# Molecular investigation of tick-borne pathogens in ticks removed from tick-bitten humans in the Republic of Korea

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## Abstract

This study investigated the presence of tick-borne pathogens in ticks removed from humans in Korea. We identified 33 ticks from three tick species, namely Amblyomma testudinarium (60.6%), Haemaphysalis longicornis (27.3%), and Ixodes nipponensis (12.1%) in order of occurrence. Tick-borne pathogens were found in 16 ticks using pathogen-specific polymerase chain reaction (PCR). From the results, 12 ticks (36.4%) tested positive for spotted fever group (SFG) Rickettsia: Rickettsia monacensis (1/12), R. tamurae (8/12), and Candidatus Rickettsia jingxinensis (3/12). Three ticks (9.1%) were positive for Anaplasma phagocytophilum. In addition, three ticks (9.1%) tested positive for Babesia gibsoni (1/3) and B. microti (2/3). In conclusion, we identified three tick species; the most common species was A. testudinarium followed by H. longicornis and I. nipponensis. SFG Rickettsia, A. phagocytophilum, and Babesia spp. were the most frequently detected pathogens in ticks removed from tick-bitten humans. R. tamurae and Ca. R. jingxinensis were firstly detected in Korea.

## Introduction

Ticks are major vectors of pathogens such as bacteria, viruses, and protozoans. These arthropods can transmit a variety of diseases to humans and animals (de La Fuente et al., 2017). Tick-borne diseases are caused by viral or bacterial pathogens transmitted through tick bites. Several tick-borne diseases such as Lyme disease (caused by*Borrelia* species), spotted fever group rickettsioses (caused by*Rickettsia* spp.), anaplasmosis (caused by *Anaplasma phagocytophilum* ), bartonellosis (caused by *Bartonella* spp.), Q fever (caused by *Coxiella burnetii* ), and babesiosis (caused by*Babesia* spp.) have been reported in the Republic of Korea (ROK) (Im et al., 2019).

The incidence of tick-borne diseases in the ROK is increasing due to global warming, increased outdoor activities, and increased international travel. The growing number of tick bites each year poses an escalating risk of tick-borne diseases (Im et al., 2019). Few studies have investigated the prevalence of tick-borne pathogens in ticks removed from tick-bitten humans in the ROK. However, it is necessary to determine the extent of tick-borne pathogens in the ROK, and to characterize them.

The present study aimed to investigate the presence of tick-borne pathogens in ticks removed from humans in the southwest provinces of the ROK. Our study detected the DNA of tick-borne pathogens from ticks using pathogen-specific nested PCR. The results of this study will contribute to the understanding of the interaction between ticks and pathogens that cause diseases in humans.

## Materials and Methods

### Ethics statement

This study was approved by the Ethics in Human Research Committee of Chosun University Hospital under an institutional review board (IRB), which approved all the experiments that used ticks removed from

tick-bitten humans (approval no. CHOSUN NON2019-001).

**Tick samples**

Ticks were removed from humans between May 2014 and September 2017 in the Jeollanam Provinces and Gwangju Metropolitan City in the ROK. All ticks were morphologically identified according to species and life stage using a microscope and standard taxonomic keys. Ticks were washed in 70% ethanol, rinsed twice with sterile phosphate-buffered saline (PBS), added to a hard tissue grinding MK28 tube (Bertin Technology, Rockville, MD, USA) containing 800 µL of PBS with 1x PC/SM (penicillin and streptomycin), ground using a FastPrep®-24 Classic instrument (MP Biomedicals, Solon, OH, USA), and stored at -80 °C until used for DNA extraction.

**DNA extraction**

We mixed 150 µL of the ground tick with 150 µL buffer ATL and 20 µL proteinase K, and incubated at 56 °C overnight for lysis; the genomic DNA was extracted using a QIAamp Tissue & Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted into 50 µL TE buffer and stored at -20 °C until PCR amplification.

**Polymerase Chain Reaction (PCR)**

To detect the presence of *Rickettsia* DNA, the outer membrane protein A gene (*ompA* ), citrate synthase gene (*gltA* ), and a 17 kDa protein gene (*17 kDa* ) of the spotted fever group*Rickettsia* species were targeted. The heat shock protein gene (*groEL* ) and the ankyrin-related protein gene (*ankA* ) were targeted to detect *A. phagocytophilum* . To detect the presence of*Borrelia* DNA, the CTP synthase gene (*pyrG* ) was targeted. The 16S-23S internal transcribed spacer region (*ITS* ) was targeted to detect *Bartonella* species. The *htpAB* -associated repetitive element (*IS1111* ) was targeted to detect*Coxiella* species. To detect the presence of *Babesia*species, 18S rDNA was targeted. To identify tick species, conventional PCR targeting the mitochondrial 16S rRNA gene (16S rDNA) was performed. All PCR primers used for detecting tick-borne pathogens, PCR conditions, and product sizes are given in Table 1. Conventional PCR (C-PCR) was performed in 20 µL reaction volumes using the AccuPowerR PCR PreMix (Bioneer Corp., Korea). Each PCR mixture consisted of 16 µL of distilled water, 1 µL of each primer (10 pmol/µL), and 2 µL of genomic DNA as template DNA. For 16S rDNA C-PCR and 18S rDNA nested PCR (N-PCR), we performed PCR using AmpliTaq Gold 360 Master Mix (Applied Biosystems, CA, USA) instead of AccuPowerR PCR PreMix.

For N-PCR, the reaction mixture was identical to that used in C-PCR, except that the first PCR product was used as template DNA, and the N-PCR primers were included. With each PCR run, a positive and a negative control (molecular grade water) were included.

All amplifications were performed in an AB thermal cycler (Applied Biosystem, Foster City, CA, USA). The amplified products were separated by electrophoresis on a 1.2% agarose gel, and stained with ethidium bromide for visualization.

**Sequencing and phylogenetic analysis**

The amplified PCR products were purified using QIAquick PCR purification kits (QIAGEN, Hilden, Germany) and sequenced with the PCR primers at Solgent Inc. (Daejeon, Korea). The sequences obtained in this study were compared for similarity with the GenBank sequences using BLAST. Gene sequences, excluding the primer regions, were aligned using the multisequence alignment program in Lasergene version 8 (DNASTAR, USA).

Phylogenetic trees were constructed using ClustalW of the MegAlign Program (DNASTAR, USA) based on the alignments of positive gene sequences using the neighbor-joining method. Bootstrap analysis (1,000 replicates) was performed according to the Kimura 2-parameter method. Pairwise alignments were performed with an open-gap penalty of 10 and a gap extension penalty of 0.5.

**Results**

### Tick identification

We obtained 33 ticks from 30 tick-bitten humans. Out of these, 15 ticks (45.5%) were adults, namely 12 females and 3 males, and 18 ticks (54.5%) were nymphs. Based on morphological examination using a microscope for tick identification, the ticks were identified as*Amblyomma testudinarium* (20, 60.6%; 7 adults and 13 nymphs),*Haemaphysalis longicornis* (9, 27.3%; 5 adults and 4 nymphs), and*Ixodes nipponensis* (4, 12.1%; 3 adults and 1 nymph), as described in Table 2. Tick identification using 16S rDNA C-PCR and DNA sequencing yielded the same results as the microscopic examination with the exception of four samples without tick DNA (shown in Table 4).

### Molecular detection of tick-borne pathogens in ticks removed from humans

We examined 33 ticks for the detection of tick-borne pathogens using pathogen-specific nested PCR. The presence of tick-borne pathogens was detected in 16 ticks. From the results, 12 ticks (36.4%) tested positive for spotted fever *Rickettsia,* namely *R. monacensis* (1 of 33, 3.0%), *R. tamurae* (8 of 33, 24.2%), and*Candidatus Rickettsia jingxinensis* (3 of 33, 9.1%). Three ticks (9.1%) were positive for *A. phagocytophilum* , while another three ticks (9.1%) were positive for either *B. gibsoni* (1 of 33, 3.0%) or *B. microti* (2 of 33, 6.0%) (Table 3). All ticks were negative for *Borrelia* spp., *Bartonella* spp., and *C. burnetii* .

Of the three *A. phagocytophilum* -positive ticks, one tick was identified as *A. testudinarium,* and two ticks were identified as*I. nipponensis* . Of the 12 SFG *Rickettsia* -positive ticks, nine ticks were identified as *A. testudinarium* , two ticks were identified as *H. longicornis,* and one tick was identified as*I. nipponensis* . The presence of *R. tamurae* was identified only in *A. testudinarium* tick. Of the three ticks detected with *Babesia* spp., two ticks were *A. testudinarium* and one tick was *H. longicornis* . Among the 33 ticks, one *I. nipponensis* (adult female) was co-infected with *A. phagocytophilum* and *R. monacensis* . In addition, co-infections of*R. tamurae* and *Babesia* spp. were identified in *A. testudinarium* (adult male) that presented in Table 4.

### Sequencing and phylogenetic analysis

The positive PCR products were sequenced and the sequencing results were aligned with the sequences obtained from the GenBank database to identify known sequences with a high degree of similarity using ClustalW. The neighbor-joining tree was constructed using the Kimura 2-parameter model (1,000 bootstrap replicates).

The partial *ankA* sequences obtained from *A. phagocytophilum* positive-tick demonstrated 99% similarity with*A. phagocytophilum* (accession no. KJ677106 and KT986059, 98% bootstrap support, Fig 1A). The partial *ankA* sequences formed a cluster with the *A. phagocytophilum* strains isolated from humans in the ROK. The partial *groEL* sequences obtained from *A. phagocytophilum* -positive ticks demonstrated 99% similarity with the*A. phagocytophilum* strain isolated from humans and dogs in the ROK (accession no. KU519286, 66% bootstrap support, Fig. 1B).

The partial 17 kDa, *ompA,* and *gltA* sequences obtained from SFG *Rickettsia*- positive ticks showed 99–100% similarity with*R. tamurae* , *R. monacensis* , and *Ca. R. jingxinensis.*A phylogenetic analysis grouped the partial *gltA* sequences with*R. tamurae* (accession no. KT753273, 86% bootstrap support, Fig. 1C), *R. monacensis* (accession no. NZ LN794217, 92% bootstrap support, Fig. 1C), and *Ca. R. jingxinensis* (accession no. KT899089, 76% bootstrap support, Fig. 1C).

The partial 18S rDNA sequences obtained from two *Babesia*species-positive ticks (Tick 12 and Tick 25) showed 99% similarity with a *B. microti* strain isolated from humans in the USA and a tick in China (accession no. KU204794 and LC314655, 100% bootstrap support, Fig. 1D). Another partial 18S rDNA sequence obtained from Tick 19 had 99% similarity with *B. gibsoni* and 100% similarity with*Babesia* spp., which were clustered with a *B. gibsoni*strain isolated from a boar in China (accession no. JX962780, 100% bootstrap support, Fig. 1D) and *Babesia* spp. from a tick in Japan (accession no. LC169083, 96% bootstrap support, Fig. 1D).

## Discussion

Recently, the risk of tick-borne disease has been associated with exposure to ticks from increasing outdoor activity. This study was performed to detect and identify the tick-borne pathogens in ticks removed from tick-bitten humans. We classified 33 ticks into three species: *A. testudinarium* (20, 60.6%; 7 adults and 13 nymphs) was the most common followed by *H. longicornis* (9, 27.3%; 5 adults and 4 nymphs) and *I. nipponensis* (4, 12.1%; 3 adults and 1 nymph). According to a tick survey study conducted by the KCDC (Korea Centers for Disease Control and Prevention) from 2013 to 2015, *H. longicornis* was the most dominant species (88.9%), followed by*H. flava* , *I. nipponensis* , *I. persulcatus* , *H. japonica* , *A. testudinarium* , and *I. granulatus* when ticks were collected from the vegetation and forests in the ROK using dry-ice bait traps and a flagging method (Song, Lee, & Ju, 2017). Interestingly, our results showed that when ticks were collected from tick-bitten humans, *A. testudinarium* was the most common.

For the molecular detection of tick-borne pathogens, we performed pathogen-specific N-PCR to detect the DNA of the tick-borne pathogens, namely SFG *Rickettsia, A. phagocytophilum, Borrelia* spp.,*Bartonella* spp., *Babesia* spp., and *C. burnetii* . Three tick samples (3 of 33, 9.1%) were positive for *A. phagocytophilum* DNA, 12 tick samples (12 of 33, 36.4%) were positive for *R. monacensis* , *R. tamurae* or *Ca. R. jingxinensis* DNA, and three ticks (3 of 33, 9.1%) were positive for*B. gibsoni* or *B. microti* DNA. Previous studies that investigated the prevalence of tick-borne infectious agents in ticks collected by dragging and flagging grass vegetation in the ROK showed that *A. phagocytophilum* was detected in 1.9% of *H. longicornis* ticks (Oh et al., 2009) and 0.1% of *I. persulcatus*ticks, and *Rickettsia* spp. were detected in 1.7% of *H. longicornis* ticks (C.-M. Kim et al., 2006). One study reported that a pool of *H. longicornis* , *H. flava,* and *I. nipponensis* ticks collected by dragging vegetation in the ROK were positive for the *Rickettsia* spp. 17 kDa antigen (60/311, 19.3%) and *ompA* gene (53/311, 17.04%) (Noh et al., 2017). In the present study, the infection prevalence of *Rickettsia* species (*R. monacensis* , *R. tamurae,* and *Ca. R. jingxinensis* ) and *A. phagocytophilum* in the ticks collected from humans was higher than that of ticks collected from the vegetation. Thus, we suggest that further study is needed to compare the infection prevalence of tick-borne pathogens, including *Rickettsia* spp.,*A. phagocytophilum,* and *Babesia* between ticks isolated from humans and ticks collected from grass vegetation.

*A. phagocytophilum* infection was first reported with serological evidence from humans in 2002, and it is currently the most frequently reported tick-borne bacterial infection in the ROK (Heo et al., 2002). The detection of *Anaplasma* spp. in ticks from grazing cattle collected from all ROK provinces has been reported (Kang et al., 2013). Another study confirmed a human granulocytic anaplasmosis (HGA) with*A. phagocytophilum* in a patient from the ROK who had a history of tick bites, clinical symptoms, and positive laboratory findings (K.-H. Kim et al., 2014). The present results showed that *A. phagocytophilum* was detected in *A. testudinarium* and *I. nipponensis* ticks. The amplicon sequences of the partial *ankA*gene in *A. testudinarium* (Tick 1) and *I. nipponensis* (Tick 29 and Tick 30) demonstrated more than 99% similarity. In the phylogenetic analysis, the sequences of the *ankA* gene from different types of ticks clustered together, showed $>$ 99% similarity with *A. phagocytophilum* strains isolated from humans in the ROK (Fig. 1A).

The first isolation of *R. monacensis* from ticks in the ROK was reported in 2013 (Lee et al., 2013). A previous study from the ROK reported that *I. nipponensis* was infected with the human pathogen*R. monacensis* and that *H. longicornis* and *H. flava*were infected with unknown SFG *Rickettsia* pathogens (Noh et al., 2017). Our results confirmed the presence of *R. monacensis* in*I. nipponensis* ticks removed from humans. In addition, our results indicated that *I. nipponensis* ticks are most likely the vectors responsible for transmitting *R. monacensis* infections in the ROK. Therefore, further studies are needed to determine the role of*I. nipponensis* in the transmission of the *R. monacensis*pathogen to humans; the blood of patients bitten by *I. nipponensis* ticks and the ticks themselves should be investigated for the presence of *R. monacensis* .

*R. tamurae* was first isolated from *A. testudinarium* ticks collected in Japan in 1993. *R. tamurae* was formally identified as a novel species by genetic and phylogenetic analyses in 2006 (Fournier, Takada, Fujita, & Raoult, 2006). In 2011, the first case of human infection was confirmed using molecular and serological analyses in Japan (Imaoka, Kaneko, Tabara, Kusatake, & Morita, 2011). The presence of SFG *Rickettsia* including *R.*

*tamurae* was found in*Amblyomma* and *Dermacentor* ticks in Thailand (Nooroong, Trinachartvanit, Baimai, & Ahantarig, 2018) and in *Haemaphysalis*ticks in Peninsular Malaysia (Kho et al., 2017). In addition, *R. tamurae* was found in *Amblyomma* ticks from an area endemic for Brazilian spotted fever in Brazil (Guedes, Leite, Pacheco, Silveira, & Labruna, 2011). Supporting these previous studies, our results showed the presence of *R. tamurae* in *A. testudinarium* ticks.

The presence of a potentially novel species of *Ca. R. jingxinensis* was proposed in *H. longicornis* nymphs from Jingxin in Northeastern China in 2016 (Liu et al., 2016) and was detected in*H. longicornis* ticks in Xi'an, China in 2017 (Guo et al., 2018). In the ROK, the pathogenicity of *Ca. R. jingxinensis* is not clear. Therefore, a further assessment of the potential pathogenicity in humans and animals is needed.

There have been no previous reports of *R. tamurae* or *Ca. R. jingxinensis* from ticks in the ROK; here, we report the first identification of *R. tamurae* and *Ca. R. jingxinensis* in ticks obtained from tick-bitten humans.

*Babesia* was first discovered in animals by Babes in 1988, and more than 100 species have been identified. In the ROK, *Babesia*spp. have been isolated from cattle and other mammals (raccoon, deer, and badger) since the 2000s (Cho et al., 2002; Han, Lee, Jang, & Na, 2010; Hong et al., 2017). *Babesia* spp. are mainly carried by*Ixodes* ticks. Previous studies using ticks collected from grass and vegetation in the ROK reported that *H. longicornis* was the most common tick species infected with *Babesia* (Kang et al., 2013; C.-M. Kim et al., 2006). Our results showed that *B. microti*was found in both *H. longicornis* and *A. testudinarium* . In the USA, the primary vector for the transmission of *B. microti* to humans is the tick *Ixodes scapularis* in the nymphal stage (Vannier & Krause, 2012). The present results suggest that further study is needed to determine the type of ticks that are the vectors for the transmission of *B. microti* to humans in the ROK.

*B. gibsoni* was first identified in nymphs of *Rhipicephalus sanguineus* ticks from infected dogs in Asia (Chao, Liao, Ho, & Shih, 2017). *B. gibsoni* was detected in *A. testudinarium* ticks in this study. The first case of human babesiosis (KO1) was reported in 2007 in the ROK, and it was highly related to Chinese ovine*Babesia* spp. (J.-Y. Kim et al., 2007). Based on the phylogenetic analysis of the 18S rDNA gene in our study, the pathogen clustered with a group of *Babesia* spp., isolated from a tick in Japan, which was diverged from the KO1 strain (Fig. 1D). The present results indicate that *Babesia* spp. may vary based on their geographical distributions.

Further investigation is needed to determine the difference between pathogens found in ticks isolated from humans and ticks collected from grass vegetation. In addition, transmission studies should be conducted to determine whether the pathogens found in ticks are the same as those found in humans bitten by those ticks. To confirm the transmission of pathogens from ticks to humans, serological testing on the blood of tick-bitten patients and their ticks will be necessary. Further experiments and correlation analysis using the blood samples of tick-bitten humans and ticks isolated from them may help predict the transmission of tick-borne diseases.

In conclusion, we confirmed three tick species carrying tick-borne pathogens; the most common species was *A. testudinarium* followed by *H. longicornis* and *I. nipponensis* . These ticks were positive for SFG *Rickettsia* , *A. phagocytophilum,* and*Babesia.* This was the first report of the presence of *R. tamurae* and *Ca. R. jingxinensis* in ticks removed from tick-bitten humans in the ROK.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Data and materials are available upon request to the corresponding author.

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**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Authorship**

All authors has made substantial contributions in the conception and design of the study, acquisition of data, analysis and interpretation of data and drafting the article or revising it critically for important intellectual content. All authors has read and approved the final version to be submitted.

Mi-Seon Bang, Choon-Mee Kim contributed equally to this work.

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**Author's contributions**

MS Bang performed the investigation and drafted the manuscript. CM Kim contributed the methodology and revised the manuscript. SH Pyun contributed the investigation. DM Kim contributed the conceptualization, supervision and revised the manuscript. NR Yun supervised. All authors read and approved the final manuscript.

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Table 1. Oligonucleotide primers and PCR conditions used for the detection of tick-borne pathogens in ticks removed from tick-bitten humans.

| **Identification** | **Target gene$^{+}$** | **Primer name** | **Nucleotide sequence (5'-3')** | **Product siz** |
|---|---|---|---|---|
| *Rickettsia* species | *ompA* | RR190.70F | ATGGCGAATATTTCTCCAAAAA | 634 |
| | | RR190.701R | GTTCCGTTAATGGCAGCATCT | |
| | | RR190.70F | ATGGCGAATATTTCTCCAAAAA | 535 |
| | | RR190.602R | AGTGCAGCATTCGCTCCCCCT | |
| | *gltA* | GLTA1F | GACGGTGATAAAGGAATCTTG | 1022 |
| | | GLTA1R | CATTTCTTTCCATTGTGCCATC | |
| | | GLTA2F | CTACGAACTTACCGCTATTAG | 446 |
| | | GLTA2R | GACCAAAACCCATTAACCTAAAC | |
| | *17 kDa* | Rr17k.1p | TTTACAAAATTCTAAAAACCAT | 539 |
| | | Rr17k.539n | TCAATTCACAACTTGCCATT | |
| | | Rr17k.90p | GCTCTTGCAACTTCTATGTT | 450 |
| | | Rr17k.539n | TCAATTCACAACTTGCCATT | |
| *Anaplasma phagocytophilum* | *groEL* | GRO607F | GAAGATGCWGTWGGWTGTACKGC | 688 |

| **Identification** | **Target gene**$^{+}$ | **Primer name** | **Nucleotide sequence (5'-3')** | **Product siz** |
|---|---|---|---|---|
| | | GRO1294R | AGMGCTTCWCCTTCWACRTCYTC | |
| | | GRO677F | ATTACTCAGAGTGCTTCTCARTG | 445 |
| | | GRO1121R | TGCATACCRTCAGTYTTTTCAAC | |
| | *ankA* | ANK-F1 | GAAGAAATTACAACTCCTGAAG | 705 |
| | | ANK-R1 | CAGCCAGATGCAGTAACGTG | |
| | | ANK-F2 | TTGACCGCTGAAGCACTAAC | 664 |
| | | ANK-R2 | ACCATTTGCTTCTTGAGGAG | |
| *Borrelia* species | *pyrG* | pyrG-1F | ATTGCAAGTTCTGAGAATA | 801 |
| | | pyrG-1R | CAAACATTACGAGCAAATTC | |
| | | pyrG-2F | GATATGGAAAATATTTTATTTATTG | 707 |
| | | pyrG-2R | AAACCAAGACAAATTCCAAG | |
| *Bartonella* species | *ITS* | ITS_OF | TTCAGATGATGATCCCAAGC | 639 |
| | | ITS_OR | AACATGTCTGAATATATCTTC | |
| | | ITS_IF | CCGGAGGGCTTGTAGCTCAG | 499 |
| | | ITS_IR | CACAATTTCAATAGAAC | |
| *Coxiella burnetii* | *IS1111* | IS111F1 | TACTGGGTGTTGATATTGC | 485 |
| | | IS111R1 | CCGTTTCATCCGCGGTG | |
| | | IS111F2 | GTAAAGTGATCTACACGA | 260 |
| | | IS111R2 | TTAACAGCGCTTGAACGT | |
| *Babesia* species | 18S rDNA | Bab5 | AATTACCCAATCCTGACACAGG | 485 |
| | | Bab8 | TTTGGCAGTAGTTCGTCTTTAACA | |
| | | Bab6 | GACACAGGGGGTAGTGACAAGA | 407 |
| | | Bab7 | CCCAACTGCTCCTATTAACCATTAC | |
| Ticks | 16S rDNA | 16S+1-F | CTGCTCAATGAATATTTAAATTGC | 450 |
| | | 16S+1-R | CGGTCTAAACTCAGATCATGTAGG | |

$^{+}$ompA, outer membrane protein A gene; gltA, citrate synthase gene; 17 kDa, 17 kDa protein gene; groEL, heat shock protein gene; ankA, ankyrin-related protein gene; pyrG, CTP synthase gene; ITS, 16S-23S internal transcribed spacer region; IS1111, htpAB-associated repetitive element; 18S rDNA, 18S ribosomal RNA gene; 16S rDNA, 16S ribosomal RNA gene

Table 2. Developmental stages and species of ticks removed from tick-bitten humans determined by both morphological identification and 16S rDNA-targeting conventional PCR

| Tick species | Tick species | *Amblyomma testudinarium* | *Heamaphysalis longicornis* | *Ixodes nipponensis* |
|---|---|---|---|---|
| Development stage | Adult female | 4 | 5 | 3 |
| | Adult male | 3 | 0 | 0 |
| | Nymph | 13 | 4 | 1 |
| | Larva | 0 | 0 | 0 |
| | Total No. (%) | 20 (60.6 %) | 9 (27.3 %) | 4 (12.1 %) |
| | | 33 (100 %) | 33 (100 %) | 33 (100 %) |

Table 3. Detection of tick-borne pathogens in ticks by pathogen-specific nested PCR

| Detected pathogens | Positive ticks numbers | /Total numbers | PCR positivity (%) |
|---|---|---|---|
| Spotted fever group *Rickettsia* species | **12** | /33 | 36.4 |
| *R. monacensis* | 1 | /33 | 3.0 |
| *R. tamurae* | 8 | /33 | 24.2 |
| *Candidatus Rickettsia jingxinensis* | 3 | /33 | 9.1 |
| *Anaplama phagocytophilum* | **3** | /33 | 9.1 |
| *Babesia* species | **3** | /33 | 9.1 |
| *B. gibsoni* | 1 | /33 | 3.0 |
| *B. microti* | 2 | /33 | 6.0 |
| *Borrelia* species | 0 | /33 | 0 |
| *Bartonella* species | 0 | /33 | 0 |
| *Coxiella burnetii* | 0 | /33 | 0 |

Table 4. Characteristics of 33 ticks using the DNA of tick-borne pathogens obtained from 30 tick-bitten humans

| Patient no. | Patient age/sex | Tick species identified by a microscopy | Development stage (sex) | Identific |
|---|---|---|---|---|
| 1 | 83/F | *A. testudinarium* | Nymph | NA |
| 2 | 46/M | *A. testudinarium* | Nymph | NA |
| 3 | 4/M | *A. testudinarium* | Nymph | *A. testud* |
| 4 | NA | *A. testudinarium* | Nymph | *A. testud* |
| 5 | 65/M | *A. testudinarium* | Nymph | *A. testud* |
| 6 | 74/M | *A. testudinarium* | Nymph | *A. testud* |
| 7 | 58/F | *A. testudinarium* | Nymph | *A. testud* |
| 8 | 52/F | *A. testudinarium* | Nymph | *A. testud* |
| 9 | 62/F | *A. testudinarium* | Nymph | *A. testud* |
| 10 | 60/M | *A. testudinarium* | Nymph | *A. testud* |
| 11 | 55/F | *A. testudinarium* | Nymph | *A. testud* |
| 12 | 30/F | *A. testudinarium* | Nymph | *A. testud* |
| 13 | 71/M | *A. testudinarium* | Nymph | *A. testud* |
| 14 | 64/M | *A. testudinarium* | Adult (female) | NA |
| 15 | NA | *A. testudinarium* | Adult (female) | *A. testud* |
| 16 | 60/F | *A. testudinarium* | Adult (female) | *A. testud* |
| 17 | 54/F | *A. testudinarium* | Adult (female) | *A. testud* |
| | | *A. testudinarium* | Adult (male) | *A. testud* |
| 18 | 53/F | *A. testudinarium* | Adult (male) | *A. testud* |
| 19 | 78/F | *A. testudinarium* | Adult (male) | *A. testud* |
| 20 | 60/F | *H. longicornis* | Nymph | *H. longic* |
| | | *H. longicornis* | Nymph | NA |
| 21 | 83/F | *H. longicornis* | Nymph | *H. longic* |
| 22 | 72/F | *H. longicornis* | Nymph | *H. longic* |
| 23 | 76/F | *H. longicornis* | Adult (female) | *H. longic* |
| | | *H. longicornis* | Adult (female) | *H. longic* |
| 24 | 77/F | *H. longicornis* | Adult (female) | *H. longic* |
| 25 | 5/M | *H. longicornis* | Adult (female) | *H. longic* |

| Patient no. | Patient age/sex | Tick species identified by a microscopy | Development stage (sex) | Identific |
|---|---|---|---|---|
| 26 | 54/F | *H. longicornis* | Adult (female) | *H. longic* |
| 27 | 77/F | *I. nipponensis* | Nymph | *I. nippon* |
| 28 | 72/M | *I. nipponensis* | Adult (female) | *I. nippon* |
| 29 | 53/F | *I. nipponensis* | Adult (female) | *I. nippon* |
| 30 | 81/F | *I. nipponensis* | Adult (female) | *I. nippon* |

Figure 1. Phylogenetic trees based on partial nucleotide sequences obtained from *A. phagocytophilum*- , spotted fever group*Rickettsia*-, and *Babesia* -positive ticks in this study and from GenBank.

(A) 560 bp of the *ankA* gene (B) 330 bp of the *groEL* gene sequences for *A. phagocytophilum* (C) 420 bp of the *gltA*gene sequences for SFG *Rickettsia* (D) 370 bp of the 18S rRNA gene sequences for *Babesia* species