infected RBCs remain circulating in peripheral blood and frequently penetrate the host's spleen [41–43]. The spleen with its lympho-reticular filter function is essential in resisting primary infections of *Babesia* sp. by eliminating infected cells from circulation. The spleen and cellular immune response play an essential role in resisting both primary and challenge infections of *Babesia* species [26]. However, the spleen is not critical for the development of immunity and its primary function is to eliminate infected cells from circulation and to stimulate phagocytosis of infected cells [36,44]. In this study, the patient had a splenectomy and red blood cells were considered to be infected with *Babesia* sp. and were not eliminated, thus resulting in the fatality, which is highly suggestive

that splenectomy is one of the critical risk factors for *Babesia* sp. symptomatic infections and potential fatality.

Inside RBCs, *Babesia* sp. begins a cycle of maturation and growth. In the initial stages of their life cycle, *Babesia* spp. are morphologically indistinguishable from *Plasmodium* sp., both appearing as ring-like parasites. Duplication occurs via budding, where one ring divides into two rings. That is commonly referred to as the “figure eight” form. Budding may recur, giving rise to the tetrad form known as a Maltese Cross [42]. Both these morphological forms are unique to *Babesia* spp. and form the basis of a microscopy-based conclusive diagnosis. Based on morphology, *Babesia* spp. are divided into the small babesias including *B. gibsoni*, *B. microti*, and *B. divergens* and large

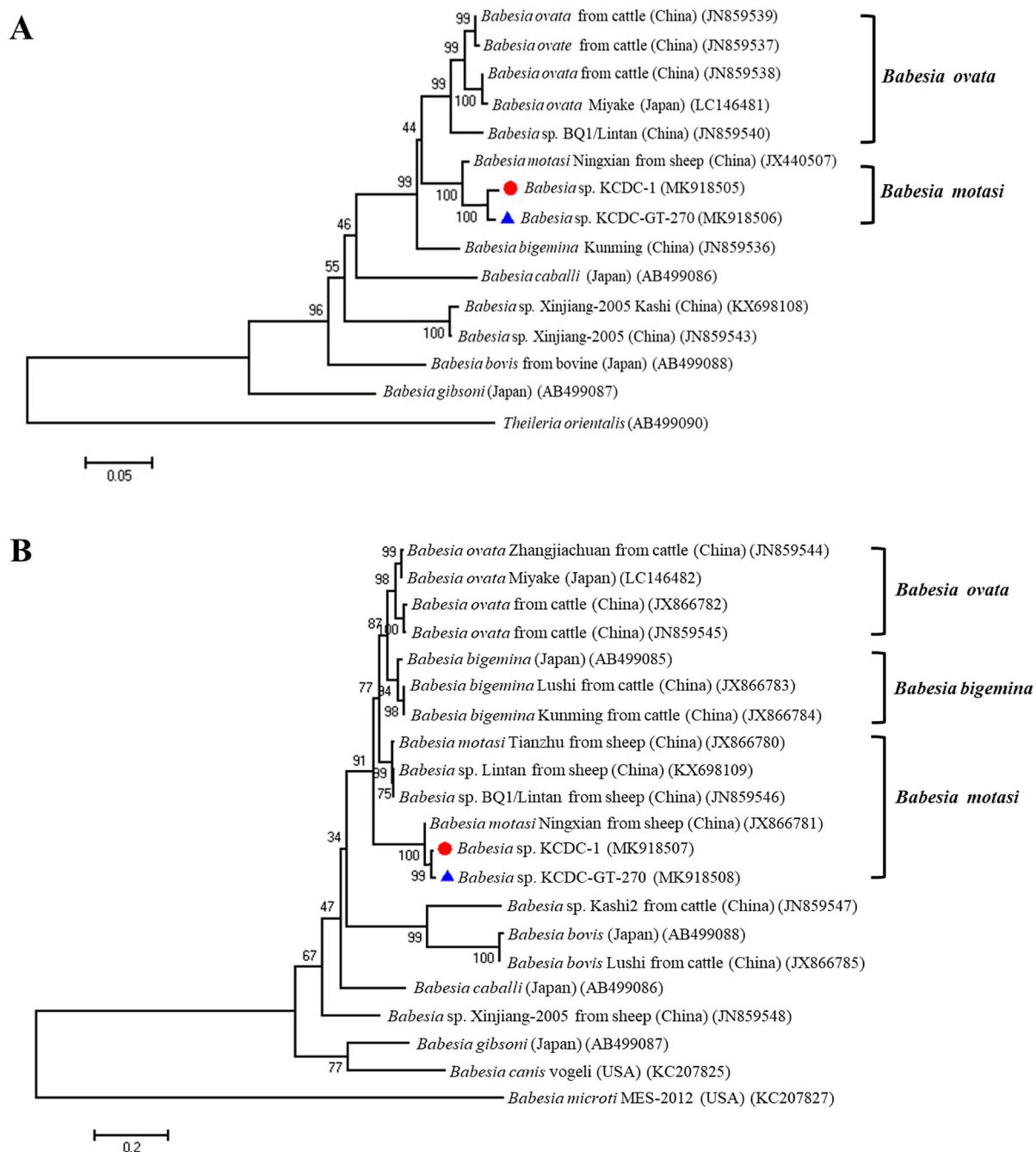


Figure 3. Phylogenetic relationships based on *COB* (A) and *COX3* (B) sequence of *Babesia* species in a human babesiosis sample and ticks, *Babesia* sp. KCDC-GT-270 and *Babesia* sp. KCDC-1, in accordance with the polymerase chain reaction-amplified sequence. The evolutionary history was inferred via the Neighbor-Joining method. The percentage of replicate trees wherein the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted using MEGA6 (● *Babesia* positive in this study) (▲ *Babesia* positives in ticks).

babesias including *B. bovis*, *B. caballi*, and *B. canis*. While small *Babesia* spp. are 1.0–2.5- μ m-long and regularly appear in the ring form and Maltese cross form, large *Babesia* spp. are 2.5–5- μ m-long and regularly appears as a unique paired pyriform [26,45]. The form of parasites detected in the patient's blood showed pleomorphic and paired pyriform rings. Hence, the *Babesia* sp. KCDC-1 was classified as large *Babesia* spp. on the basis of appearance and size.

Regarding the molecular characteristics of the parasite obtained from the patient's blood, we performed

nested PCR for 18S rDNA and the β -tubulin gene of *B. microti*, which has been regarded as the most probable cause of human babesiosis and has been detected in wild mice and other mammals in Korea [46,47]. When we sequenced full-length sequence of 18S rDNA of *Babesia* sp. [37,38], the sequence was identical to that of *Babesia* sp. KO1 (DQ346955) in Korea. Phylogenetic analysis, *Babesia* sp. KCDC-1 in our study formed a clade including several ovine *Babesia* spp. reported previously in China [48]. Based on the analysis of 18S rDNA amplification from the collected ticks, two

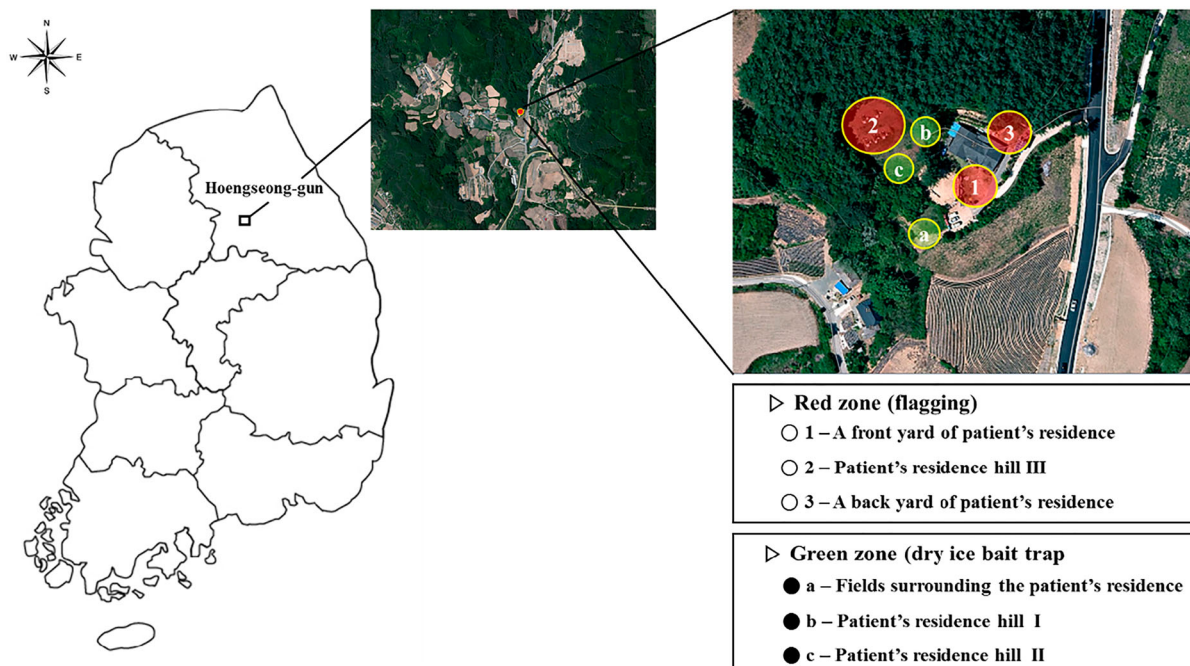


Figure 4. Location of the sampling site and collection methods surrounding the *Babesia*-positive patient's residence in Hoengseong-gun, Gangwon-do, Korea.

sequences of *Babesia* spp. KCDC-GT-270 and KCDCGT-272 were identical to that of *Babesia* sp. KO1 (DQ346955) and *Babesia* sp. Hebei-2005 (DQ159074). Previous studies confirmed that the occurrence of additional viable sites in *COB* and *COX3* allowed for an improved interspecies differentiation of *Babesia* species [22,23]. The phylogenetic analysis for both *COB* and *COX3* indicates that *Babesia* sp. KCDC-1 and KCDC-GT-270 were highly identical and formed a clade including ovine *B. motasi* Ningxian in China (JX440507) based on *COB* and ovine *B. motasi* Ningxian in China (JX866781) based on *COX3*. This indicated that the identified *Babesia* parasites might be

B. motasi, and this is the first study to detect *B. motasi* in human babesiosis and *H. longicornis* in Korea.

The *B. microti* Group is a varied group of globally distributed parasites of various lineages, such as the USA, Kobe, Hobetsu, and Munich [49,50]. The major lineage of the *B. microti* Group, the US lineage, includes *B. microti sensu stricto*, a causal agent of human babesiosis in the northeastern and upper mid-western US, where most human cases worldwide have been reported [9]. While pathogenicity may vary among parasite populations, the *B. microti* US lineage parasites are infectious to humans with or without a splenectomy. Recently, the US lineage parasites have

Table 2. Species, stage, gender, and number of collected ticks in *Babesia*-positive patient's residence.

Collecting site	<i>Haemaphysalis longicornis</i>				<i>Haemaphysalis flava</i>		Total No. (%)
	Female	Male	Nymph	Larva	Male	Larva	
A front yard of patient's residence	148	1	26	30	–	237	442 (74.0)
A back yard of patient's residence	7	–	2	–	–	–	9 (1.5)
Patient residence hill III	18	–	6	37	–	63	124 (20.8)
Fields surrounding the patient's residence	3	–	–	–	–	–	3 (0.5)
Patient residence hill I	5	–	4	–	1	–	10 (1.7)
Patient residence hill II	5	–	3	1	–	–	9 (1.5)
No	186	1	41	68	1	300	597

Table 3. Detection of *Babesia* DNA in the collected ticks via PCR.

Genus/species	Gender/stage	No.	Number (%) of ticks containing DNA of			
			<i>Babesia</i> spp (18S rRNA)	<i>Babesia</i> spp (<i>CoB/CoX-3</i>)	<i>B. microti</i> (β -tubulin)	<i>B. divergens</i> (18S rRNA)
<i>Haemaphysalis longicornis</i>	Adult	187	1 (0.5)	1 (0.5)	1 (0.5)	–
	Nymph	41	1 (2.4)	–	–	–
	Larva	68	–	–	–	–
<i>Haemaphysalis flava</i>	Adult	1	–	–	–	–
	Larva	300	–	–	–	–
No.		597	2 (0.3)	1 (0.2)	1 (0.2)	0

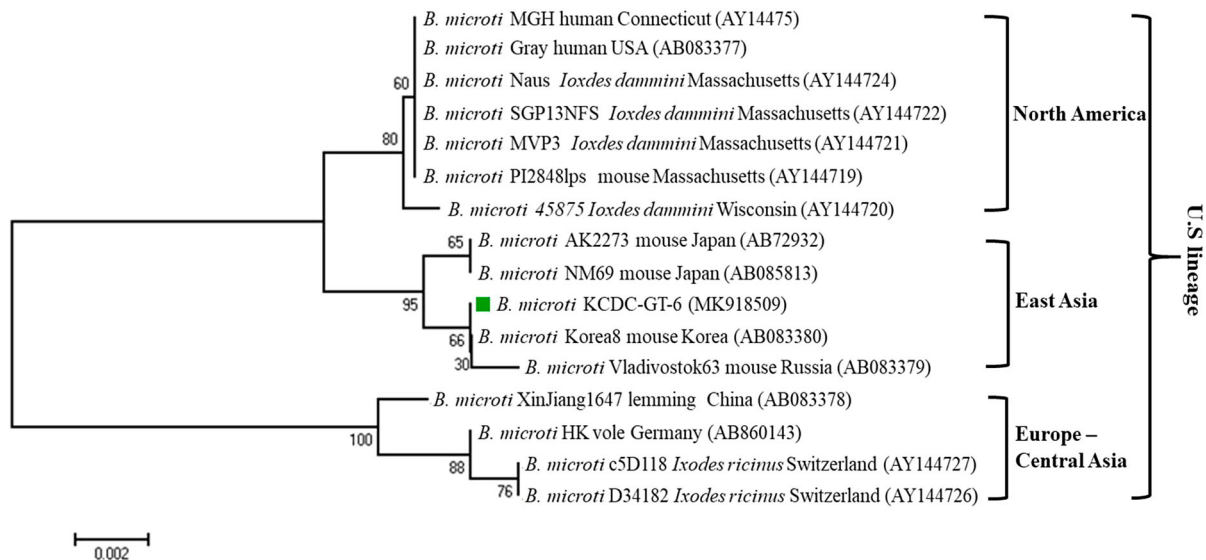


Figure 5. Phylogenetic relationships based on the β -tubulin gene sequence of *Babesia microti* in a tick, *B. microti* KCDC-GT-6, in accordance with the polymerase chain reaction-amplified sequence. The evolutionary history was inferred via the Neighbor-Joining method. The percentage of replicate trees wherein the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted using MEGA6 (■ *B. microti* positive in ticks).

been established to genetically vary in their β -tubulin gene sequences [51]. Moreover, parasites of the US lineage are phylogenetically clustered into 3 different sub-lineages, North America, Europe-Central Asia, and East Asia, each of which reflects the geographic origin of the parasites [9]. Previous reports from Korea have shown that *B. microti* detected in small mammals and wild animals closely aligned with the US strain [46,47,51]. Similarly, in this study, the *B. microti* detected in *H. longicornis* were closely related with the US lineage and clustered into East Asia with Korea8 from a mouse in Korea and Vladivostok63 from mice in Russia. This study is the first to detect *B. microti*, of the US lineage in *H. longicornis* in Korea.

Babesia parasites are transmitted by the bite of ticks that have distinct geographical distributions [52]. *Ixodes* spp. ticks are common in the USA and Europe [53,54]. The primary vector of *B. microti* is *I. scapularis*. Other vectors of various strains of *B. microti* include *I. spinipalpis*, *I. angustus*, *I. muris*, and *I. ricinus*. Furthermore, the vector of *B. divergens* and *B. venatorum* are *I. ricinus* and *I. persulcatus*, respectively [14]. In Asia, tick-borne pathogens were detected in both *Haemaphysalis* and *Ixodes* ticks [16,55–57]. In particular, *H. longicornis* is the primary tick species in China and essentially serves as a vector for several pathogens that cause anaplasmosis, babesiosis, and rickettsiosis [58]. In Korea, *H. longicornis* is the most commonly tick species infected with *Babesia*, collected from grass and vegetation [59,60]. The present results indicate that most of the collected ticks around the patient's residence were *H. longicornis* and *H. flava*. Correspondingly, we confirmed that two of the

H. longicornis were infected with *B. motasi* and the one was infected with *B. microti*. Recent reports indicate that *H. longicornis* is the potential carrier for *B. microti* to the vertebrate host and is assumed to serve as a tick vector of *B. motasi*-like isolates [10]. The present results suggest that *H. longicornis* ticks may serve as the predominant vector of *B. motasi* and *B. microti* in Gangwon-do, Korea.

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SHH, JRR, SHC and SEL conceived the study and designed the experiments. SHH performed the experiment. SYK and BGS performed tick collection and species identification. CRC, THU, CNK and YGK performed the acquisition and interpretation of the clinical data. SHH and SEL analysed the data and wrote the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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