ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 54, No. 1: 87-91, February 2016
http://dx.doi.org/10.3347/kjp.2016.54.1.87

▣ BRIEF COMMUNICATION

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

Jun-Gu Kang[1], Sungjin Ko[1], Heung-Chul Kim[2], Sung-Tae Chong[2], Terry A. Klein[3], Jeong-Byoung Chae[1], Yong-Sun Jo[1], Kyoung-Seong Choi[4], Do-Hyeon Yu[5], Bae-Keun Park[6], Jinho Park[7], Joon-Seok Chae[1,*]

[1]*Laboratory of Veterinary Internal Medicine, BK21 PLUS Program for Creative Veterinary Science Research, Research Institute for Veterinary Science and College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea;* [2]*5th Medical Detachment, 168th Multifunctional Medical Battalion, 65th Medical Brigade, Unit 15247, APO AP96205-5247, USA;* [3]*Public Health Command District-Korea, 65th Medical Brigade, Unit 15281, APO AP 96205-5281, USA;* [4]*College of Ecology and Environmental Science, Kyungpook National University, Sangju 37224, Korea;* [5]*College of Veterinary Medicine, Chonnam National University, Gwangju 61186, Korea;* [6]*College of Veterinary Medicine, Chungnam National University, Daejeon 34134, Korea;* [7]*College of Veterinary Medicine, Chonbuk National University, Iksan 54596, Korea*

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 *gro*EL 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-

• Received 12 September 2015, revised 23 October 2015, accepted 26 November 2015.
* Corresponding author (jschae@snu.ac.kr)

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])					
			A. phagocytophilum	*A. bovis*	*Bartonella* spp.	Double infection[b]	Double infection[c]	Triple infection[d]
H. flava	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
H. longicornis	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. nipponensis	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. persulcatus	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)

[a]PCR-positive pathogens was calculated by minimum infection rate (MIR), number of positive pools/total number of individual ticks tested.
[b]Two adult ticks assayed separately demonstrated a double infection with *A. phagocytophilum* and *A. bovis*.
[c]Two adult ticks assayed separately demonstrated a double infection with *A. phagocytophilum* and *Bartonella* spp.
[d]One 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* (KWDAP1, 2, 3, and 4) from KWD spleen tissues [8]. In addition, the 5/363 (1.4%) amino acid sequences of *A. phagocytophilum gro*EL (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 gro*EL (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*.

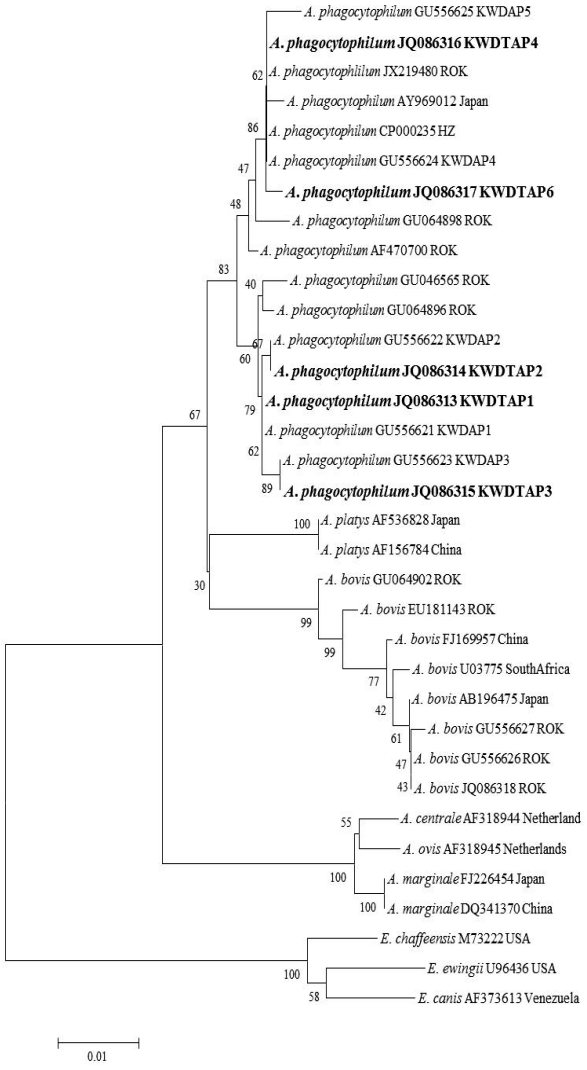

Fig. 1. Phylogenetic relationships for *Anaplasma phagocytophilum* (bold letters) detected from ticks collected from Korean water deer and *Anaplasma* and *Ehrlichia* species based on partial nucleotide sequences of 16S rRNA gene fragments (926 bp). The neighbor-joining method was used for constructing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

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.

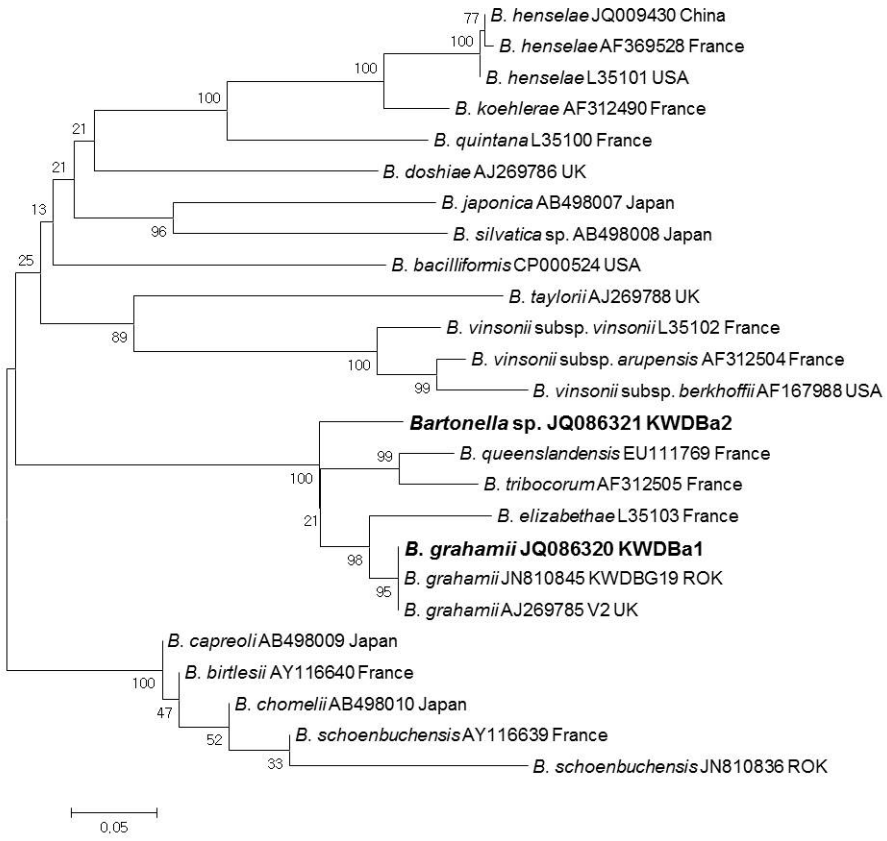

Fig. 2. Phylogenetic relationships for *Bartonella grahamii* and *Bartonella* spp. (bold letters) detected from ticks collected from Korean water deer and *Bartonella* spp. based on partial nucleotide sequences of ITS gene fragments (484 bp). The neighbor-joining method was used for constructing the 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 pathogens in KWD and associated ticks that pose potential serious threats to medical and veterinary health in the ROK.

ACKNOWLEDGMENTS

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (project no. PJ010092)", Rural Development Administration, the Republic of Korea. This research was also supported by a National Research Foundation of Korea Grant funded by the Korean Government (2011-0015349), the Public Health Command District-Korea, and the Armed Forces Health Surveillance Branch, Global Emerging Infections Surveillance and Response System (AFHSC-GEIS), Silver Spring, Maryland, USA.

Disclosure: The opinions expressed in this article are those of the authors and do not reflect official policy or positions of the US Department of the Army, the US Department of De-

fense, or the US Government.

CONFLICT OF INTEREST

No competing financial interests exist.

REFERENCES

1. Bengis RG, Leighton FA, Fischer JR, Artois M, Mörner T, Tate CM. The role of wildlife in emerging and re-emerging zoonoses. Rev Sci Tech 2004; 23: 497-511.
2. Belongia EA, Reed KD, Mitchell PD, Kolbert CP, Persing DH, Gill JS, Kazmierczak JJ. Prevalence of granulocytic *Ehrlichia* infection among white-tailed deer in Wisconsin. J Clin Microbiol 1997; 35: 1465-1468.
3. Little SE, Stallknecht DE, Lockhart JM, Dawson JE, Davidson WR. Natural coinfection of a white tailed deer (*Odocoileus virginianus*) population with three *Ehrlichia* spp. J Parasitol 1998; 84: 897-901.
4. Petrovec M, Bidovec A, Sumner JW, Nicholson WL, Childs JE, Avsic-Zupanc T. Infection with *Anaplasma phagocytophila* in cervids from Slovenia: evidence of two genotypic lineages. Wien Klin Wochenschr 2002; 31: 641-647.
5. Kawahara M, Rikihisa Y, Lin Q, Isogai E, Tahara K, Itagaki A, Hiramitsu Y, Tajima T. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma entral*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Appl Environ Microbiol 2006; 72: 1102-1109.
6. Lee M, Yu D, Yoon J, Li Y, Lee J, Park J. Natural co-infection of *Ehrlichia chaffeensis* and *Anaplasma bovis* in a deer in South Korea. J Vet Med Sci 2009; 71: 101-103.
7. National Institute of Biological Resources of Korea (NIBR). Survey and resource management of wildlife. National Institute of Biological Resources of Korea, Incheon, Korea. 2011, pp. 24.
8. Kang JG, Ko S, Kim YJ, Yang HJ, Lee H, Shin NS, Choi KS, Chae JS. New genetic variants of *Anaplasma phagocytophilum* and *Anaplasma bovis* from Korean water deer (*Hydropotes inermis argyropus*). Vector-Borne Zoonotic Dis 2011; 11: 929-938.
9. Ko S, Kim SJ, Kang JG, Won S, Lee H, Shin NS, Choi KS, Youn HY, Chae JS. Molecular detection of *Bartonella grahamii* and *B. schoenbuchensis*-related species in Korean water deer (*Hydropotes inermis argyropus*). Vector-Borne Zoonotic Dis 2013; 13: 415-418.
10. Chen SM, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J Clin Microbiol 1994; 32: 589-595.
11. Kim CM, Kim JY, Yi YH, Lee MJ, Cho MR, Shah DH, Klein TA, Kim HC, Song JW, Chong ST, O'Guinn ML, Lee JS, Lee IY, Park JH, Chae JS. Detection of *Bartonella* species from ticks, mites and small mammals in Korea. J Vet Sci 2005; 6: 327-334.
12. Oh JY, Moon BC, Bae BK, Shin EH, Ko YH, Kim YJ, Park YH, Chae JS. Genetic identification and phylogenetic analysis of *Anaplasma* and *Ehrlichia* species in *Haemaphysalis longicornis* collected from Jeju Island, Korea. J Bacteriol Virol 2009; 39: 1-11.
13. Kang JG, Kim HC, Choi CY, Nam HY, Chae HY, Chong ST, Klein TA, Ko S, Chae JS. Molecular detection of *Anaplasma*, *Bartonella*, and *Borrelia* species in ticks collected from migratory birds from Hong-do Island, Republic of Korea. Vector-Borne Zoonotic Dis 2013; 13: 215-225.
14. Birtles RJ, Harrison TG, Saunders NA, Molyneux DH. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int J Syst Bacteriol 1995; 45: 1-8.
15. Bown JK, Bennett M, Begon M. Flea-borne *Bartonella grahamii* and *Bartonella taylorii* in bank voles. Emerg Infect Dis 2004; 10: 684-687.
16. Billeter SA, Levy MG, Chomel BB, Breitschwerdt EB. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Med Vet Entomol 2008; 22: 1-15.
17. Yamaguti N, Tipton VJ, Keegan HL, Toshioka S. Ticks of Japan, Korea, and the Ryukyu Islands. Brigham Young University Science Bulletin, Biological Series 1971; 15: 1-226.
18. Korea Centers for Disease Control and Prevention. Infectious Diseases Surveillance Yearbook. 2014, pp. 27.