



Prevalence of *Anaplasma* and *Bartonella* spp. in Ticks Collected from Korean Water Deer (*Hydropotes inermis argyropus*)

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Abstract: Deer serve as reservoirs of tick-borne pathogens that impact on medical and veterinary health worldwide. In the Republic of Korea, the population of Korean water deer (KWD, *Hydropotes inermis argyropus*) has greatly increased from 1982 to 2011, in part, as a result of reforestation programs established following the Korean War when much of the land was barren of trees. Eighty seven *Haemaphysalis flava*, 228 *Haemaphysalis longicornis*, 8 *Ixodes nipponensis*, and 40 *Ixodes persulcatus* (21 larvae, 114 nymphs, and 228 adults) were collected from 27 out of 70 KWD. A total of 89/363 ticks (266 pools, 24.5% minimum infection rate) and 5 (1.4%) fed ticks were positive for *Anaplasma phagocytophilum* using nested PCR targeting the 16S rRNA and *groEL* genes, respectively. The 16S rRNA gene fragment sequences of 88/89 (98.9%) of positive samples for *A. phagocytophilum* corresponded to previously described gene sequences from KWD spleen tissues. The 16S rRNA gene fragment sequences of 20/363 (5.5%) of the ticks were positive for *A. bovis* and were identical to previously reported sequences. Using the ITS specific nested PCR, 11/363 (3.0%) of the ticks were positive for *Bartonella* spp. This is the first report of *Anaplasma* and *Bartonella* spp. detected in ticks collected from KWD, suggesting that ticks are vectors of *Anaplasma* and *Bartonella* spp. between reservoir hosts in natural surroundings.

Key words: *Anaplasma* spp., *Bartonella* spp., tick, Korean water deer

The close association of wild animals and birds and their associated arthropod vectors with human populations and domestic livestock, travel, international trading of livestock, and illegal wildlife trading assist in the spread of infectious diseases worldwide [1]. Deer serve as reservoirs of several vector-borne pathogens that impact on human and veterinary health throughout much of the world, including the Republic of Korea (ROK) [2-6]. In the ROK, the Korean water deer (KWD, *Hydropotes inermis argyropus*) populations have rapidly increased from 1982 to 2011, in part, as a result of reforestation programs and the absence of predators and prohibition of hunting [7]. Consequently, KWD are often observed grazing

on vegetation near/in rural villages, agricultural areas (e.g., rice paddies), and even in metropolitan cities in close association with human populations, livestock, and poultry farms. A previous survey identified a high prevalence of *Anaplasma phagocytophilum*, *Anaplasma bovis*, and *Bartonella grahamii* in KWD spleen tissues, suggesting that KWD are reservoirs of these pathogens [8-9].

A. phagocytophilum is the causative agent of human granulocytic anaplasmosis and transmitted mainly by *Ixodes* ticks in many parts of the world [10]. In the ROK, *A. phagocytophilum* was identified by PCR in deer, rodents, and ticks belonging to the genera *Haemaphysalis* and *Ixodes* that were collected by tick drag and from migratory birds [8,11,12]. *B. grahamii* was first isolated from rodents in the United Kingdom and is transmitted mainly by *Ctenocephalides nobilis nobilis*, a flea often associated with rodents [14,15]. However, recently *Bartonella* spp. have been detected in several tick species by PCR in other parts of the world [16]. In the ROK, *Bartonella* spp. have been de-

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tected in ticks, e.g., *Haemaphysalis flava*, *Haemaphysalis longicornis*, *Ixodes nipponensis*, and *Ixodes turdus*, collected by tick drag and from migratory birds [11,13].

The purpose of this study was to identify tick-borne pathogens, e.g., *A. phagocytophilum* and *Bartonella* spp., in ticks that were collected from KWD that serve as suspected natural reservoirs of tick-borne pathogens and pose serious vector-borne disease threats to wildlife, domestic animals, and human populations.

The Conservation Genome Resource Bank for Korean Wildlife examined KWD carcasses in the ROK from March 2008-May 2009. A total of 27/70 (38.6%) of the KWD carcasses were positive for ticks. Ticks were removed and placed individually in cryovials containing 70% ethyl alcohol and labeled with a unique identification number that corresponded to the KWD collection data. Ticks were provided to the 5th Medical Detachment, 168th Multifunctional Medical Battalion, 65th Medical Brigade, Yongsan US Army Garrison, Seoul, where they were identified [17] and pooled according to tick species, life stages, sex (adults), and KWD collection numbers and then provided to the Laboratory of Veterinary Internal Medicine, Seoul National University where they were tested for selected pathogens.

Individuals and pools of ticks were homogenized mechanically using a Beadbeater TissueLyser II (QIAGEN, Hilden, Germany) with lysis buffer, proteinase K, and 5 mm stainless steel beads (30 frequencies/s for 5 min), followed by incubation at 56°C overnight and then centrifugation at 12,000 g for 10 min at 4°C. The genomic DNA extraction was performed using DNeasy® Tissue Kits (QIAGEN) according to the manufacturer's instructions. PCR and nested-PCR were performed using generic-specific primer sets for *Bartonella* and *Ehrlichia/Anaplasma*, and species-specific primers for *A. phagocytophilum*, *A. bovis*, and *B. grahamii* as described by Kang et al. [13].

PCR products were purified using QIAquick Gel Extraction kits (QIAGEN) and were cloned with pGEM®-T Easy Vectors (Promega Corp.). Plasmid DNA for sequencing was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega Corp.) according to the manufacturer's instructions. Purified recombinant plasmid DNA was sequenced by dideoxy termination with an automatic sequencer (ABI 3730xl capillary DNA sequencer, Foster City, California, USA). The DNA sequences were evaluated with Chromas software (Ver 2.33), aligned using Clustal X (Ver 2.1), and then examined

Table 1. Prevalence of tick-borne pathogens in fed ticks collected from Korean water deer (*Hydropotes inermis argyropus*) in the ROK using 16S rRNA primer sets

Species	Stage	No. of pools (no. of ticks)	No. of PCR-positive samples (prevalence [%] ^a)					
			<i>A. phagocytophilum</i>	<i>A. bovis</i>	<i>Bartonella</i> spp.	Double infection ^b	Double infection ^c	Triple infection ^d
<i>H. flava</i>	Larva	1 (1)	0	0	0	0	0	0
	Nymph	8 (10)	3 (30.0)	0	2 (20.0)	0	0	0
	Male	47 (47)	14 (29.8)	3 (6.4)	1 (2.1)	3 (6.4)	0	0
	Female	29 (29)	13 (44.8)	2 (6.9)	2 (6.9)	1 (3.4)	0	0
	Subtotal	85 (87)	30 (34.5)	5 (5.8)	5 (5.8)	4 (4.6)	0	0
<i>H. longicornis</i>	Larva	2 (19)	2 (10.5)	0	0	0	0	0
	Nymph	26 (104)	8 (7.7)	1 (1.0)	2 (1.9)	1 (1.0)	1 (1.0)	0
	Male	45 (45)	7 (15.6)	2 (4.5)	0	1 (2.2)	0	0
	Female	60 (60)	21 (35.0)	9 (15.0)	3 (5.0)	5 (8.3)	1 (1.7)	0
	Subtotal	133 (228)	38 (16.7)	12 (5.3)	5 (2.2)	7 (3.0)	2 (0.9)	0
<i>I. nipponensis</i>	Male	5 (5)	1 (20.0)	0	0	0	0	0
	Female	3 (3)	2 (66.7)	1 (33.3)	0	1 (33.3)	0	0
	Subtotal	8 (8)	3 (37.5)	1 (12.5)	0	1 (12.5)	0	0
<i>I. persulcatus</i>	Larva	1 (1)	0	0	0	0	0	0
	Male	15 (15)	8 (53.3)	0	0	0	0	0
	Female	24 (24)	10 (41.7)	2 (8.3)	1 (4.2)	1 (4.2)	0	1 (4.2)
	Subtotal	40 (40)	18 (45.0)	2 (5.0)	1 (2.5)	1 (2.5)	0	1 (2.5)
Total	Larva	4 (21)	2 (9.5)	0	0	0	0	0
	Nymph	34 (114)	11 (9.6)	1 (0.9)	4 (3.5)	0	0	0
	Male	112 (112)	30 (26.8)	5 (4.5)	1 (0.9)	4 (3.6)	0	0
	Female	116 (116)	46 (39.7)	14 (12.1)	6 (5.2)	9 (7.8)	0	1 (0.9)
	Total	266 (363)	89 (24.5)	20 (5.5)	11 (3.0)	13 (3.6)	2 (0.6)	1 (0.3)

^aPCR-positive pathogens was calculated by minimum infection rate (MIR), number of positive pools/total number of individual ticks tested.

^bTwo adult ticks assayed separately demonstrated a double infection with *A. phagocytophilum* and *A. bovis*.

^cTwo adult ticks assayed separately demonstrated a double infection with *A. phagocytophilum* and *Bartonella* spp.

^dOne adult tick demonstrated a triple infection with *A. phagocytophilum*, *A. bovis*, and *Bartonella* spp.

with a similarity matrix. The phylogenetic trees of nucleotide sequences were developed using the MEGA6 program. GenBank accession numbers for the 16S ribosomal (r) RNA and ITS region sequences and specific genospecies sequences related to bacterial pathogens for sequence comparisons are shown in Figs. 1, 2.

A total of 363 (21 larvae, 114 nymphs, and 228 adults) fed ticks belonging to 2 genera and 4 species, *H. flava* (n=87), *H. longicornis* (n=228), *I. nipponensis* (n=8), and *I. persulcatus*

(n=40), were collected from 27/70 (38.6%) KWD carcasses (Table 1). The minimum infection rate of *A. phagocytophilum* was 24.5% (89/363 ticks, 266 pools) for ticks using nested PCR targeting the 16S rRNA gene (Table 1). The 16S rRNA sequences (n=89) of *A. phagocytophilum* were classified into 5 genotypes that showed 98.7-100% similarity to the *A. phagocytophilum* HZ strain isolated from a human (CP000235) (Fig. 1). Four of 5 genotypes (KWDTAP1, 2, 3, and 4) corresponded to previously detected 16S rRNA sequences of *A. phagocytophilum* (KWDTAP1, 2, 3, and 4) from KWD spleen tissues [8]. In addition, the 5/363 (1.4%) amino acid sequences of *A. phagocytophilum* groEL (KWDTAPg, JQ086319) obtained from 2 *H. flava*, 2 *I. nipponensis*, and 1 *I. persulcatus* adult ticks were identical to each other and corresponded to the amino acid sequence of *A. phagocytophilum* groEL (ADO34908) that was previously reported from KWD spleen tissue. All *A. bovis* 16S rRNA gene sequences (JQ086318) from ticks were identical and corresponded to the *A. bovis* sequence (GU556626) previously identified from KWD spleen tissues. A total of 13 (3.6%) of the ticks demonstrated dual infections with *A. phagocytophilum* and *A. bovis*.

Using the ITS specific nested PCR, a total of 11 *Bartonella* sequences were classified into 2 genotypes (KWDBa1 and KWDBa2) (Fig. 2). KWDBa1 (JQ086210, n=4) was identified as *B. grahamii* V2 strain (AJ269785) and corresponded to the previous ITS sequence (JN810845) identified from KWD spleen tissues. KWDBa2 (JQ086321, n=7), which showed 92.7% similarity to *B. grahamii* V2 strain (AJ269785), was identified as *Bartonella* spp. (Fig. 2). In addition, 2 (0.6%) adult ticks each demonstrated double infections with *A. phagocytophilum* and *Bartonella* spp., while 1 (0.3%) individual adult tick demonstrated triple infections with *A. phagocytophilum*, *A. bovis*, and *Bartonella* spp.

KWD and the Korean spotted deer (*Cervus nippon mantchuricus*) are strongly suspected as reservoirs of *Anaplasma* and *Bartonella* species in the ROK [6,11,13]. This study supports evidence that ticks become infected while blood feeding on deer and are vectors of *Anaplasma* and *Bartonella* species that are present in deer populations in the ROK. However, there is insufficient correlation of *Anaplasma* and *Bartonella* spp. transmission between KWD and blood feeding ticks since some attached ticks collected from *A. phagocytophilum* positive KWD were negative for *A. phagocytophilum*, while attached ticks collected from negative KWD were positive for *A. phagocytophilum*. These results indicate that blood feeding does not always accompany ingestion of these vector-borne pathogens.

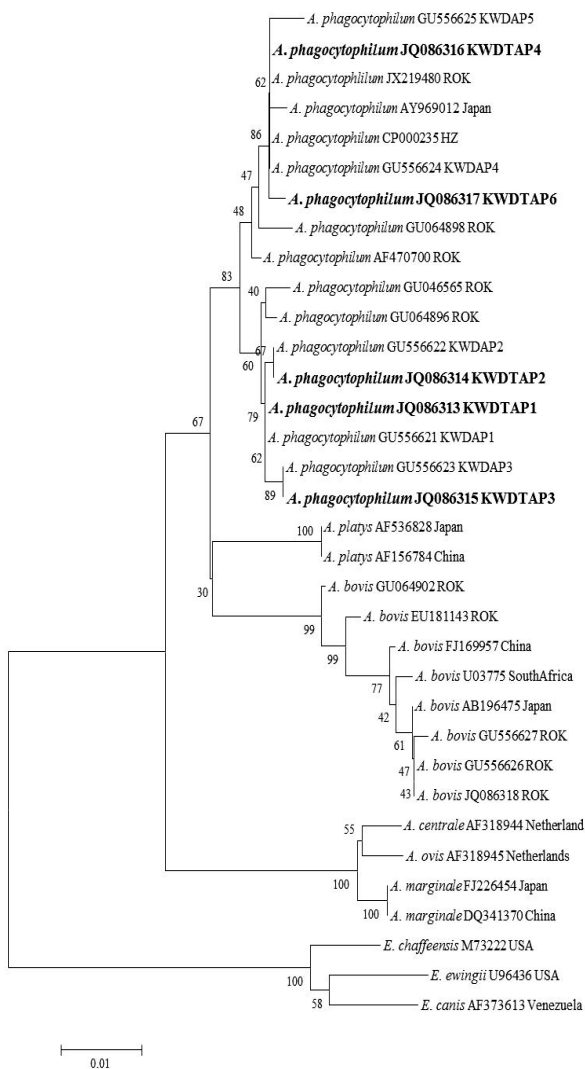


Fig. 1. Phylogenetic relationships for *Anaplasma phagocytophilum* (bold letters) detected from ticks collected from Korean water deer and *Anaplasma* and *Ehrlichia* species based on partial nucleotide sequences of 16S rRNA gene fragments (926 bp). The neighbor-joining method was used for constructing the phylogenetic tree. The numbers at the nodes are the proportions of 1,000 bootstrap iterations that support the topology shown.

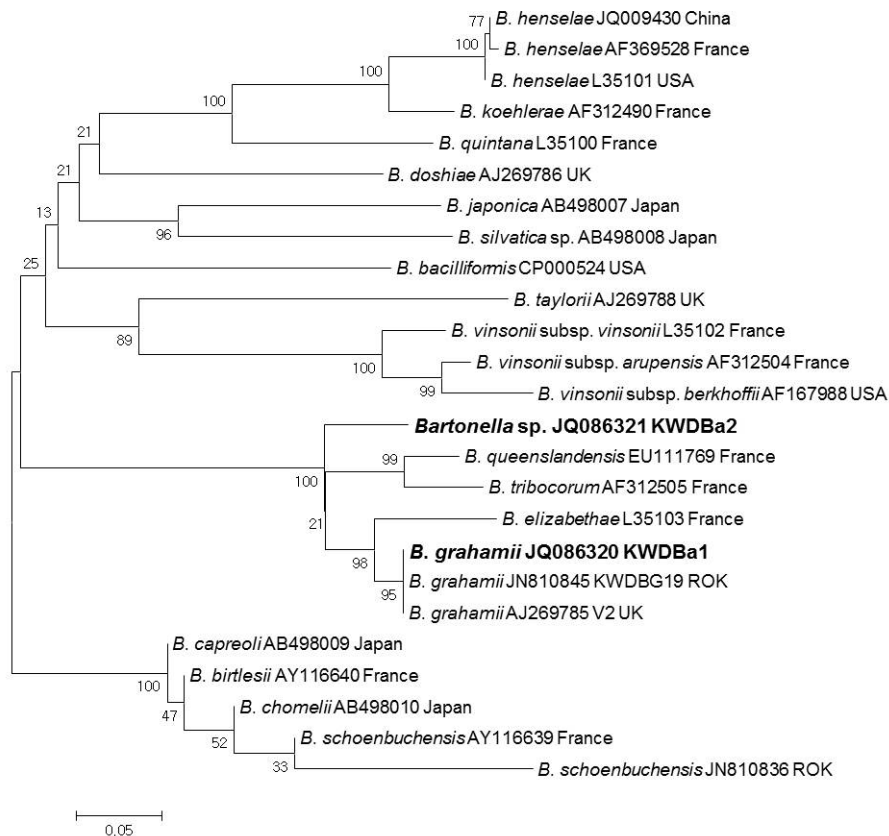


Fig. 2. Phylogenetic relationships for *Bartonella grahamii* and *Bartonella* spp. (bold letters) detected from ticks collected from Korean water deer and *Bartonella* spp. based on partial nucleotide sequences of ITS gene fragments (484 bp). The neighbor-joining method was used for constructing the phylogenetic tree. The numbers at the nodes are the proportions of 1,000 bootstrap iterations that support the topology shown.

Bartonella spp. are mainly transmitted by fleas, but recently *Bartonella* DNA has been detected in unfed *Haemaphysalis* and *Ixodes* spp. collected by tick drag and from partially fed and recently attached ticks collected from migratory birds in the ROK [11,13]. Molecular evidence supports the potential transmission of *Bartonella* spp. since it is transmitted by transstadial transmission as indicated by positive unfed ticks that were collected by tick drag. Tick bites among ROK populations are largely unreported with an average of only 40 tick bites annually, while there were 55 cases of severe fever with thrombocytopenia syndrome reported for 2014 [18]. Therefore, the potential risk associated with the transmission of *A. phagocytophilum* and *Bartonella* spp. in ticks that fed on infected KWD to ROK civilian and military populations and livestock is unknown.

In summary, evidence supports the maintenance of *A. phagocytophilum*, *Bartonella* spp. and other tick-borne patho-

gens in KWD and associated ticks that pose potential serious threats to medical and veterinary health in the ROK.

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CONFLICT OF INTEREST

No competing financial interests exist.

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