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Original Article

Genetic Identification and Phylogenetic Analysis of *Anaplasma* and *Ehrlichia* Species in *Haemaphysalis longicornis* Collected from Jeju Island, Korea

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A total of 1,395 *Haemaphysalis longicornis* ticks collected from Jeju Island of Korea were examined by 16S rRNA gene-based nested PCR for the presence of infection with *Anaplasma* and *Ehrlichia* species. Template DNAs to detect the tick-borne pathogens were prepared from a total 506 tick pools. Eight genera of *Anaplasma* and six *Ehrlichia* by 16S rRNA gene PCR and sequencing analysis were identified. *A. phagocytophilum* was the most prevalent (27 [1.9%]) by nested PCR, followed by *A. bovis* (5 [0.4%]), *E. chaffeensis* (4 [0.2%]), and *A. centrale* (1 [0.1%]). In the phylogenetic analysis based on 16S rRNA sequences, eight genera of *Anaplasma* group (> 99.4% homology) and six *Ehrlichia* group (> 99.5% homology) were close to deposited *A. marginale* strains (AF309867, AF414874, and FJ226454) and *Ehrlichia* sp. (DQ324547), respectively. Three *Anaplasma* species groups *A. phagocytophilum* (group A), *A. bovis* (group B), and *A. centrale* (group C) and one *Ehrlichia* species *E. chaffeensis* (group D) were determined by comparing with *Anaplasma* and *Ehrlichia* related sequences. First, twenty-eight *A. phagocytophilum* clones belonging to group A were divided into 7 genotypes. The sequence similarity among genotypes A1 to A4 was very high (> 99.6%). Genotype B2 was close to *A. bovis* from Korea (99.7%). Genotype D1 was close to known *E. chaffeensis* strains (M73222, AF147752, and AY350424) and their similarity value was 99.7%. In conclusion, the genera of *Anaplasma*/*Ehrlichia*, *A. phagocytophilum*, and *E. chaffeensis* identified in predominant *H. longicornis* ticks were ubiquitous throughout the Jeju Island. The various native groups have been found through sequence identities and phylogenetic analysis.

Key Words: *Haemaphysalis longicornis*, Tick-borne pathogens, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*

INTRODUCTION

Anaplasmosis and ehrlichiosis which cause tick-borne rickettsial diseases in both humans and domestic animals were distributed in the world. *Anaplasma* genospecies group (*A. phagocytophilum*, *A. bovis*, *A. platys*, *A. marginale*, and *A. centrale*) and *Ehrlichia* genospecies group (*E. chaffeensis*, *E. ewingii*, and *E. canis*) are well known to be pathogenic to human, domestic, and pet animals with acute febrile diseases (1~5). Particularly, *A. phagocytophilum* and *E. chaffeensis*, etiologic agents of human granulocytic anaplasmosis (HGA) and human monocytic ehrlichiosis (HME), have been reported mainly from USA and Europe (6, 7). In

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Asia, the emergence of intracellular organisms such as *Borrelia*, *Bartonella*, *Anaplasma*, *Ehrlichia*, and *Rickettsia* species have been reported frequently from China, Japan, and Korea (8~12). It is known to cause infection or co-infection with only one or mixed infectious agents in ticks and rodents of the various Asian countries. In Korea, *A. phagocytophilum* and *E. chaffeensis* were first determined by Heo *et al* (4) in 2002 by indirect immunofluorescence assay (IFA), Western blotting, and TaqMan real-time PCR from Korean patients. Further studies have been done in *Haemaphysalis* sp. and *Ixodes* sp. ticks, dogs, ruminants, and wild animals until the present one (11, 13~15). *H. longicornis* and *Ixodes* spp. ticks collected from Korea was the most predominant (>91.8%) and the prevalence of tick-borne pathogens by TaqMan PCR and species-specific PCR was frequent for some bacteria, including *A. platys*, *E. chaffeensis*, and *Rickettsia* sp. in *H. longicornis* ticks for the most part (10). However, study for bacterial etiologic agents from Jeju Island which is at a distance from Korean inland areas is rare.

Recently, diagnostic methods for detecting of rickettsial pathogens have rapidly changed. Molecular methods that are based on 16S rRNA gene sequence and the nested PCR technique utilizing first PCR product used as template have been reported (11, 15, 16). However, PCR amplicons by *Anaplasma*/*Ehrlichia* associated 16S rRNA gene PCR was not easily amplified with DNAs isolated from ticks and animal.

The aim of this study was to investigate genetic identification and phylogenetic analysis of tick-borne pathogens in *H. longicornis* ticks collected from Jeju Island of Korea.

MATERIALS AND METHODS

Survey area and sample collection

Unfed ticks were collected by dragging and flagging method using 1 m^2 flannel flags, in the Jeju Special Self-Governing Province, Korea. From June 2007 to August 2008, a total of 1,395 ticks were collected from 72 sampling sites into the three survey areas (East, West, and North) which were mostly the grass vegetation, followed by the stock farm, forest, Hallasan National Park, and no livestock (Fig. 1) (Table 1). The tick identification was classified into the various developmental stages according to the standard taxonomic method described by Yamaguti *et al* (17).

DNA preparation and identification of tick DNA

After collection, the ticks were placed in bottles containing 70% ethyl alcohol. The tick samples were disrupted for 5 min with 3.2 mm stainless steel beads (BioSpec Products, New Haven, UK) in 1.5 ml tubes by TissueLyser II (Universal laboratory mixer-mill disruptor, Qiagen GmbH, Qiagen Strasse Hilden, Germany). Total DNA was isolated from whole tick tissues by using DNeasy Blood & Tissue kit (Qiagen). The extracted DNA were electrophoresed through 0.8% agarose gel and the quality of the tick DNA was evaluated by PCR based on the *H. longicornis* tick 5.8S ribosomal DNA spacer (internal transcribed spacer 2, ITS2) with primers HITS2-F (5'-GGTGCTCGAGACT-CGTTTTG-3') and HITS2-R (5'-ATTCGCGGTTTACGA-GAGAA-3'). Briefly, PCR reaction mixture contained 10 pmol of each primer, 1 U of HiPi^{TM} Plus *Taq* DNA polymerase (ELPIS Biotech, Daejeon, Korea), 20 mM of each dNTP, 50 mM Tris-HCl (pH 9.0), 1.6 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.1% Tween 20 and 2 µl template tick genomic DNA in a final volume of 25 µl. The PCR amplification was performed in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) with following program: an initial denaturation at 94℃ for 5 min, followed by 35 cycles consisting of denaturation at 94℃ for 1 min, annealing at 59℃ for 1 min, and extension at 72℃ for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. To confirm the specificity of the tick DNA, total DNAs extracted from the *H. flava* and *Ixodes persulcatus* were used as negative controls (Fig. 2).

Detection of *Anaplasma* and *Ehrlichia* sp. by PCR

To detect both the 16S rRNA of *Anaplasma* and *Ehrlichia* species, PCR was conducted using primer sets AE1-F (5'-AAGCTTAACACATGCAAGTCGAA-3') and AE1-R (5'-AGTCACTGA CCCAACCTTAAATG-3') in this study.

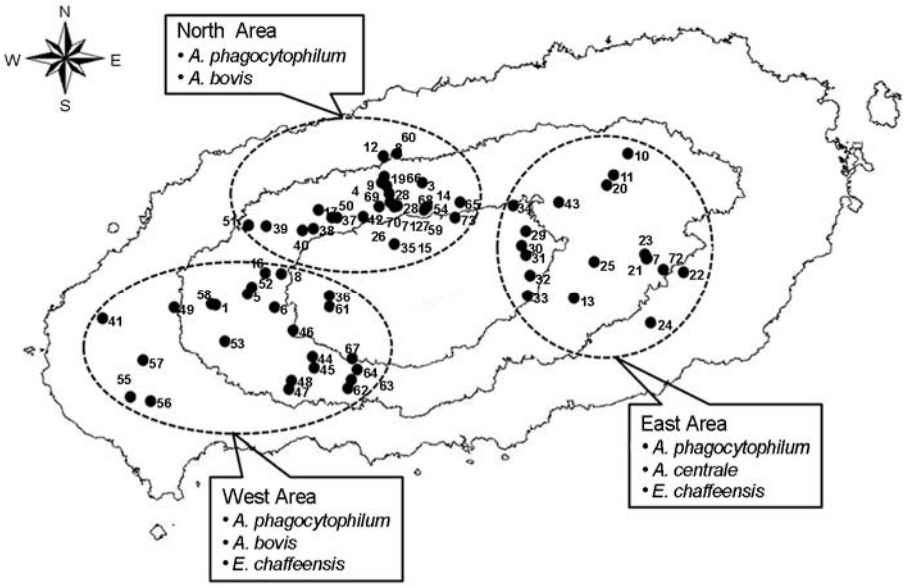

Figure 1. Map of Jeju Island, Korea. Seventy-two tick collection sites indicated by black dots are classified into three areas (East, West, and North) and tick-borne pathogens identified in Jeju Island are indicated by squares.

PCR reaction mixture was performed using conditions mentioned above. Briefly, the PCR amplification was performed with an initial denaturation at 94℃ for 5 min, followed by 40 cycles consisting of denaturation 94℃ for 1 min, 1 min of annealing at 59℃, and 2 min of extension at 72℃. To determine the prevalence of *Anaplasma*/*Ehrlichia* genomic species, nested PCR was performed using 1 μl of corresponding primary products (16S rRNA PCR amplicons) as templates. Species-specific primer pairs for PCR were as follows: *A. phagocytophilum* primers AP-F (5'-GTCGAACGGATTATTCTTTATAGCTTGC-3') and AP-R (5'-CCCTTCCGTTAAGAAGGATCTAATCTCC-3') and *E. chaffeensis* primers EC-F (5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3') and EC-R (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') according to the method described by Kim *et al* (10). The annealing for second PCR was performed at 56℃. The genomic DNA samples extracted from *A. phagocytophilum* provided by J. Stephen Dumler (Johns Hopkins University School of Medicine, Baltimore, MD, USA) and *E. chaffeensis* Arkansas strain provided by J. Dawson (CDC, Atlanta, GA, USA) were used as a positive control for detecting tick-borne pathogens *Anaplasma* and *Ehrlichia* species, respectively.

Cloning, sequencing analysis, and phylogenetic analysis

The ~1,406 bp of 16S rRNA PCR products, ~926 bp *A. phagocytophilum* nested PCR products and ~390 bp *E. chaffeensis* nested PCR products were eluted from the agarose gel using the Qiagen gel extraction kit (Qiagen), and cloned into the PCR cloning vector pGEM-T easy vector (Promega Co., Madison, WI, USA) and were transformed into competent *Escherichia coli* JM109 cells; *E. coli* containing recombinant plasmids were extracted using the SV Minipreps DNA purification system (Promega) and identified by PCR amplification. The sequence analysis of the purified recombinant plasmid DNA was performed with T7 and SP6 promoter primer set using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data were analyzed using Chromas software (Ver 2.33, http://www.technelysium.com.au/chromas.html) and were aligned using CLUSTAL X (Ver 2.0, http://www.clustal.org/). Aligned sequences were examined with a similarity matrix. To access the relationships between individual pathogens, the phylogenetic tree was constructed by the UPGMA (unweighted pair group method with arithmetic mean) method with 1,000 bootstrap resamplings using MEGA software (http://www.megasoftware.net/).

Nucleotide sequence accession numbers

The GenBank accession numbers of 16S rRNA gene sequences and specific genospecies sequences related to *Anaplasma*/*Ehrlichia* pathogens for sequence comparisons

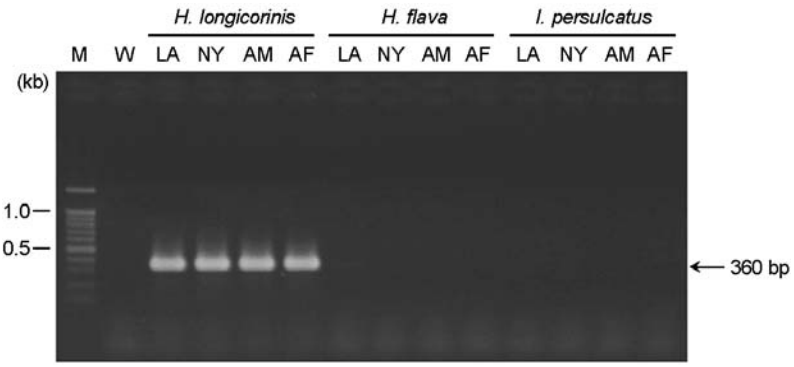

Figure 2. Agarose gel electrophoresis of *H. longicornis* tick 5.8S rRNA internal transcribed spacer 2 gene (360 bp amplicons). Lane M, 100 base pair size marker. Abbreviation of each lane is as follows: W, water control; LA, larva; NY, nymph; AM, adult male; AF, adult female.

were as follows; *Anaplasma* sp. 201-3 from Japan (EU-368723), *Anaplasma* sp. NS108 from Japan (AB454076), *Anaplasma* sp. from China (FJ69956), *A. phagocytophilum* from USA (AY055469), *A. phagocytophilum* from USA (AY055469), *A. phagocytophilum* from UK (AY082656), *A. phagocytophilum* from Sweden (AY527213), *A. phagocytophilum* from Korea (AF470699), *A. phagocytophilum* from Korea (AF470701), *A. platys* from Japan (AY077619), *A. bovis* from Korea (AF470698), *A. bovis* from Japan (AB196475), *A. centrale* from USA (AB211164), *A. centrale* from The Netherlands (AF318944), *A. centrale* from France (AF283007), *A. ovis* from China (EF587237), *A. ovis* from The Netherlands (AF318945), *A. marginale* from Japan (FJ226454), *A. marginale* from China (AJ633048), *A. marginale* from Australia (AF414874), *A. marginale* from USA (AF309867), *Ehrlichia* sp. FN147 from Japan (AB-196303), *Ehrlichia* sp. from China (DQ324547), *Ehrlichia* sp. HF565 from Japan (AB024928), *Ehrlichia* sp. from Japan (AB028319), *E. canis* from USA (M73226), *E. canis* from Spain (AY394465), *E. chaffeensis* from Korea (AY-350424), *E. chaffeensis* 0214 form Korea (DQ402484), *E. chaffeensis* from China (AF147752), *E. chaffeensis* from USA (M73222), *Candidatus Ehrlichia shimanensis* from Japan (AB074459), *E. ewingii* from USA (AY093440), and *E. ovina* from The Netherlands (AF318946) (Fig. 3~5). In the present study, the 22 unique DNA sequences of *Anaplasma* and *Ehrlichia* identified from *H. longicornis* ticks were deposited in GenBank under the accession numbers (GU046563~GU046565, GU064895~GU064903, and GU075695~GU075704) (Fig. 3~5).

RESULTS

Identification and prevalence of the tick

All ticks collected from Jeju Island were identified as *H. longicornis* by their morphological patterns. As a result, of the total 1,395 ticks (506 pools) from 72 sampling sites in three survey areas (East, West, and North); nymph was the most frequently collected, which account for 1,043 ticks (74.8%), followed by 200 female adult ticks (14.3%), 149 male adult ticks (10.7%), and 3 larva ticks (0.2%). A total of 506 tick pools were first detected by PCR using specific primer sets for amplifying the 360 bp fragment of the *H. longicornis* 5.8S rRNA ITS2 gene and compared with the classified other genus ticks, *H. flava* and *I. persulcatus* (Fig. 2).

Prevalence and regional distribution of tick-borne pathogens by species-specific PCR

Both *Anaplasma* and *Ehrlichia* 16S rRNA genes associated with genus and genomic species were detected by conventional PCR and species-specific nested PCR assay with *H. longicornis* tick DNAs collected from Jeju Island, Korea. First, we amplified a total of 506 tick pool DNA extracts (total 1,395 ticks) by conventional PCR using 16S rRNA primer pairs for genus *Anaplasma*/*Ehrlichia* and as a result, fifty-one *Anaplasma*/*Ehrlichia* related pathogens were evaluated. Of these pathogens, fourteen 16S rRNA gene PCR amplicons, which were positives for 8 (0.6%) *Anaplasma* species and 6 (0.4%) *Ehrlichia* species by analyzing the sequence alignments based on the GenBank

Table 1. Prevalence of total ticks (number of pools) and tick-borne pathogens identified from the three main survey areas during 2007 and 2008 in Jeju Island, Korea

Survey areas (n=72[a])	Stages	No. of ticks (no. of pools[b])	No. of PCR-positive samples (%[c])					
			Anaplasma spp.	*Ehrlichia* spp.	*A. phagocytophilum*	*A. bovis*	*A. centrale*	*E. chaffeensis*
East (n=23)	Larva	3 (1)	0	0	0	0	0	0
	Nymph	342 (49)	5 (1.5)	1 (0.3)	3 (0.9)	0	0	1 (0.3)
	Male	70 (70)	1 (1.4)	0	2 (2.9)	0	1 (1.4)	1 (1.4)
	Female	79 (79)	0	0	1 (1.3)	0	0	1 (1.3)
	Subtotal	494 (199)	6 (1.2)	1 (0.2)	6 (1.2)	0	1 (0.2)	3 (0.6)
West (n=25)	Nymph	473 (70)	1 (0.2)	1 (0.2)	6 (1.3)	0	0	1 (0.2)
	Male	51 (51)	1 (2.0)	0	6 (11.8)	1 (2.0)	0	0
	Female	69 (69)	0	1 (1.5)	3 (4.4)	3 (4.4)	0	0
	Subtotal	593 (190)	2 (0.3)	2 (0.3)	15 (2.5)	4 (0.7)	0	1 (0.2)
North (n=24)	Nymph	228 (37)	0	0	3 (1.3)	0	0	0
	Male	28 (28)	0	0	0	0	0	0
	Female	52 (52)	0	3 (5.8)	3 (5.8)	1 (1.9)	0	0
	Subtotal	308 (117)	0	3 (1.0)	6 (2.0)	1 (0.3)	0	0
Total	Larva	3 (1)	0	0	0	0	0	0
	Nymph	1,043 (156)	6 (0.6)	2 (0.2)	12 (1.4)	0	0	2 (0.2)
	Male	149 (149)	2 (1.4)	0	8 (5.4)	1 (0.7)	1 (0.7)	1 (0.7)
	Female	200 (200)	0	4 (2.0)	7 (3.5)	4 (2.0)	0	1 (0.5)
	Total	1,395 (506)	8 (0.6)	6 (0.4)	27 (1.9)	5 (0.4)	1 (0.1)	4 (0.2)

[a] Number of collection sites from the three main survey areas of Jeju Island.
[b] Number of pools for developmental tick stages is as follows, three ticks per pool for larva; five to 7 ticks per pool for nymph; one tick per pool for male and female adults.
[c] PCR-positive pathogens are calculated by minimum infection rate (MIR). MIR = Number of positive pools/total number of ticks tested × 100

database for accession numbers (Table 1). The most commonly isolated pathogen in *H. longicornis* ticks was *A. phagocytophilum* (27 [1.9%]), followed by *A. bovis* (5 [0.4%]), *E. chaffeensis* (4 [0.2%]), and *A. centrale* (1 [0.1%]) (Table 1). The prevalence of infection with *A. phagocytophilum* according to the minimum infection rate (MIR) from the three survey areas was 15 (2.5%) in the west, followed by the north area 6 (2.0%), and east area 6 (1.2%). The MIR of *A. phagocytophilum* according to tick stage was 8 (5.4%) in the male adult ticks, followed by the female adult ticks 7 (3.5%), and nymph 12 (1.4%). By survey areas, *E. chaffeensis* was detected in the east and west area, whereas genera of *Anaplasma* were detected in all survey areas. Eight *Anaplasma* positive were 6 (0.6%) nymph pools and 2 (1.3%) male adults and six *Ehrlichia* positive were 2 (0.2%) nymph pools and 4 (2.0%) female adults, respectively. Twenty-four (1.7%) tick-borne PCR positive pathogens identified in the west area was the most prevalent, followed by the east area (17 [1.2%]), and the north area (10 [0.7%]). *A. bovis* and *E. chaffeensis* were mostly found in the west (4 [0.3%]) and in the east (3 [0.2%]), respectively.

Phylogenetic analysis of *Anaplasma* and *Ehrlichia* species

To identify the *Anaplasma* and *Ehrlichia* species detected in ticks, nucleotide sequences of fourteen PCR amplified products were analyzed and compared with the fragment of

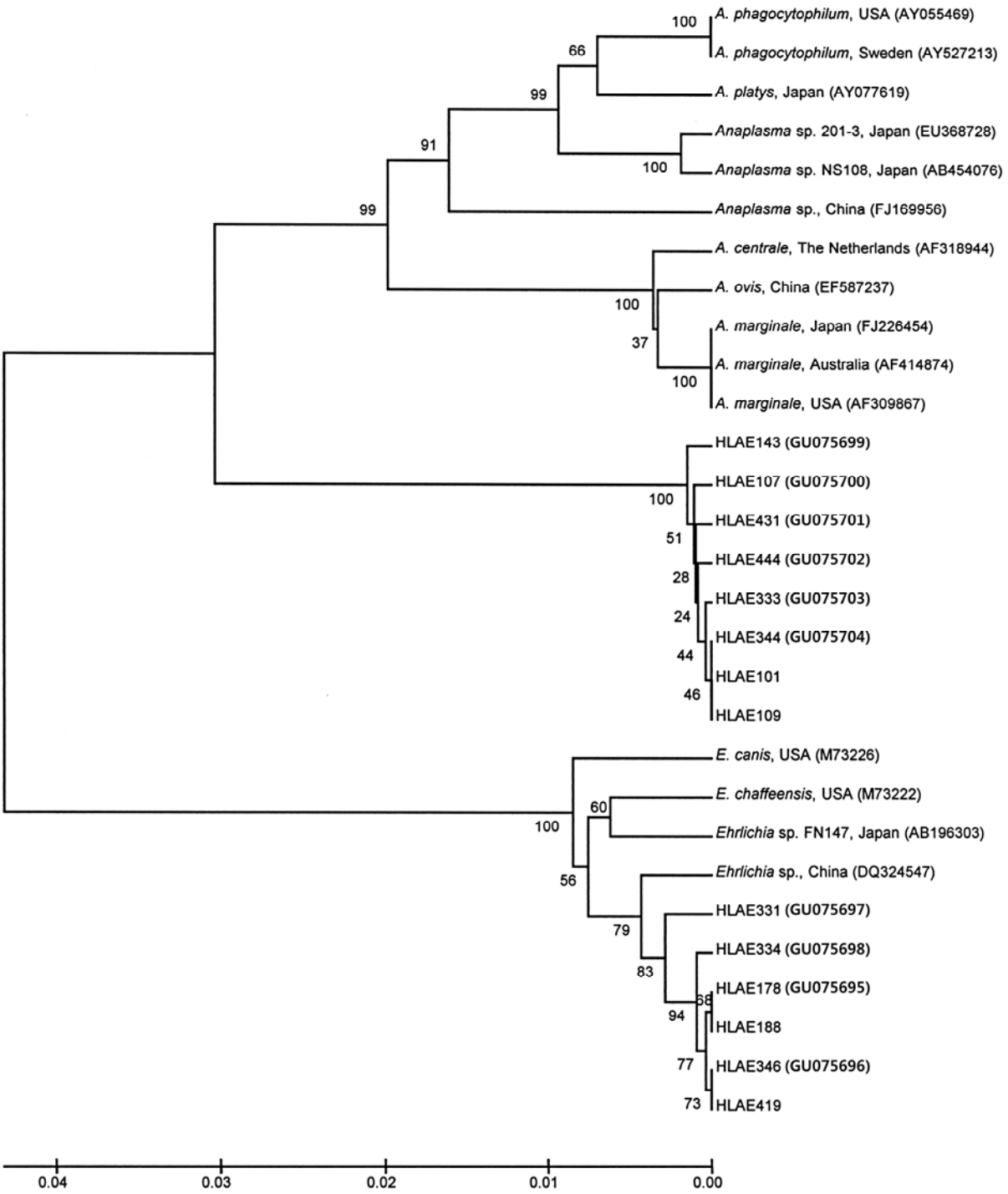

Figure 3. Phylogenetic tree based on 1,406 bp sequences of *Anaplasma* and *Ehrlichia* species collected in Jeju Island of Korea. The phylogenetic tree was constructed based on the alignment of 16S rRNA gene sequences by CLUSTAL W and followed by the unweighted pair group method with arithmetic mean (UPGMA) method with 1,000 bootstrap resamplings using MEGA software. The GenBank accession numbers are in parentheses.

16S rRNA sequences and its internal sequences of various *Anaplasma* and *Ehrlichia* species obtained from the GenBank database. Through phylogenetic analysis, the 16S rRNA gene sequences were divided into two high homolo-

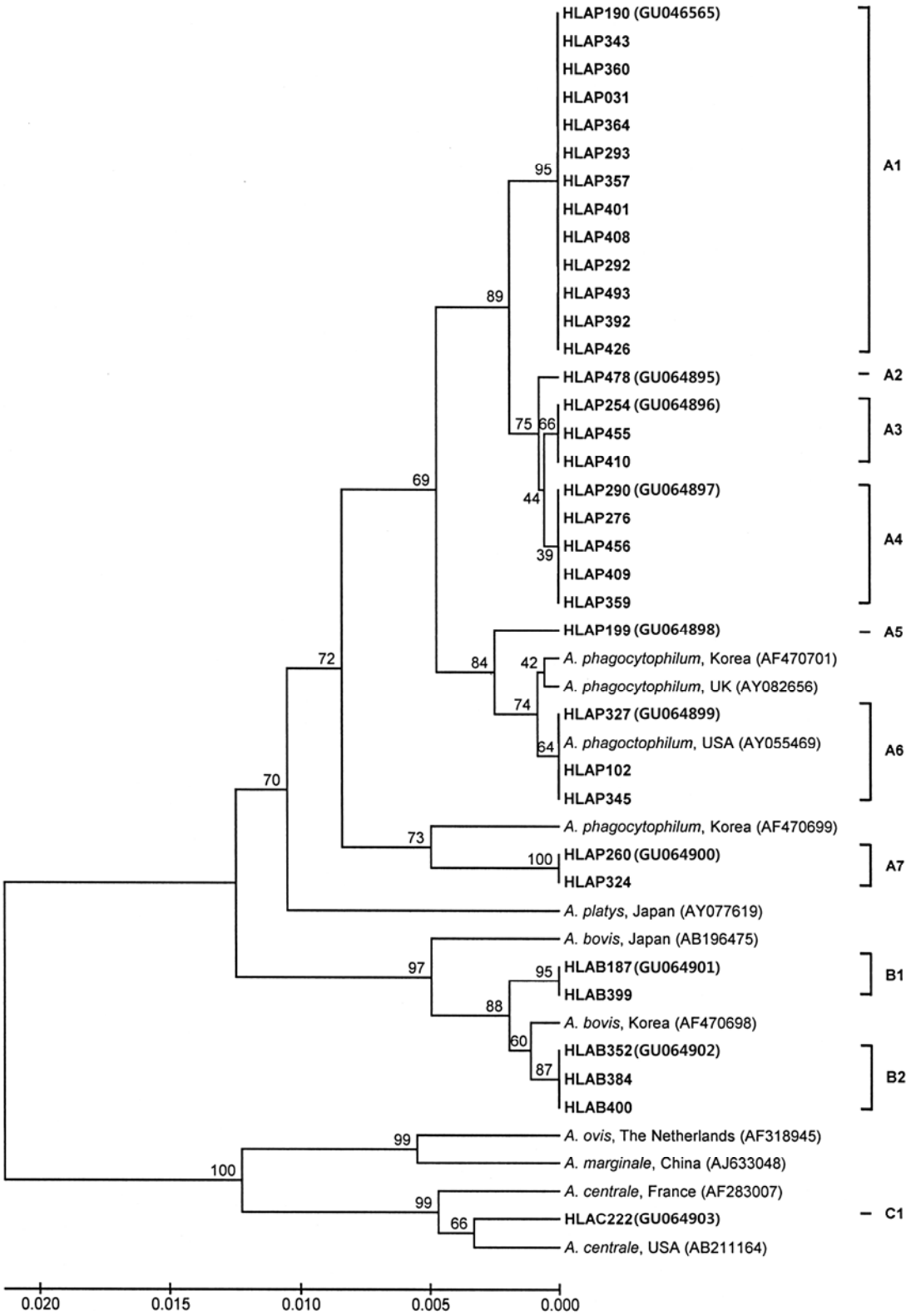

Figure 4. Phylogenetic tree based on 926 bp gene sequence of *A. phagocytophilum*, *A. bovis*, and *A. centrale*. The phylogenetic tree was constructed based on the alignment of *Anaplasma* gene sequences obtained from species-specific nested PCR assay, by CLUSTAL W and followed by the UPGMA method with 1,000 bootstrap resamplings using MEGA software.

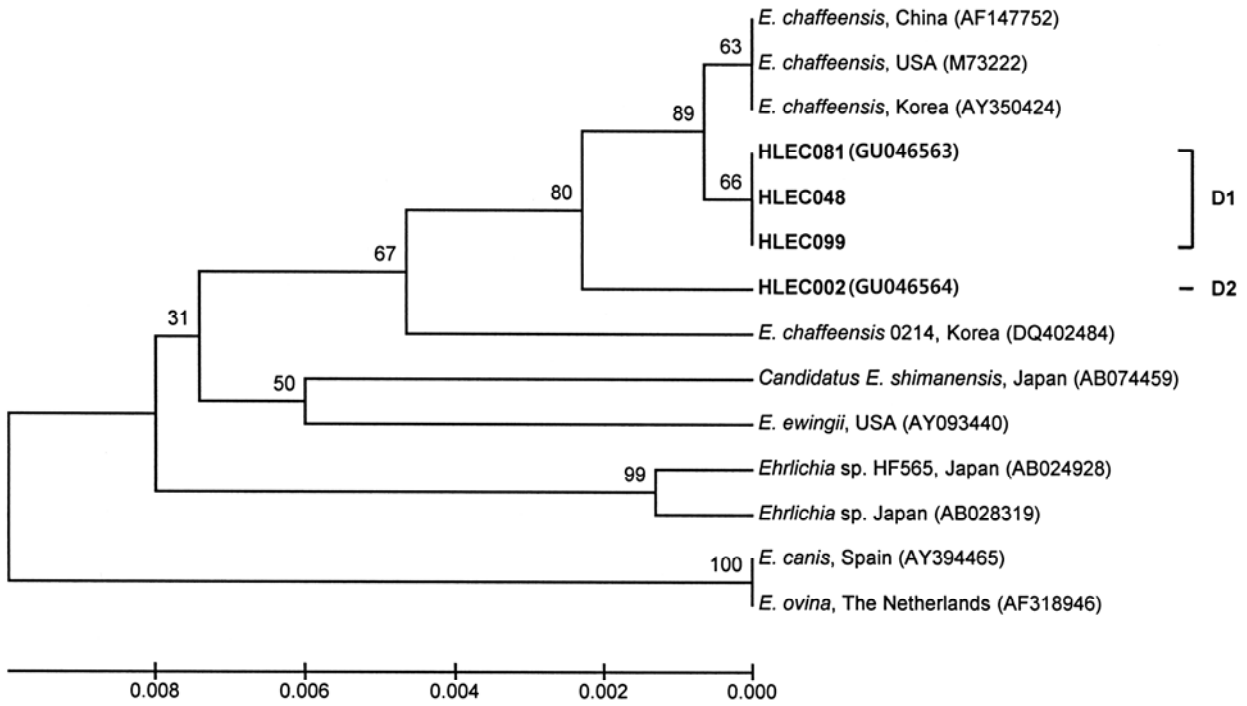

Figure 5. Phylogenetic tree based on 390 bp sequence of *E. chaffeensis*. The phylogenetic tree was constructed based on the alignment of *Ehrlichia* gene obtained from species-specific nested PCR assay, by CLUSTAL W and followed by the UPGMA method with 1,000 bootstrap resamplings using MEGA software.

gous groups that were identified as 8 *Anaplasma* sp. and 6 *Ehrlichia* sp. by comparing those of other gene sequences of *Anaplasma* and *Ehrlichia* species available in GenBank database (Fig. 3). The sequence similarity among 8 *Anaplasma* group ranged from 99.4% to 99.7% and was very similar to *A. marginale* (FJ226454, AF414874, and AF-309867), which ranged from 93.9% to 94.3. The similarity level among 6 *Ehrlichia* group ranged from 99.5% to 99.9% and was homologous to *Ehrlichia* sp. from China (DQ-324547) ranged from 98.8% to 99.0% (Fig. 3).

Fig. 4 shows that the phylogenetic tree of the 3 *Anaplasma* species groups, including 28 *A. phagocytophilum* (group A), 5 *A. bovis* (group B), and 1 *A. centrale* (genotype C), identified by *A. phagocytophilum* specific PCR amplicons and compared with *Anaplasma* related species available in GenBank. Group A belongs to the 28 *A. phagocytophilum* clones which ranged from 97.9% to 98.4% and divided into 7 genotypes by the sequence similarity. In case of genotype A6 belonging to the three clones showed identical sequences (100%) to known *A. phagocytophilum* originated from USA (AY055469). The homogeneity among four genotypes A1, A2, A3, and A4 was high (> 99.6%). Genotype A7 including two clones was the most similar to *A. phagocytophilum* from Korea (AF470699) with 98.9% homology. The sequence similarity between the two genotypes (B1 and B2) classifying to *A. bovis* was 99.4% and these were highly similar with 99.1% and 99.7% similarity to known *A. bovis* sequences from Japan (AB196475) and from Korea (AF470698), respectively. Genotype C (HLAC222) belonging to one *A. centrale* showed 99.2% homology to known *A. centrale* (AB211164, USA) deposited in GenBank (Fig. 4). The phylogenetic tree among *E. chaffeensis* clones is shown in Fig. 5. Two genotypes D1 and D2 were identified as partial sequence of *E. chaffeensis* 16S rRNA gene by sequence comparison. D1 and D2 showed 99.7% and 99.2% homology to known *E. chaffeensis* sequences (AY350424, M73222, and AF-147752) from GenBank, respectively (Fig. 5).

DISCUSSION

Until now, the most prevalent tick species associated to tick-borne pathogens are *Haemaphysalis* spp. followed by *Ixodes* spp. throughout Korea. Especially, *H. longicornis* tick among *Haemaphysalis* spp. is known to be the most commonly collected species in previous reports (10, 13). The present study revealed one tick species *H. longicornis* in 72 collection sites from Jeju Island despite the emergence of several tick spp. from Korea. It is speculated that the geographical distribution of only one tick species collected is thought to be due to natural environment of forest, river, shrub-undergrowth, and the particular climate different from the inland area of Korea.

The methods for the identification of tick-borne pathogens in ticks have been used by real-time quantitative RT-PCR (TaqMan) or nested PCR. However, 16S rRNA genes from ticks and rodents had not been easily amplified in the previous Korean reports. Previous PCR primers ECC and ECB described by Kim *et al* (10) were used to amplify primary segment of the 16S rRNA using template DNA of nested PCR for detection of *Anaplasma*/*Ehrlichia*-related genospecies groups. In the present study we could not find exact coincide with the numbers of 16S rRNA gene positive compared with the numbers of detected *Anaplasma*/*Ehrlichia* genospecies by nested PCR. Although TaqMan PCR method (10) for detecting tick-borne pathogens revealed a high specificity in screening *Anaplasma*/*Ehrlichia* 16S rRNA genes, Kim *et al* (10) did not yet confirm their sequence analysis in domestic ticks. However, the PCR detection and sequence analysis for genus *Anaplasma* and *Ehrlichia* genes in our results were successfully identified with modified primer pairs. The majority of *Anaplasma* and *Ehrlichia* 16S rRNA PCR amplicons were detected on gel electrophoresis but several negatives of those of positive amplicons also amplified in the nested PCR for genospecies identification. The difference for these PCR results is probably thought to be either due to a variation in the pathogen-related DNA quantities because of the size of each tick stage or a variation in the number of tick pools during genomic DNA preparation. Both *Anaplasma* and *Ehrlichia* 16S rRNA sequences analyzed from *H. longicornis* ticks were highly homologous among their groups and were somewhat different compared with those of other countries Japan, China, USA, and Europe. Therefore, genus *Anaplasma*/*Ehrlichia* spp. identified in *H. longicornis* ticks can explain the native tick-borne pathogens in Jeju Island of Korea.

Ticks of genera *Haemaphysalis* and *Ixodes* collected from Korea were found to be infected with several tick-borne pathogens tested (*A. phagocytophilum*, *A. platys*, *E. chaffeensis*, *E. ewingii*, and *E. canis*) (13, 18, 19). To the best of our knowledge, four tick-borne pathogens *A. phagocytophilu*m, *A. bovis*, *A. centrale*, and *E. chaffeensis* were identified in Jeju Island of Korea. Especially, tick-borne pathogens infected with *A. phagocytophilum* were ubiquitous or common among the collection sites and also emerged in all tick stage except for larva, in agreement with previous data that some *A. phagocytophilum* strains were detected in *H. longicornis* ticks collected in Jeju Island of Korea (13). Thus, *H. longicornis* may be an epidemiologically important vector tick related to *A. phagocytophilum* infection found in this area.

E. chaffeensis, *E. canis*, and *E. ewingii* are etiologic agents of canine ehrlichiosis and HME. *E. chaffeensis* has been mostly detected in *H. longicornis* ticks collected from the inland areas of Korea, such as Seoul, Gyeonggi province, and Demilitarized zone (DMZ) (19, 20). However, *E. chaffeensis* infection has been rarely reported in human, pet animal and other tick species until recently (4, 15). As a result, in contrast to the most predominant *A. phagocytophilum* genotypes, *E. chaffeensis* pathogens identified from Jeju Island were also not common consistent with previous studies mentioned above.

A. bovis infection is usually associated with some symptoms including fever, lymphadenopathy, depression, and loss of conditioning from the ruminants such as cattle (21). In this study five *A. bovis* were identified by the nested PCR and sequencing analysis for detection of *A. phagocytophilum* from 506 tick pools. In Korea, one *A. bovis*-like organism analyzed by *A. phagocytophilum* specific PCR have been reported previously in *H. longicornis* tick

from Gyeonggi province (20). Recently, *A. bovis* was first found in the cattle blood of Yonaguni Island, Okinawa, Japan in 2006 (21), since those of the pathogen infected with *A. bovis* detected from deer on the Hokkaido and Honshu Island, Japan, collected during 2001 and 2002 (16). Although it has not provided epidemiologically indisputable evidences and those of pathogen were found in each different local area, ticks pathogens infected might be disseminated by tick-mediated transmission between mammalian hosts or by migration between regions from climate change.

A. bovis are widely distributed in Jeju Island even though they were poor in the present study. Consequently, tick-borne pathogens classified into similar or identical genotype groups by the sequence alignment and phylogenetic analysis in this study would be either widespread or infected to human and animals through their life cycle and disease transmission between autochthonous *H. longicornis* ticks infected with these obligate intracellular etiological agents in the nature.

Our results suggest that the genetic diversity of genus *Anaplasma*/*Ehrlichia* and their genospecies present in Jeju Island of Korea. Consequently, it is required to compare the epidemiological characterization based on the 16S rRNA sequence analysis and phylogenetic analysis of *E. chaffeensis* agents identified in the human and wild animals living in Jeju Island and moreover, it is necessary to clarify the pathogenicity factors of *A. phagocytophilum*, *A. bovis*, *A. centrale*, and *E. chaffeensis* detected in this study to both human and domestic animals in Korea.

REFERENCES

1) Dumler JS, Bakken JS. Ehrlichial diseases of humans: emerging tick-borne infection. Clin Infect Dis 1995;20: 1102-10.
2) Murphy GL, Ewing SA, Whitworth LC, Fox JC, Kocan AA. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet Parasitol 1998;79:325-39.
3) Inokuma H, Ohno K, Onishi T, Raoult D, Brouqui P. Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. J Vet Med Sci 2001;63:815-7.
4) Heo EJ, Park JH, Koo JR, Park MS, Park MY, Dumler JS, Chae JS. Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. J Clin Microbiol 2002;40:3082-5.
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 centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major Islands in Japan. Appl Environ Microbiol 2006;72:1102-9.
6) Bakken JS, Dumler JS. Human granulocytic ehrlichiosis. Clin Infect Dis 2000;31:554-60.
7) Dumler JS, Choi KS, Garcia-Garcia JC, Barat NS, Scorpio DG, Garyu JW, Grab DJ, Bakken JS. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. Emerg Infect Dis 2005;11:1828-34.
8) Uchida T, Yan Y, Kitaoka S. Detection of *Rickettsia japonica* in *Haemaphysalis longicornis* ticks by restriction fragment length polymorphism of PCR product. J Clin Microbiol 1995;33:824-8.
9) Shimada Y, Inokuma H, Beppu T, Okuda M, Onishi T. Survey of *Ixodid* tick species on domestic cats in Japan. Vet Parasitol 2003;111:231-9.
10) Kim CM, Yi YH, Yu DH, Lee MJ, Cho MR, Desai AR, Shringi S, Klein TA, Kim HC, Song JW, Baek LJ, Chong ST, O'Guinn ML, Lee JS, Lee IY, Park JH, Foley J, Chae JS. Tick-borne rickettsial pathogens in ticks and small mammals in Korea. Appl Environ Microbiol 2006;72:5766-76.
11) Sun J, Liu Q, Lu L, Ding G, Guo J, Fu G, Zhang J, Meng F, Wu H, Song X, Ren D, Li D, Guo Y, Wang J, Li G, Liu J, Lin H. Coinfection with four genera of bacteria (*Borrelia*, *Bartonella*, *Anaplasma*, and *Ehrlichia*) in *Haemaphysalis longicornis* and Ixodes sinensis ticks from China. Vector Borne Zoonotic Dis 2008;8:791-5.
12) Jilintai, Seino N, Hayakawa D, Suzuki M, Hata H, Kondo S, Matsumoto K, Yokoyama N, Inokuma H. Molecular survey for *Anaplasma bovis* and *Anaplasma phagocytophilum* infection in cattle in a pastureland

where sika deer appear in Hokkaido, Japan. Jpn J Infect Dis 2009;62:73-5.

13) Kim CM, Kim MS, Park MS, Park JH, Chae JS. Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. Vector Borne Zoonotic Dis 2003;3:17-26.
14) Lee JH, Park HS, Jung KD, Jang WJ, Koh SE, Kang SS, Lee IY, Lee WJ, Kim BJ, Kook YH, Park KH, Lee SH. Identification of the spotted fever group rickettsiae detected from *Haemaphysalis longicornis* in Korea. Microbiol Immunol 2003;47:301-4.
15) Yu DH, Li YH, Yoon JS, Lee JH, Lee MJ, Yu IJ, Chae JS, Park JH. *Ehrlichia chaffeensis* infection in dogs in South Korea. Vector Borne Zoonotic Dis 2008;8:355-8.
16) 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 centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Appl Environ Microbiol 2006; 72:1102-9.
17) Yamaguti N, Tipton VJ, Keegan H, Toshioka S. Ticks of Japan, Korea, and the Ryukyu islands. Brigham Young Univ Sci Bull Biol Ser 1971;15:1-126.
18) Chae JS, Kim CM, Kim EH, Hur EJ, Klein TA, Kang TK, Lee HC, Song JW. Molecular epidemiological study for tick-borne disease (*Ehrlichia* and *Anaplasm*a spp.) surveillance at selected U.S. military training sites/installations in Korea. Ann N Y Acad Sci 2003; 990:118-25.
19) Lee SO, Na DK, Kim CM, Li YH, Cho YH, Park JH, Lee JH, Eo SK, Klein TA, Chae JS. Identification and prevalence of *Ehrlichia chaffeensis* infection in *Haemaphysalis longicornis* ticks from Korea by PCR, sequencing and phylogenetic analysis based on 16S rRNA gene. J Vet Sci 2005;6:151-5.
20) Chae JS, Yu do H, Shringi S, Klein TA, Kim HC, Chong ST, Lee IY, Foley J. Microbial pathogens in ticks, rodents and a shrew in northern Gyeonggi-do near the DMZ, Korea. J Vet Sci 2008;9:285-93.
21) Ooshiro M, Zakimi S, Matsukawa Y, Katagiri Y, Inokuma H. Detection of *Anaplasma bovis* and *Anaplasma phagocytophilum* from cattle on Yonaguni Island, Okinawa, Japan. Vet Parasitol 2008;154:360-4.