Prevalence of Anaplasma, Bartonella and Borrelia Species in Haemaphysalis longicornis collected from goats in North Korea

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North Korea is located on the northern part of the Korean Peninsula in East Asia. While tick-borne pathogens of medical and veterinary importance have been reported from China and South Korea, they have not been reported from North Korea. To screen for zoonotic tick-borne pathogens in North Korea, ticks were collected from domestic goats. A total of 292 (27 nymph, 26 male, 239 female) Haemaphysalis (H.) longicornis were collected and assayed individually for selected tick-borne pathogens. A total of 77 (26.4%) were positive for Anaplasma bovis, followed by Bartonella (B.) grahamii (15, 5.1%), Anaplasma phagocytophilum (12, 4.1%), Bartonella henselae (10, 3.4%), and Borrelia spp. (3, 1.0%) based on 16S ribosomal RNA and ITS species-specific nested polymerase chain reaction. Using the groEL-based nested PCR, a total of 6 and 1 H. longicornis were positive for B. grahamii and B. henselae, respectively. All products were sequenced and demonstrated 100% identity and homology with previously reported sequences from other countries in GenBank. This is the first report of the detection of tick-borne pathogens in the North Korea and suggests that farm animals may act as reservoirs for zoonotic tick-borne pathogens.

Keywords: Anaplasma, Bartonella, Borrelia, Haemaphysalis longicornis, North Korea

Introduction

Ticks are recognized as primary arthropod vectors of infectious disease agents that pose significant medical and veterinary health issues [7]. The incidence of tick-borne diseases (TBDs) is increasing worldwide as more pathogens are recognized [27], especially in northeastern Asian countries such as China [24,37,41], the South Korea [4,15,16,22] and Japan [35]. The transmission of zoonotic pathogens involving vertebrate hosts and ticks that interact in a constantly changing environment is often difficult to control because of zoonotic/domestic host(s), habitat distributions and transmission cycles in areas in which pastured domestic stock and wild animals coexist [32].

North Korea, which is located on the northern part of the Korean Peninsula in East Asia, is bound by China to the northeast, South Korea to the southeast, the Yellow Sea to the southwest and the Korean peninsula strait to the southeast. Tick-borne zoonotic pathogens from various sources, including wild deer [2,16,19], wild rodents [4,18], domestic animals [9,17,19] and ticks [18,30] have been reported near the southern boundary of the demilitarized zone (DMZ) separating South and North Korea. Regional prevalence and geographic and host distributions are important factors that must be understood to develop and initiate prevention strategies. Despite the geographical, political, and epidemiological importance of North Korea, screening for tick-borne pathogens has not been conducted, largely due to political and academic isolation.

In this study, tick-borne disease surveillance was conducted at a goat farm to identify tick species infesting goats and associated tick-borne zoonotic pathogens that may adversely impact veterinary and medical health in North Korea.
addition, comparative phylogenetic analysis of zoonotic pathogens detected in North Korea was conducted.

**Materials and Methods**

**Survey area and tick sampling**

Blood-feeding ticks were collected from goats from April–May 2009 at farms along the coast in Rajin, Rason special economic zone, North Korea, to assess the prevalence of selected tick-borne pathogens among goats that were pastured from March–May of 1999–2009 at nearby mountainous areas. The goats grazed on indigenous shrubs, grasses, and other herbaceous vegetation, where they were exposed to questing ticks. The herd was composed of LaMancha, Erdene Black Cashmere goats from Mongolia, and Black Spanish goats from the United States. Ticks were collected from goats by placing fine tweezers placed around the mouth part, slowly removing the attached tick, and then placing up to 10 ticks in vials containing 70% ethanol. Ticks were transported to Seoul National University, where they were identified microscopically to stage of development and species according to Yamaguti et al. [38].

**DNA preparation and polymerase chain reaction (PCR)**

Identified ticks were individually homogenized mechanically using a Beadbeater TissueLyser II (Qiagen, Germany) with lysis buffer, proteinase K, and 5 mm stainless steel beads (30 frequencies/sec for 5 min), after which they were incubated at 56°C overnight and then centrifuged at 12,000 × g for 10 min at 4°C. Following centrifugation, the supernatant was used for genomic DNA extraction, which was performed with a DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instructions.

### Table 1. Nucleotide sequences of polymerase chain reaction (PCR) primers and conditions for amplification of Anaplasma, Bartonella and Borrelia species genes

| Species and target genes | Name of PCR primers and conditions | Primer sequences (5′ - 3′) | PCR product size (bp) | References |
|--------------------------|------------------------------------|---------------------------|----------------------|------------|
| **Anaplasma and Ehrlichia spp. 16S rRNA** | AE1-F AE1-R | AAGCTTAACACATGCAAGTCGAA AGTCACGTACCACACCTAAATG | 1406 | [28] |
| | Conditions 94/1 56/1 72/1.5 | | | |
| **Anaplasma (A.) phagocytophilum 16S rRNA** | EE3 EE4 | TGGCAACGGATTATTCTTTA TAGCTTGC CCCCCTCGTAAAGAAGATCAATCTCC | 926 | [1] |
| | Conditions 94/0.5 56/0.5 72/0.75 | | | |
| **A. bovis 16S rRNA** | ABKl AB1r | TAGCTTGCATGGGGGACAA TCTCCGGGAATCAGTCTG | 547 | [16] |
| | Conditions 94/0.5 59/0.5 72/0.5 | | | |
| **Borrelia spp. 16S rRNA** | B1 B8 | CAGTGCCTTAAAGCAGC CCTTAAATACCTTCCTCCC | 1427 | [29] |
| | Conditions 94/1 58/1 72/1.5 | | | |
| **Bartonella spp. ITS** | QHVE1 QHVE3 | TTCAGATGATGATCCCAAGC AACATGCTGTAATATCCTT | 735 | [20] |
| | Conditions 94/0.75 55/0.75 72/0.75 | | | |
| **Bartonella spp. groEL** | QHVE12 QHVE14 | GCAGCTAAGAATCTCAGCATT GCAATCAGTACAGC | 484-569 | [33] |
| | Conditions 94/0.5 59/0.75 72/0.75 | | | |
| | HspF1d BbHS1630.n | GAATNAGAATAAGTNGAA AAACACGCTCCCTTC | 1489 | [40] |
| | Conditions 94/1.5 54/1.5 72/1.5 | | | |

ITS, internal transcribed spacer.
Fig. 1. Map of Rason (Rajin-Sunbong) special economic zone (black dotted circle) in North Korea. Ticks were collected from mountain-pastured goats at farms at which they were housed along the coast in Rajin.

Fig. 2. Phylogenetic relationships for *Anaplasma bovis* detected from ticks in North Korea (bold letters) and *Anaplasma* and *Ehrlichia* species from other countries based on partial nucleotide sequences of the 16S rRNA gene fragment (547 bp). The neighbor-joining method was used to construct the phylogenetic tree. The numbers at the nodes are the proportions of 1,000 bootstrap iterations that support the topology shown. ROK, Republic of Korea; DPRK, Democratic People’s Republic of Korea.
PCR and nested-PCR were performed using specific primer sets for *Anaplasma (A.) phagocytophilum, A. bovis, and Bartonella and Borrelia* spp. (Table 1). *A. phagocytophilum* genomic DNA used for the positive control was provided by J. Stephen Dumler (Johns Hopkins University School of Medicine, Baltimore, MD, USA). *Bartonella (B.) henselae* (ATCC49882) and *B. grahamii* (ATCC700132) were purchased from the American Type Culture Collection. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. All PCR assays were repeated a minimum of three times for confirmation.

**Cloning, nucleotide sequencing and phylogenetic analysis**

PCR amplicons were purified using QIAquick Gel Extraction Kits (Qiagen) and cloned using pGEM-T Easy Vectors (Promega, USA). The recombinant plasmids were transformed into *Escherichia coli* DH5α, then plated onto LB agar plates containing 100 μg/mL ampicillin. The recombinant plasmid DNA was purified using the Wizard Plus SV Miniprep DNA Purification System (Promega), and sequenced using a T7 and SP6 promoter primer set by dideoxy termination with an automatic sequencer (3730xl capillary DNA Analyzer; Applied Biosystems, USA). Phylogenetic analysis of the obtained sequences was conducted using Clustal X software (ver. 2.0) [21] and the neighbor-joining method with the MEGA 4.0 program [35]. Bootstrap values for the consensus tree were based on analysis of 1,000 replications.

**Nucleotide sequence accession numbers**

The obtained sequences in the present study were submitted to GenBank (The National Center for Biotechnology Information, USA), and accession numbers for the 16S ribosomal RNA (rRNA), *groEL*, and internal transcribed spacer (ITS) gene sequences are shown in Figs. 2–6.

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**Fig. 3.** Phylogenetic relationships for *Anaplasma phagocytophilum* (bold letter) detected from ticks in North Korea (bold letters) and related *Anaplasma* and *Ehrlichia* species from other countries based on partial nucleotide sequences of the 16S rRNA gene fragment (925 bp). The neighbor-joining method was used to construct the phylogenetic tree. The numbers at the nodes are the proportions of 1,000 bootstrap iterations that support the topology shown.
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Results

A total of 292 (27 nymphs, 26 males, 239 females) Haemaphysalis (H.) longicornis Neumann were collected from domestic goats and assayed individually using generic-specific nested PCR primer sets for Anaplasma, Bartonella, Borrelia, and Ehrlichia species. A total of 77 ticks (26.4%) were positive for A. bovis, followed by B. grahamii (15, 5.1%), A. phagocytophilum (12, 4.1%), B. henselae (10, 3.4%), and Borrelia spp. (3, 1.0%) (Table 2). Ehrlichia spp. were not detected. Eleven ticks had mixed infections of two pathogens: A. phagocytophilum and B. henselae (1, 0.3%), A. bovis and B. henselae (5, 1.7%), A. bovis and B. grahamii (4, 1.4%), and B. grahamii and Borrelia spp. (1, 0.3%).

A. bovis and A. phagocytophilum 16S rRNA genes were detected by species-specific nested PCR. The genome sequences were analyzed and compared with partial 16S rRNA gene sequences to demonstrate genetic relationships between Anaplasma spp. reported from ticks elsewhere. All 77 A. bovis 16S rRNA gene sequences (KC422268) were identical, as were those of A. bovis detected in deer from South Korea (GU556626) and Japan (AB196475) (Fig. 2). All A. phagocytophilum 16S rRNA gene sequences (KC422267) were identical, and they demonstrated 99.6% similarity to A. phagocytophilum from a deer in Japan (AB196721) and a South African dog (AY570540) (Fig. 3).

Bartonella spp. ITS genes were detected by nested PCR assay. The product sizes of B. henselae and B. grahamii, including non-coding sequences, were 569 bp and 484 bp, respectively. The genome sequences were analyzed and compared with partial ITS gene sequences to demonstrate genetic relationships between Bartonella spp. All 12 B. henselae ITS gene sequences from North Korea (KC422265) were identical and were also identical to B. henselae sequences detected in humans from France (AF312496) and the United States (L35101) (Fig. 4). Moreover, B. grahamii ITS gene sequences (KC422266) from North Korea were identical, as were those of B. grahamii from a mouse from the UK.
**Fig. 5.** Phylogenetic relationships for *Bartonella grahamii* and *Bartonella henselae* detected from ticks in North Korea (bold letters) and *Bartonella* species from other countries based on partial protein sequences of the 409 amino acid *groEL* gene fragment. The neighbor-joining method was used to construct the phylogenetic tree. The numbers at the nodes are the proportion of 1,000 bootstrap iterations that support the topology shown.

(AJ269785) and a South Korean deer (JN810847) (Fig. 4).

*groEL* specific nested PCR revealed that the product size of six *B. grahamii* (KC422270) and one *B. henselae* (KC422271) *groEL* gene was 888 bp and that the gene encoded 295 amino acids of *groEL*. The six *B. grahamii* sequences of the *groEL* amino acids were identical and corresponded to *B. grahamii* mice from the UK (BAG12709) and Japan (AAD04238) (Fig. 5). One *B. henselae* sequence of the *groEL* amino acids corresponded to *B. henselae* from the United States (AAD04238) and France (AAK97288).

A total of three ticks were positive for *Borrelia* spp. using the 16S rRNA genes, and these were identical and demonstrated 99.0% homology to *B. theileri* (NR025874) (Fig. 6).

**Discussion**

Little information is available regarding the causative agents of tick-borne diseases of veterinary and medical importance in North Korea, in part because of strict access limitations [41]. Preliminary data based on a high prevalence of known and potential (not identified to species) pathogens observed in *H. longicornis* collected from goats that were pastured in the mountainous terrain in North Korea illustrate the need for further studies investigating the impact of tick-borne diseases among domestic animals, as well as humans.

In South Korea, *H. longicornis* is the tick most commonly collected from grasses and herbaceous vegetation habitats, and these have been shown to have high tick-borne infection rates [4,18,28]. *A. bovis*, the causative agent of benign bovine rickettsiosis, is widely found in ticks collected from cattle and surrounding vegetation throughout much of Africa and Asia [9,11,26]. In South Korea, *A. bovis* has been identified in *H. longicornis* collected from deer, birds, cattle, and vegetation (tick drag) [15-17,22,28]. In Japan, *A. bovis* infection rates among sika deer and co-pastured cattle were 23% and 15%, respectively [14]. While *A. bovis* is a pathogen of veterinary...
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Table 2. Prevalence of Anaplasma (A.) phagocytophilum, A. bovis, Bartonella spp., and Borrelia spp. targeting the 16S rRNA gene fragment from Haemaphysalis longicornis ticks collected from goats in North Korea

| Stages  | Number of ticks | Number of amplicon sequences (%) |
|---------|----------------|----------------------------------|
|         | A. bovis | A. phagocytophilum | Bartonella (B.) henselae | B. grahamii | Borrelia spp. | A. phagocytophilum and B. henselae* | A. bovis and B. henselae* | A. bovis and B. grahamii* | B. grahamii and Borrelia spp.* |
| Nymph   | 27      | 2 (7.4)            | 0                      | 0          | 0            | 0              | 0        | 0        | 0             |
| Male    | 26      | 4 (15.4)           | 0                      | 0          | 0            | 0              | 0        | 0        | 0             |
| Female  | 239     | 71 (29.7)          | 12 (5.0)               | 10 (4.2)   | 15 (6.3)     | 3 (1.3)         | 1 (0.4)  | 5 (2.1)  | 4 (1.7)       |
| Total   | 292     | 77 (26.4)          | 12 (4.1)               | 10 (3.4)   | 15 (5.1)     | 3 (1.0)         | 1 (0.3)  | 5 (1.7)  | 4 (1.4)       |

*Dual infection.

importance, it is not known to cause human disease [31]. A. phagocytophilum, the causative agent of human granulocytic anaplasmosis, was detected in H. longicornis collected from goats in North Korea and has also been detected from H. longicornis and wild deer in South Korea [10,16,22,28] and from goats, cattle, dogs, and ticks collected from vegetation in China [41]. Our survey showed high infection rates of A. bovis (27.3%) among H. longicornis collected from goats in North Korea, indicating that they are of veterinary concern, while A. phagocytophilum (4.3%) poses a medical threat to goat herders and others exposed to questing ticks in North Korea. Both A. bovis and A. phagocytophilum showed a high degree of...
homology to species from neighboring countries, indicating that they have widespread distribution throughout eastern Asia.

Evidence suggests that many Bartonella spp. (e.g., B. henselae, B. vinsonii, B. bovis, B. elizabethae, B. washoensis, and B. claridgeiae) are the causative agents of various diseases in people and/or animals [5]. While the cat flea (Ctenocephalides felis) and rodent flea (Ctenocephalides nobilis) have been identified as the primary vectors of B. henselae and B. grahamii, respectively [5,12]. H. longicornis collected from goats in North Korea demonstrated high infection rates. Bartonella spp. are distributed worldwide and transmitted by a wide variety of vectors, including ticks, flies, body lice, mites, and sandflies [5,36]. More recently, various species of ticks have been shown to be positive for Bartonella spp. by PCR [3], and B. henselae has been shown to be transmitted through the salivary contents of Ixodes spp. [8]. In a previous study, Bartonella DNA was detected by PCR in H. flava, H. longicornis, I. nipponensis, and I. turdus ticks collected from vegetation (tick drag) and/or from rodents captured in rural areas and US military training sites in South Korea [18]. In the present study, B. grahamii and B. henselae ITS gene fragments were detected and DNA sequences corresponded to B. grahamii and B. henselae ITS gene sequences in GenBank. Since there were insufficient ITS gene sequences available in GenBank for a comprehensive comparison, additional phylogenetic analysis using the groEL gene was conducted by nested PCR. A total of 7/25 (28%) of the groEL gene sequences (1 B. henselae and 6 B. grahamii) were positive, and their translocated amino acids sequences corresponded to B. grahamii and B. henselae sequences reported from other countries in GenBank, respectively. Thus, molecular evidence implicates ticks as potential vectors of Bartonella species in Korea.

Borrelia species are transmitted by Ixodid ticks, and have recently been detected by PCR from H. longicornis in China, H. flava in Japan, and from I. persulcatus, I. nipponensis, and I. turdus in South Korea [4,13,15,30,34]. H. longicornis in North Korea were found to be positive for Borrelia spp. by PCR using 16S rRNA primer sets and gene sequences compared with those available in GenBank. Prior to this survey, Borrelia spp. had not been detected from Haemaphysalis spp. and I. nipponensis collected from the Korean Peninsula [4,23,30]. While Lyme borreliosis was not a reportable disease in South Korea until 2010, from 2005–2012, there were 11 autochthonous cases reported, mostly in the northern part of South Korea, which were suspected to be due to the greater abundance of I. persulcatus [25]. However, based on the results of the present study, H. longicornis, the predominant tick species in grasslands, pastures, and gravesites in forested areas in South Korea [6], is a potential vector of Borrelia species.

In conclusion, various species of ticks are known vectors or implicated as vectors Anaplasma, Borrelia, Bartonella, and Ehrlichia spp. A rapidly expanding number of reservoir-adapted pathogens have been discovered among a wide range of species of ticks as known or suspected vectors of zoonotic tick-borne pathogens. The results of the present study implicates H. longicornis as a potential vector of Anaplasma, Bartonella, and Borrelia spp. in the North Korea and indicates that it will impact veterinary and medical health in North Korea.

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Conflict of Interest

There is no conflict of interest.

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