

Detection of *Bartonella* species from ticks, mites and small mammals in Korea

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We investigated the prevalence of *Bartonella* infections in ticks, mites and small mammals (rodents, insectivores and weasels) collected during 2001 through 2004, from various military installations and training sites in Korea, using PCR and sequence analysis of 16S rRNA, 23S rRNA and *groEL* heat shock protein genes. The prevalence of *Bartonella* spp. was 5.2% ($n = 1,305$ sample pools) in ticks, 19.1% ($n = 21$) in mesostigmatid mites and 13.7% ($n = 424$ individuals) in small mammals. The prevalence within the family Ixodidae was, 4.4% ($n = 1,173$) in *Haemaphysalis longicornis* (scrub tick), 2.7% ($n = 74$) in *H. flava*, 5.0% ($n = 20$) in *Ixodes nipponensis*, 11.1% ($n = 9$) in *I. turdus*, 33.3% ($n = 3$) in *I. persulcatus* and 42.3% ($n = 26$) in *Ixodes* spp. ticks. In rodents, the prevalence rate was, 6.7% ($n = 373$) in *Apodemus agrarius* (striped field mouse) and 11.1% ($n = 9$) in *Eothenomys regulus* (Korean red-backed vole) and in an insectivore, *Crociodura lasiura*, 12.1% ($n = 33$). Neither of the two weasels were positive for *Bartonella* spp. Phylogenetic analysis based on amino acid sequence of a portion of the *groEL* gene amplified from one *A. agrarius* spleen was identical to *B. elizabethae* species. We demonstrated the presence of *Bartonella* DNA in *H. longicornis*, *H. flava* and *I. nipponensis* ticks, indicating that these ticks should be added to the growing list of potential tick vectors and warrants further detailed investigations to disclose their possible roles in *Bartonella* infection cycles.

Key words: *Bartonella*, Korea, mites, PCR, rodents, ticks

Introduction

Bartonella species are pathogens of emerging and re-emerging significance, causing a wide array of clinical syndromes in human and animal hosts [9]. During recent years, an increasing number of *Bartonella* species have been isolated and characterized, and the genus currently consists of 19 species including two subspecies [9]. At least 9 species (*B. bacilliformis*, *B. quintana*, *B. henselae*, *B. elizabethae*, *B. clarridgeiae*, *B. grahamii*, *B. washoensis*, *B. koehlerae*, *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis*) are now known to potentially infect humans and/or animals [9,27,31].

As for many vector-borne disease agents, a wide range of mammalian reservoir hosts including rodent and arthropod vectors, such as sand flies, fleas and body lice, are involved in the natural cycle of various *Bartonella* spp. [16]. Different species of rodents (*Apodemus* spp., *Rattus rattus*, and *Microtus* spp.) also act as a primary vertebrate reservoir of *Bartonella* spp. [16]. Ticks such as *Ixodes pacificus* or *I. scapularis* (USA.), *I. ricinus* (The Netherlands and Italy) and *I. persulcatus* (Western Siberia) were found to be frequently infected with *Bartonella* spp. [1,5,6,14,25,28,29]. However the direct role of ticks in *Bartonella* transmission is not yet established. Recently, a wide variety of biting flies collected from animals were found infected with *B. henselae* and *B. bovis* [7]. In another study, biting *Hippoboscidae* flies were found infected with *Bartonella* parasites and suggested to be involved in transmission of *Bartonella* in ruminant animals [11] indicating that our knowledge regarding the biology of *Bartonella* infections is rapidly expanding. In Korea, *Haemaphysalis longicornis* is the predominant species of ticks in terms of its significance to human and veterinary medicine. Our previous studies have demonstrated

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that *H. longicornis* and *I. persulcatus* ticks are frequently infected with *Anaplasma phagocytophilum* (human granulocytic ehrlichiosis agent) and *Ehrlichia chaffeensis* (human monocytic ehrlichiosis agent). The role of these ticks as potential vectors for *Ehrlichia* and *Anaplasma* infections in humans in Korea is suspected [3,12,17,26].

Recently, serologic evidence of antibodies against *B. henselae* and *B. quintana* was reported for the first time in Korean patients suffering from lymphadenopathy [4]. However, the infection of possible tick vectors or rodents with *Bartonella* spp. has not been previously reported in Korea. The association between the natural hosts, vectors, and *Bartonella* spp. generally determines the spectrum of the most probable hosts (natural or incidental) and geographic distribution of *Bartonella* organisms. The presence of *Bartonella*-specific antibodies in human subjects in Korea has indirectly raised the possibility that the *Bartonella* bacteria may be circulating among the rodent and insectivore populations (natural reservoir hosts) or possibly in tick or mite vectors in Korea. Thus, it is important to disclose the prevalence of *Bartonella* species in various ticks and rodents populations that may be involved in the natural cycle of *Bartonella* infections in humans and/or animals in Korea. The present study aimed at PCR-based detection and identification of *Bartonella* spp. among tick and mite vectors as well as small mammals in Korea.

Materials and Methods

Tick, mite and small mammal sampling

Ticks were collected by dragging and flagging grassland and forest ground cover and by removing the ticks attached on various wild rodents at the U.S. and Korean military installations and training sites in Korea. During the year 2001 to 2003, a total of 1,979 ticks were collected from wild rodents ($n=297$ ticks) and vegetation ($n=1,682$ ticks). Based on microscopic examination, ticks were identified to species and classified morphologically by developmental stages [32]. Subsequently, different species of ticks were pooled to form 1,305 sample pools ($n=1-27$ ticks per sample pool in which large size pools mainly contained nymphs) consisting of 40 tick sample pools from wild

rodents and 1,265 tick sample pools from vegetation followed by storage at -70°C in 1.5 ml eppendorf tubes. Ninety four Mesostigmatid mites were collected from wild rodents and insectivores and pooled into 21 samples ($n=4-5$ mites per sample pool). During the year 2002 to 2004, spleen samples from 424 small mammals (389 wild rodents, 33 insectivores and two weasels) live captured by Sherman traps from the similar locations were assayed for the presence of *Bartonella* parasites. After reaching the laboratory, the animals were sacrificed in accordance with animal protocols, the abdominal cavity opened aseptically, and the spleen was collected and stored individually at -70°C .

DNA and PCR amplification

For the extraction of PCR amplifiable DNA, each tick, mite, and rodent, insectivore and weasel spleens were mechanically homogenized using sterile scissors. The genomic DNA was extracted using DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's instructions. In the first study, PCR based screening of ticks and mite pool samples collected during the year 2001 to 2003 was performed using primer pair BTNi-F and BTNi-R amplifying a 356 bp fragment of the 16S rRNA gene of *Bartonella* spp. (Table 1). The PCR mixture (20 μl volume) consisted of 4 pmol of each primer (BTNi-F and BTNi-R), 200 μM dNTPs, PCR buffer-I (SuperBio, Korea) and 1 U of SuperTaq DNA polymerase (SuperBio, Korea). The amplification conditions consisted of initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min and a final extension at 72°C for 7 min.

In a second study, PCR based screening of the spleen samples of small mammals collected during the year 2002 to 2004 was performed using primers (BAT23-F and BAT23-R) targeting 23S rRNA gene of *Bartonella* spp (Table 1). The PCR reaction was run in a final volume of 20 μl . The PCR mixture consisted of 4 pmol of each primer (BAT23-F and BAT23-R), 200 μM dNTPs, PCR buffer-I (SuperBio, Korea) and 1 U of SuperTaq DNA polymerase (SuperBio, Korea) PCR conditions included initial denaturation at 95°C for 3 min followed by 35-three step cycles of 95°C for 1 min, 55°C for 90 s, 72°C for 1 min and one cycle of final extension at 72°C for 3 min.

Table 1. Oligonucleotide primers and PCR assays

Primer*	Target gene	Oligonucleotide sequences (5'-3')	Nucleotide position	Product size (bp)	Type of sample
BTNi-F	16S rRNA	TTAGAGTGAGCGGCAAAC	78-95	356	Ticks and mites
BTNi-R		TACCGTCATTATCTTCACCG	433-414		
BAT23-F	23S rRNA	GATAGCGMACCAGTACCGTG	362-381	917	Small mammal spleen
BAT23-R		CGACTCACCCCTGCTCAGATT	1232-1251		
BTNgroEL1	groEL	GAAGATGTGGAAGGTGAA	505-522	336	Small mammal spleen
BTNgroEL2		TCACGGTCATAGTCAGAAG	849-821		

*The sequences of the oligonucleotide primers were derived from the respective gene sequences of *B. henselae* complete genome sequence available in the GenBank database (accession number NC005956).

Table 2. PCR-based analysis of ticks with nested-PCR targeting 16S rRNA for genus-specific identification of *Bartonella*

Developmental stages	<i>Haemaphysalis longicornis</i>	<i>H. flava</i>	<i>Ixodes nipponensis</i>	<i>I. turdus</i>	<i>I. persulcatus</i>	<i>Ixodes</i> spp.	Total
No. positive/No. examined (%) [§]							
Larva*	18/67	0/11	0	0	0	11/24	29/102 (28.4)
Nymph [†]	16/993	0/53	1/13	1/8	0	0/2	18/1,069 (1.7)
Adult male	3/30	0/7	0/4	0	1/2	0	4/43 (9.3)
Adult female	15/83	2/3	0/3	0/1	0/1	0	17/91 (18.7)
Total (%)	52/1,173 (4.4)	2/74 (2.7)	1/20 (5.0)	1/9 (11.1)	1/3 (33.3)	11/26 (42.3)	68/1,305 [‡] (5.2)

*2-27 ticks per pool (748 ticks), [†]1-3 ticks per pool (1,097 ticks), [‡]A total of 1,979 ticks was pooled into 1,305 samples that included tick samples collected from both, wild rodents (297 ticks pooled into 40 sample pools) and grassland (1,682 ticks pooled into 1,265 sample pools). [§]Parenthesis show percent positive. ^{||}*Ixodes* spp. ticks were collected from wild rodents. Subsequently, >1,800 Ixodid ticks collected from rodents captured in the same locality were identified as *I. nipponensis* (data not shown).

In another independent study, the 336 bp fragment of *Bartonella*-specific *groEL* gene was amplified from a rodent spleen sample. The PCR was performed in a total volume of 25 µl and the PCR mixture consisted of 4 pmol of each primer (BTNgroEL1 and BTNgroEL2), 200 µM dNTPs, PCR buffer-I (SuperBio, Korea) and 1 U of SuperTaq DNA polymerase (SuperBio, Korea). The reaction conditions consisted of initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 90 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min. PCR products were electrophoresed in a 1% (w/v) agarose gel, visualized by staining with ethidium bromide and photographed by a still video documentation system (Gel Doc-2000; Bio- Rad, USA).

Cloning, nucleotide sequencing and phylogenetic analysis

PCR products were purified using Wizard Plus DNA purification system (Promega, USA) and cloned in a pGEMT-easy vector (Promega, USA) as per the instructions provided by the manufacturer. The cloned DNA was sequenced by cycle sequencer using ABI Prism 377 DNA sequencer (Genotech, Korea). The nucleotide sequences of the 16S rRNA, 23S rRNA and *groEL* gene amplified from representative ticks, mites, and rodent and insectivore samples were registered in the GenBank databases. Sequence homology searches were made at the National Center for Biotechnology Information (NCBI, USA) BLAST network service. Comparative analysis of the nucleotide or amino acid sequences determined in the present study with those of existing sequences in the GenBank database was done using multiple sequence alignment with hierarchical clustering (8). Phylogenetic analysis was done using Multiple Sequence Alignment Program (AlinX, Vector NTI Suit v. 7.0; InforMax, USA).

Results

Identification of tick and rodent species

A total of 1,979 ticks collected from small mammals ($n = 297$ ticks) and vegetation ($n = 1,682$ ticks) were

Table 3. Identification of *Bartonella* species in spleen of small mammals by PCR targeting 23S rRNA

Species	No. tested	PCR positive	Prevalence (%)
<i>Apodemus agrarius</i>	373	53	14.2
<i>Crocidura lasiura</i>	33	4	12.1
<i>Eothenomys regulus</i>	9	1	11.1
<i>Apodemus peninsulae</i>	3	0	0
<i>Rattus rattus</i>	2	0	0
<i>Mustela sibirica</i>	2	0	0
<i>Cricetulus triton nestor</i>	1	0	0
<i>Mus musculus</i>	1	0	0
Total	424	58	13.7

identified and classified into two genera and five species [*Haemaphysalis longicornis* ($n = 1,549$), *H. flava* ($n = 115$), *Ixodes nipponensis* ($n = 20$), *I. turdus* ($n = 9$), *I. persulcatus* ($n = 3$), and *Ixodes* spp. ($n = 283$)]. The collection of the 1,979 ticks was then pooled into 1,305 sample pools that included both, 297 ticks collected from wild rodents (pooled into 40 sample pools) and 1,682 ticks collected from grassland (pooled into 1,265 sample pools). The summary of the identified ticks along with their developmental stages examined in this study is shown in Table 2. *H. longicornis* was the single most abundant species and most of the ticks irrespective of species identified were collected during their nymphal stage of development (Table 2). A collection of 424 small mammals were identified and classified into five genera and six species of rodents, one species of insectivore, and two weasels (*Mustela sibirica*) (Table 3). *Apodemus agrarius* (striped field mouse) species accounted for 88% (373/424) of the small mammals sampled. Other species of rodents captured were identified as *A. peninsulae* ($n = 3$), *Eothenomys regulus* ($n = 9$), *Rattus rattus* ($n = 2$), *Cricetulus triton nestor* ($n = 1$) and *Mus musculus* ($n = 1$) and one insectivore species, *Crocidura lasiura* ($n = 33$). All the mite samples included in this study were Mesostigmatid mites.

Table 4. Homology comparison of the *Bartonella* sp. 16S rRNA gene fragment (356 bp) sequences*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		98.3	98.3	97.8	98.1	96.4	99.2	97.8	97.8	98.0	98.3	98.9	98.9	98.1	98.6	98.6	98.6	98.3	98.3	96.7
2	6		97.2	97.8	97.5	95.6	97.5	96.7	96.7	97.5	98.1	97.8	97.5	97.5	98.3	97.0	97.2	96.9	97.2	96.4
3	6	10		97.5	96.7	96.4	97.5	97.2	97.8	98.3	97.5	99.2	97.5	96.4	97.5	96.9	97.5	96.9	96.9	96.1
4	5	7	9		96.9	95.8	97.0	96.1	96.4	97.8	97.2	97.5	97.2	97.2	97.2	96.4	97.2	96.6	96.1	95.3
5	1	3	7	6		97.5	98.3	99.2	98.9	96.4	97.2	97.2	97.2	99.4	96.9	99.4	97.2	97.5	98.0	97.8
6	8	10	6	9	8		96.4	97.5	97.8	96.4	95.8	97.2	96.4	97.2	95.6	97.5	96.6	96.1	96.4	96.6
7	0	4	5	7	1	9		98.1	98.1	97.2	97.5	98.1	98.1	97.8	97.8	98.9	97.8	97.5	97.5	96.9
8	2	6	4	9	3	8	2		99.7	96.9	96.9	97.8	96.9	98.6	97.2	99.2	96.9	96.9	98.0	98.3
9	2	6	3	8	4	7	2	1		97.2	96.7	98.1	96.9	98.3	96.9	99.2	97.2	96.9	98.0	98.3
10	6	6	4	6	6	7	6	4	3		97.2	98.6	97.5	96.4	97.8	96.7	97.5	96.6	96.7	96.4
11	4	6	9	10	5	9	5	6	7	7		98.0	97.8	97.2	98.0	96.9	97.5	97.2	96.9	96.1
12	4	6	2	8	4	5	4	2	9	4	6		98.0	97.2	98.0	97.5	98.0	97.5	97.5	96.7
13	4	7	8	9	4	8	4	2	2	8	7	7		97.2	97.5	97.8	99.2	97.8	97.5	95.8
14	1	4	8	6	1	8	2	1	5	7	6	5	5		97.5	98.9	97.2	97.8	98.0	97.8
15	4	5	9	10	6	10	4	5	6	6	7	6	8	5		97.2	97.2	97.5	97.2	98.1
16	0	4	5	7	1	9	0	2	2	6	5	4	3	2	4		97.8	97.5	98.6	98.0
17	4	7	7	8	5	8	4	6	5	7	7	6	2	6	8	4		97.2	97.5	95.8
18	6	9	10	11	3	9	6	5	5	11	9	9	8	3	8	4	9		97.5	96.1
19	1	5	5	8	3	7	1	2	2	4	5	2	4	4	4	1	5	4		96.9
20	6	5	7	10	6	11	6	4	4	6	7	6	9	5	0	6	10	8	4	

*Percent identity between sequences of 16S rRNA gene fragment is shown as the upper matrix. The lower matrix shows the number of nucleotide differences. 1, *Bartonella* sp.-FY-*Ixodes turdus*-Korea (AY920919) and *Bartonella* sp. YS-mite-Korea (AY920920); 2, *Bartonella* sp.-OR-*H. longicornis*-Korea (AY920922); 3, *Bartonella* sp.-008KTC-*H. longicornis*-Korea (AY920921); 4, *B. alsatica*-382 (AJ002139); 5, *B. birtlesii* (AF204274); 6, *B. clarridgeiae*-CIP104882 (X97822); 7, *B. doshiae*-R18 (Z31351); 8, *B. grahamii*-V2 (Z31349); 9, *B. henselae*-FR96-Bk38 (AJ223779); 10, *B. phoceensis*-16120 (AY515119); 11, *B. quintana* (AJ250247); 12, *B. rattimassiliensis*-15908 (AY515120); 13, *B. schoenbuchensis*-R6 (AJ278190); 14, *B. taylorii* M6 (Z31350); 15, *B. tribocorum*-IBS 506 (AJ003070); 16, *B. vinsonii*-Baker (Z31351); 17, *B. weissii*-FC7049UT (AF199502); 18, *B. bacilliformis*-LA6.3 (Z70003); 19, *B. koehlerae* C-29 (AF076237); 20, *Rochalimaea* (*Bartonella*) *elizabethae* (L01260). The nucleotide sequences of strain FY and strain YS were 100% identical.

Detection of *Bartonella* sp. from ticks and mites

The infection rates with *Bartonella* spp. observed in various species of ticks at different developmental stages of life are shown in Table 2. Using PCR primer sets targeting *Bartonella*-specific 16S rRNA, 68 (5.2%) out of 1,305 tick pool samples tested PCR positive (Table 2). These PCR positive tick samples included *H. longicornis* ($n = 52$), *H. flava* ($n = 2$), *I. nipponensis* ($n = 1$), *I. turdus* ($n = 1$), *I. persulcatus* ($n = 1$) and *Ixodes* spp. ($n = 11$). A specific PCR product of 356 bp was observed in 13 (32.5%) out of 40 tick pool samples collected from rodents and 55 (4.3%) out of 1,265 tick pool samples collected from grassland. In general, at least one sample from all genera and species of ticks collected in this study were found infected with *Bartonella* spp (Table 2). Out of 21 Mesostigmatid mite pool samples, four pools (19%) were found PCR positive by PCR targeting *Bartonella*-specific 16S rRNA gene fragment. In all cases, the PCR positive results were identified by production of a unique 356 bp PCR amplicon (data not shown). In order to further confirm the presence of *Bartonella* among these samples, the PCR products obtained from representative samples from *I. turdus* ticks

(strain FY), Mesostigmatid mites (strain YS) and two samples from *H. longicornis* ticks (strains 008KTC and OR) were sequenced and registered in GenBank (accession numbers AY920919 to AY920922). The homology level between nucleotide sequences from four samples determined in this study varied from 97.2% to 100%. The nucleotide sequences of strain FY and strain YS were 100% identical. Comparative analysis of nucleotide sequences of four strains determined in this study revealed 95.6% to 99.2% homology when compared with the 16S rRNA sequences of 17 known *Bartonella* species available in the GenBank database which confirmed the presence of *Bartonella* among these samples (Table 4). Briefly, the nucleotide sequence of strain FY and YS had 99.2% homology with that of *B. doshiae* while strain 008KTC and OR had close homology to *B. rattimassiliensis* (99.2%) and *B. tribocorum* (98.3%), respectively (Table 4).

Detection of *Bartonella* sp. from small mammals

Out of 424 spleen samples collected from small mammals (rodents and insectivores), 58 (13.7%) revealed production of a unique amplicon of 917 bp when the PCR was

Table 5. Homology comparison of the *Bartonella* sp. 23S rRNA gene fragment (917 bp) sequences*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		98.9	98.2	99.1	99.0	98.8	98.5	98.4	98.3	97.9	97.6	97.3	97.3	96.8	96.4
2	10		98.6	98.9	99.0	99.2	99.3	98.6	98.7	98.2	97.8	97.9	97.8	97.4	96.8
3	17	13		98.2	98.3	98.3	98.2	97.8	98.1	97.2	97.1	97.0	96.9	96.4	96.0
4	8	10	17		98.8	98.6	98.5	98.2	98.5	97.7	97.5	97.3	97.1	96.6	96.2
5	9	9	16	11		98.9	98.9	98.6	98.7	98.3	97.6	97.4	97.6	97.4	96.9
6	11	7	16	12	10		99.2	99.2	98.7	97.6	97.7	97.9	97.6	97.2	96.7
7	13	6	17	13	10	7		98.8	98.8	98.0	97.6	98.0	97.7	97.3	97.1
8	14	12	20	16	12	7	11		98.4	97.5	97.3	97.5	97.1	96.6	96.4
9	16	11	18	13	11	11	11	14		97.8	97.8	98.4	97.6	97.2	96.8
10	19	16	26	21	16	22	18	23	20		97.8	97.2	97.7	97.5	96.8
11	22	20	27	23	22	21	22	34	20	20		97.1	98.7	98.0	97.6
12	25	19	28	25	24	19	18	23	14	26	27		96.8	96.4	96.0
13	25	20	28	27	22	22	21	27	22	21	11	29		98.8	98.7
14	29	24	33	30	24	26	25	30	26	23	18	33	11		97.7
15	33	27	37	34	29	30	27	33	29	29	22	37	11	21	

*Percent identity between sequences of 23S rRNA gene fragment is shown as the upper matrix. The lower matrix shows the number of nucleotide differences. 1, *Bartonella* sp. wild rodent Y-KG [Korea] AY920925; 2, *Bartonella* sp. wild rodent J2-KG [Korea] AY920924; 3, *Bartonella* sp. wild rodent J1-KG [Korea] AY920923; 4, *Bartonella elizabethae* [USA] AF410940; 5, *Bartonella henselae* Huston-1 [USA] AF410943; 6, *Bartonella doshiae* R18 [USA] AF410939; 7, *Bartonella birtlesii* N40 [USA] AF410944; 8, *Bartonella grahamii* V2 NCTC 12860 [USA] AF410942; 9, *Bartonella vinsonii* subsp. *arupensis* [USA] AF410937; 10, *Bartonella quintana* VR358 [USA] AF410946; 11, *Bartonella bacilliformis* KC584 [USA] L39095; 12, *Bartonella vinsonii* subsp. *berkhoffii* 93CO-1 [USA] AF410941; 13, *Bartonella weissii* 99-BO1 [USA] AF410947; 14, *Bartonella clarridgeiae* ATCC 700095 AF410938 15, *Bartonella* sp. Deer 159-660-1 [USA] AF410945.

performed with genus-specific primers targeting the *Bartonella*-specific 23S rRNA. Both weasels were negative for *Bartonella* spp. infections. Rodent and insectivore species that tested PCR positive were *Apodemus agrarius* (53/373), *Eothenomys regulus* (1/9) and *Crocidura lasiura* (4/33). To confirm the presence of *Bartonella* spp. among these PCR positive samples, PCR products obtained from three representative *A. agrarius* spleen samples (strains J1-KG, J2-KG, and Y-KG) were sequenced and registered in GenBank under the accession numbers AY920923-AY920925. The homology level between the three 23S rRNA sequences determined in this study varied from 98.2% to 98.9%. The nucleotide sequence similarity searches revealed 96% to 99.2% homology when the sequences determined in this study were compared with 12 *Bartonella* species sequences available in the GenBank database (Table 5). Briefly, the nucleotide sequence of strain J1-KG, J2-KG and Y-KG had close homology to *B. henselae* or *B. doshiae* (98.3%), *B. birtlesii* (99.3%), and *B. elizabethae* (99.1%), respectively (Table 5).

Further study was carried out in which a DNA sample extracted from one *A. agrarius* mouse spleen (K286) was subjected to PCR amplification of 336 bp fragment of *Bartonella*-specific *groEL* gene. Comparative analysis of the deduced amino acid sequence of PCR amplified *groEL* gene (*Bartonella* sp. K286, accession number AY920926) with the *Bartonella* sequences representing 17 known *Bartonella* species available in the GenBank database

indicated that the *groEL* sequence of K286 strain was 100% identical or similar to that of *B. elizabethae*. Subsequent phylogenetic analysis also revealed close clustering of the K286 strain with that of *B. elizabethae* (Fig. 1). Based on the deduced amino acid sequence homology and phylogenetic analysis, K286 strain was identified as *B. elizabethae*.

Discussion

Molecular evidence regarding the role of various arthropod vectors and vertebrate reservoir hosts in *Bartonella* infections continues to accumulate at an exponential rate [2]. This is reporting the detection of *Bartonella* infections in ticks, mites, rodents and insectivore populations in Korea. It has been established that the prevalence of *Bartonella* infections in different species of ticks varies considerably in different parts of the world. For instance, *I. pacificus* and *I. scapularis* in the US, *I. ricinus* in The Netherlands and Italy and *I. persulcatus* ticks in Western Siberia have been reported to be frequently infected with *Bartonella* spp. [1,5,14,25,28,29]. In Korea, *Haemaphysalis* spp. and *Ixodes* spp. of ticks are known reservoirs of *A. phagocytophilum* (human granulocytic ehrlichiosis agent) and *E. chaffeensis* (human monocytic ehrlichiosis agent), respectively [3,21,26]. Since *H. longicornis* is the predominant species of tick found in Korea, the majority (76.5%) of the PCR positive ticks in this study were identified as *H. longicornis* at various stages of their developmental life cycle.

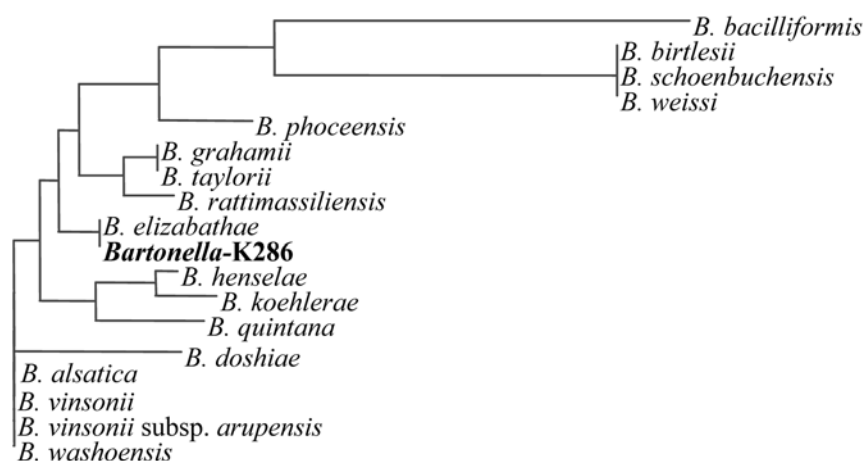


Fig. 1. Phylogenetic tree of *groEL* amino acid sequences from 17 representative *Bartonella* species. The tree was generated by using computer software Vector NTI Suite v. 7.0 (InforMax, USA). Sequences were obtained from GenBank database. Accession numbers: AY920926 (*Bartonella* sp. K286); AAD04243 (*Rochalimaea-Bartonella elizabethae*); CAA78859 (*B. bacilliformis*); AAK69694 (*B. birtlesii*); AAM77030 (*B. schoenbuchensis*); AAC24233 (*B. weissii*); AAS89952 (*B. phoceensis*); AAD04242 (*B. grahamii*); AAK97286 (*B. taylorii*); AAS89951 (*B. rattimassiliensis*); AAB69094 (*B. henselae*); AAM77029 (*B. koehlerae*); AAB69095 (*B. quintana*); AAK97211 (*B. alsatica*); AAD04241 (*B. doshiae*); AAD04244 (*B. vinsonii*); AAK97285 (*B. vinsonii* subsp. *arupensis*); AAL89757 (*B. washoensis*).

In addition, at least one sample from each *Ixodes* species of tick examined in this study was found to harbor *Bartonella* spp. The *Bartonella* infection in *I. nipponensis* tick reported in this study is particularly important because; (i) *I. nipponensis* is one of the most important cause of tick bite related ailments in humans in Korea and 19 such cases in humans have so far been documented [18], (ii) *I. nipponensis* is one of the primary vectors and reservoir of *Borrelia* spp. including *B. burdgorferi*, *B. afzelii* and *B. valaisiana* in Korea [20], and (iii) we recently identified large numbers (>1,800) of *I. nipponensis* ticks collected from the tick infested wild rodents in Korea (unpublished data), indicating that apart from *H. longicornis*, *I. nipponensis* is now emerging as a major tick species prevalent in Korea.

To our knowledge this is the first evidence of *Bartonella* infection in *I. nipponensis*, *H. longicornis* and *H. flava*, indicating that these ticks should also be added to the growing list of possible tick vectors for *Bartonella* spp. It appears that human patients with a history of tick bites in Korea should always be examined for the possibility of *Bartonella* infections. In the present study, four (19%) mesostigmatid mites were found infected with *Bartonella* spp. Further screening of large number of mites in the future will be helpful to disclose the association of different mite species with the *Bartonella* infection in Korea.

To find out the most closely related homolog for the *Bartonella* species that may be present in the tick and mite sample pools, we performed sequence analysis of a PCR amplicons obtained from 4 representative tick and mite sample pools and compared these sequences with the available sequences of *Bartonella* in the GenBank database. Our analysis of the 16S rRNA sequences from these tick and

mite samples revealed high degrees (95.6% to 99.2%) of similarity with the sequences of 17 known *Bartonella* species available in the database. These results confirmed the presence of *Bartonella* infections among the tick and mite pooled samples. Due to a high degree of sequence similarity, it was difficult for us to make any specific inferences regarding the identification of a particular species of *Bartonella* among these ticks. However, sequences of strain FY and YS had close homology to *B. doshiae* (99.2%) while strain OR and strain 008KTC had close homology to *B. tribocorum* (98.3%) and *B. rattimiliensis* (99.2%), respectively. This variability in sequence homology obtained from different samples indicates that different species of *Bartonella* spp. may be present among ticks.

Apart from ticks, *Bartonella* infections are widely distributed in rodent and insectivore populations that act as a significant reservoir of *Bartonella* spp. associated with human diseases [10]. The primers targeting 23S rRNA in this study were selected for genus-specific identification of *Bartonella* spp. from small mammals which resulted in identification of *Bartonella* infection in 13.7% of the captured rodents and insectivores. In countries like the U.S., Sweden, and Greece, 42.2, 16.5 and 31.3% rodents were found infected with *Bartonella* spp., respectively [15,19,30]. As indicated in these studies, numerous *Bartonella* species are found in rodents and insectivores. *Apodemus flavicollis* was the most commonly captured species in Sweden (110/236) and in Greece (61/70). However, in our study the highest PCR positive rate was obtained from *A. agrarius* (53/373), the most common rodent species captured (373/424) during the year 2002 to 2004. Among the other captured species that tested PCR positive in this study were

Crocidura lasiura (4/33) and *Eothenomys regulus* (1/9). The identity or similarity of the 23S rRNA sequences from three representative rodent and insectivore spleen samples determined in this study ranged from 96.4% to 99.1% when compared with 12 *Bartonella* sequences available in the GenBank database. We did not investigate further to identify the species of *Bartonella* among these PCR positive rodent samples. However, the prevalence of *Bartonella* infections in this study was much higher in ticks collected on rodents (32.5%) than ticks collected from vegetation (4.3%). This, however be due to the fact that only one species, *I. nipponensis*, was taken from the rodents and insectivores, whereas, *Haemaphysalis* spp. accounted for >90% of the ticks taken from vegetation. Interestingly, the principal tick species that may be involved in the natural cycles of *B. elizabethae* infection is not yet known. Therefore, it is more likely that ticks are positive as they fed on bacteremic rodents.

The comparison of deduced amino acid sequence of the amplified *groEL* gene (strain K286) revealed 100% identity or similarity with *B. elizabethae*. Phylogenetic analysis performed with the *groEL* sequences from the 17 representative *Bartonella* species revealed that strain K286 clustered closely with *B. elizabethae*. Although, we have amplified a small stretch of *groEL* (336 bp) gene, the above analysis of the amino acid sequence determined in this study confirms the infection of *A. agrarius* with *B. elizabethae* or a very similar species. Our knowledge of the transmission chain of *Bartonella* species carried by rodents to humans or animals is still rudimentary. However, it is well established that the *Bartonella* spp. from rodents, e.g., *B. elizabethae*, can infect aberrant hosts including domestic cats [13], dogs [24] and certain groups like elite orienteers suffering from myocarditis [23] and intravenous heroin addicts [22]. *B. elizabethae* infections in the striped field mouse, *A. agrarius*, reported in this study is important because it makes up to >75% of the total population of field mice in Korea [20]. The species-specific detection of *Bartonella* from other rodents, insectivores, ticks and mites was not attempted in our present study. It is possible that apart from *B. elizabethae*, different *Bartonella* species may also be present among the rodent/insectivore samples that were PCR positive in this study. In future investigations, screening ticks, mites, fleas and rodent populations in Korea for the possibility of *Bartonella* infection should be therefore continued to provide useful information to elucidate their role in the epidemiology of disease resulting from *Bartonella* spp. infections. Future studies involving different molecular targets like *groEL* (encoding 65 kDa heat shock protein), *gltA* (encoding citrate synthetase) or *rpoB* (encoding RNA polymerase beta subunit) genes will be necessary to determine *Bartonella* species prevalence in Korea.

In conclusion, the results of this study indicate that there was a diversity of *Bartonella* spp. present in ticks, mites,

rodents and insectivores in Korea. Infections with *Bartonella* spp. in ticks, mites and small mammals in general, and specifically *B. elizabethae* in *A. agrarius*, warrants further investigations on possibilities of human or animal infections in Korea.

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