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Microbial pathogens in ticks, rodents and a shrew in northern Gyeonggi-do near the DMZ, Korea

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A total of 1,618 ticks [420 individual (adults) and pooled (larvae and nymphs) samples], 369 rodents (*Apodemus agrarius*, *Rattus norvegicus*, *Tscherskia triton*, *Mus musculus*, and *Myodes regulus*), and 34 shrews (*Crocidura lasiura*) that were collected in northern Gyeonggi-do near the Demilitarized Zone (DMZ) of Korea during 2004-2005, were assayed by PCR for selected zoonotic pathogens. From a total of 420 individual and pooled tick DNA samples, *Anaplasma (A.) phagocytophilum* (16), *A. platys* (16), *Ehrlichia (E.) chaffeensis* (63), *Borrelia burgdorferi* (16), and *Rickettsia* spp. (198) were detected using species-specific PCR assays. Out of 403 spleens from rodents and shrews, *A. phagocytophilum* (20), *A. platys* (34), *E. chaffeensis* (127), and *Bartonella* spp. (24) were detected with species-specific PCR assays. These results suggest that fevers of unknown causes in humans and animals in Korea should be evaluated for infections by these vector-borne microbial pathogens.

Keywords: Bartonella, Borrelia, Rickettsia, rodents, *Crocidura lasiura*, tick-borne pathogens

Introduction

Korea is a northeast Asian peninsular country with four clearly demarked seasons. Seventy percent of the land area is mountainous, with interspersed fertile river valleys. Ticks are commonly collected during the early spring through late autumn, while are few ticks are collected during the cold winter season. Many wild animals inhabit the Demilitarized Zone (DMZ) and the area adjacent to it, and these animals are hosts to ticks and serve as reservoirs for tick-borne pathogens [17]. The Korean and US military have numerous small to large training sites near the DMZ where large populations of small mammals (rodents and insectivores) and occasional deer, wild pigs, and other small mammals are found [32]. Additionally, tourist activity is expected to increase in the area in the near future, which may increase the risk of human exposure to ticks and the pathogens they harbor [5,16].

Ectoparasites (e.g. ticks and fleas) are vectors of a number of pathogens that are important to humans and also veterinary practice. Ticks are harmful ectoparasites that directly or indirectly cause a variety of disease states in their host. Ticks are known vectors of protozoa, rickettsiae, bacteria, and viruses, that may cause serious and life-threatening illnesses in human. Screening ticks for disease-causing pathogens using molecular epidemiological tools provides useful data on the distribution and prevalence of tick-borne pathogens. Moreover, with increases in the mean global annual temperatures of 1°C since the 1880s [10], it is predicted that the temperate Korean climate may be altered to a subtropical climate. These environmental changes may potentially alter the distribution of wild animals and the arthropod vectors and the pathogens they transmit. Tick-borne encephalitis was previously thought to not exist in Korea, but recent evidence from molecular testing of ticks and rodents suggests that it is present in Korea [19]. Many of the pathogenic agents transmitted by ticks, including *Ehrlichia* spp., *Anaplasma* spp., Borrelia spp., *Bartonella* spp., and *Rickettsia* spp., are known to be human and animal pathogens worldwide [8,20,29].

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Recent seroepidemiological findings documented the presence of human monocytic ehrlichiosis and human granulocytic anaplasmosis in Korea [11,26]. Molecular evidence of *Ehrlichia* and *Anaplasma* spp. was identified in ticks collected from animals and grass vegetation in Korea [17,21]. Additionally, a spotted fever group Rickettsia, similar to *Rickettsia* (*R.*) *japonica*, was identified in *Haemaphysalis* (*H.*) *longicornis* ticks by PCR, and antibodies to these organisms were detected in human patients with acute febrile illness [14].

The United States Forces Korea rodent- and tick-borne disease surveillance program was initiated to provide ecological and epidemiological information on potential risks of infection for personal who occupy or train in various environments near the DMZ. This is especially important when considering recent serological evidence that confirmed the presence of *Ehrlichia* (*E.*) *chaffeensis* and *Anaplasma* (*A.*) *phagocytophilum* [11,26].

The purpose for this study was to identify vector-borne pathogens in ticks, rodents and shrews in order to provide more accurate risk assessment of tick-borne pathogens that may affect human and animal health in Korea.

Materials and Methods

Study sites

Ticks were collected in the field by dragging and flagging grass vegetation and forested ground cover (fallen leaves, clumps of grasses and scattered shrubs). Ticks also were removed from various wild rodents (*Apodemus agrarius*, *Rattus norvegicus*, *Tscherskia triton*, *Mus musculus*, and *Myodes regulus*) and a shrew (*Crocidura lasiura*) that were live-trapped at US military installations and training sites in northern Gyeonggi-do near the DMZ (Fig. 1).

Tick collections

During March through September 2004, a total of 1,618 ticks were collected from grass vegetation and forest leaf litter (933 ticks) and wild rodents (685 ticks) at 17 sites (Fig. 1). Based on microscopic examination, ticks were identified to species and developmental stage characterized. Adult ticks were stored and assayed individually, while the nymphs and larvae were pooled (1-6 and 1-30 ticks per pool, respectively) into 420 sample pools (62 from wild rodents and 358 from grass vegetation and forest leaf litter) and stored at -70°C until they were assayed.

Tissue samples

A total of 403 small mammals (369 wild rodents and 34 shrews) belonging to six species, six different genera, and two families were live captured at US military installations and training sites in northern Gyeonggi-do near the DMZ in Korea from August 2004 through June 2005 using Sherman traps (3" × 5" × 9" folding traps; H.B. Sherman Traps, USA). The live-caught rodents and shrews were transported to Korea University where they were euthanized in accordance with the Korea University animal use protocol, their abdominal cavities opened aseptically, and spleen samples collected and stored individually at -70°C until assayed.

DNA extraction

DNA was extracted from pools of larvae, nymphs and individual adult ticks. A total of 747 and 174 nymphs were collected by tick drag/flag and from rodents and a shrew, respectively, and these were placed in 215 pools according to collection site, while DNA was extracted from 186 individual adult ticks (76 males and 110 females) and 19 pools of larvae with using DNeasy tissue kits (Qiagen, Germany) (Table 1). Individual ticks and pools of ticks were mechanically homogenized using sterile scissors and a manual homogenizer (General Biosystem, Korea). DNA extraction was performed using DNeasy tissue kits (Qiagen, Germany) in accordance with instructions provided by the manufacturer.

Detection of tick-borne pathogens by PCR

Purified DNA was used for the detection of tick-borne pathogens using conventional and nested PCR [16]. PCR assays using genomic DNAs and species-specific primers, as previously described, were used to identify selected zoonotic pathogens [18].

Nested PCR: The nested PCR technique was used for the detection of *A. phagocytophilum* by amplifying a 926 bp fragment of *A. phagocytophilum*-specific 16S rRNA gene in a total volume of 25 μl as previously described [4]. Species-specific primers for *A. platys*, *E. chaffeensis*, *E. ewingii*, and *E. canis* were used in the nested PCR assays [23,24]. The primers ECC and ECB were used to amplify

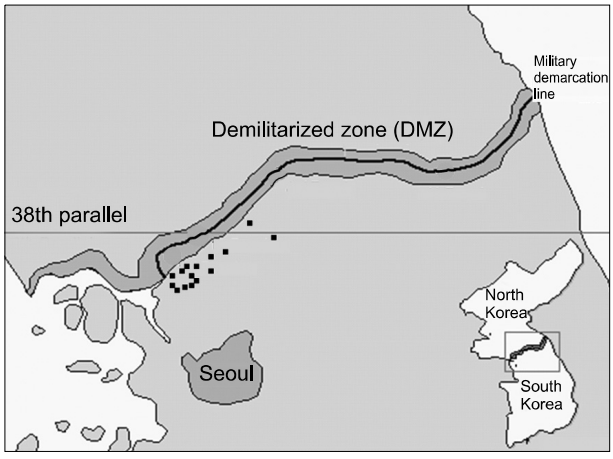

Fig. 1. Collection sites were conducted in northern Gyeonggi-do near the Demilitarized Zone of Korea. The small black squares indicate sample collection sites.

Table 1. The total number of ticks and the number of individuals (adults) and pools (larvae and nymphs) assayed, and the number of pools PCR positive by stage and gender (adults) for selected rickettsial pathogens

Species	Stages	No. ticks	No. pools[†]	No. of PCR positive (%*)									
				A. phagocytophilum	*A. platys*	*E. canis*	*E. chaffeensis*	*E. ewingii*	*E. muris*	*Bartonella* spp.	*Borrelia burgdorferi*	*Rickettsia japonica*	*Rickettsia* spp.[‡]
Haemaphysalis longicornis	Nymph	421	92	1 (0.2)	5 (1.2)	0	13 (3.1)	0	0	0	0	0	68 (16.2)
	Male	52	52	1 (1.9)	1 (1.9)	0	16 (30.8)	0	0	0	0	0	38 (73.1)
	Female	97	97	4 (4.1)	1 (1.0)	0	26 (26.8)	0	0	0	0	0	35 (36.1)
	Subtotal	570	241	6 (1.1)	7 (1.2)	0	55 (9.6)	0	0	0	0	0	141 (24.7)
Haemaphysalis flava	Nymph	276	62	0	1 (0.4)	0	6 (2.2)	0	0	0	0	0	15 (5.4)
	Male	19	19	0	2 (10.5)	0	0	0	0	0	0	0	0
	Female	11	11	0	2 (18.2)	0	0	0	0	0	0	0	0
	Subtotal	306	92	0	5 (1.6)	0	6 (2.0)	0	0	0	0	0	15 (4.9)
Ixodes nipponensis	Larvae[§]	511	19	0	1 (0.2)	0	0	0	0	0	3 (0.6)	0	8 (1.6)
	Nymph[§]	174	43	9 (5.2)	2 (1.1)	0	2 (1.1)	0	0	0	13 (7.5)	0	27 (15.5)
	Nymph	50	18	0	1 (2.0)	0	0	0	0	0	0	0	7 (14.0)
	Male	5	5	1 (20.0)	0	0	0	0	0	0	0	0	0
	Female	2	2	0	0	0	0	0	0	0	0	0	0
	Subtotal	742	87	10 (1.4)	4 (0.5)	0	2 (0.3)	0	0	0	16 (2.2)	0	42 (5.7)
Total	Larvae	511	19	0	1 (0.2)	0	0	0	0	0	3 (0.6)	0	8 (1.6)
	Nymph	921	215	10 (1.1)	9 (0.6)	0	21 (2.3)	0	0	0	13 (1.4)	0	117 (12.7)
	Male	76	76	2 (2.6)	3 (3.9)	0	16 (21.5)	0	0	0	0	0	38 (50.0)
	Female	110	110	4 (3.6)	3 (2.7)	0	26 (23.6)	0	0	0	0	0	35 (31.8)
	Total (%)	1,618	420	16 (1.0)	16 (1.0)	0	63 (3.9)	0	0	0	16 (1.0)	0	198 (12.2)

*Percent = No. of PCR positive/No. of ticks ×100. [†]Ticks were pooled in groups of 1-5 ticks (nymphs) and 2-30 ticks (larvae), and the adults were individually assayed. [‡]Spotted fever group of Rickettsia. [§]Ticks collected from small mammals. All other ticks assayed were collected by tick drags.

Table 2. The number of mixed infections observed in ticks collected from grass vegetation and forest leaf litter and small mammals

Species	Stages	*E. chaffeensis/ Rickettsia spp.*	*A. phagocytophilum/ E. chaffeensis*	*A. phagocytophilum/ Rickettsia spp.*	*Rickettsia spp./ A. platys*	*B. burgdorferi/ Rickettsia spp.*	*E. chaffeenis/ B. burgdorferi*	*A. phagocytophilum/ B. burgdorferi*	*A. platys/E. chaffeensis/ Rickettsia spp.*	*B. burgdorferi/ Rickettsia spp./A. phagocytophilum*	*A. phagocytophilum/ A. platys/ Rickettsia spp.*	Total
Haemaphysalis longicornis (n = 570/241)	Nymph (n = 421/92)	7	0	1	0	0	0	0	4	0	0	12
	Male (n = 52/52)	10	1	0	0	0	0	0	1	0	0	12
	Female (n = 97/97)	12	0	1	0	0	0	0	0	0	0	13
	Total	29	1	2	0	0	0	0	5	0	0	37
Haemaphysalis flava (n = 306/92)	Nymph (n = 276/62)	3	0	0	0	0	0	0	0	0	0	3
	Male (n = 19/19)	0	0	0	0	0	0	0	0	0	0	0
	Female (n = 11/11)	0	0	0	0	0	0	0	0	0	0	0
	Total	3	0	0	0	0	0	0	0	0	0	3
Ixodes nipponensis (n = 742/87)	Larva pools (n = 511/19)	0	0	0	1	1	0	0	0	0	0	2
	Nymph (n = 224/61)	0	2	2	2	5	2	1	0	2	1	17
	Male (n = 5/5)	0	0	0	0	0	0	0	0	0	0	0
	Female (n = 2/2)	0	0	0	0	0	0	0	0	0	0	0
	Total	0	2	2	3	6	2	1	0	2	1	19

The numbers in parenthesis are the number of ticks/the number of pooled DNAs and/or single DNAs.

all *Ehrlichia* spp. [6,7]. The primers EPLAT5 and EPLAT3 were used for *A. platys*-specific amplification [22], the primers HE1 and HE3 were used for *E. chaffeensis*-specific amplification [3], the primers EE52 and HE3 were used for *E. ewingii*-specific amplification [23], and the primers ECAN5 and HE3 were used for *E. canis*-specific amplification [23].

Conventional PCR: Identification of *Bartonella* spp., *E. muris*, *Borrelia* (*B.*) *burgdorferi*, *R. rickettsii*, and *R. japonica* was performed using conventional PCR with the species-specific primers [9,30,33]. The citrate synthase gene (*glt*A) was selected for the identification of *E. muris* [14]. The primers BhCS (781p) and BhCS (1137n) were used for *Bartonella* spp. amplification [24]. The *glt*A gene was used for the identification of *Bartonella* spp. The *osp*C gene was selected for the identification of *B. burgdorferi*. A pair of synthesized oligonucleotide primers derived from the gene sequence encoding the 190 kDa antigen of *R. rickettsii*, Rr190.70p and Rr190.602n, as described by Regnery *et al*. [30], was used for the PCR amplification of *R. rickettsii* DNA. Species-specific primers, Rj10 and Rj5, were used for the *R. japonica* 17 kDa antigen gene fragment [9]. PCR reactions were performed using 50-100 ng of template DNA, a species-specific primer set, and the PCR mixture. The PCR products were electrophoresed in 1% agarose gel; they were then stained with ethidium bromide and photographed using a still video documentation system (Gel Doc 2000; BioRad, USA).

Isolation of *Bartonella* sp.

Small mammal spleens were collected in 2 ml tubes and maintained on dry ice for transportation and subsequently used for culture isolation. The spleens were homogenized and then plated on fresh chocolate agar and allowed to incubate in 5% CO_2 at 35^{o}C for up to 4 weeks. The single colonies that grew were scraped for identification of *Bartonella* spp. The isolates were then confirmed as *Bartonella* spp. by PCR and DNA sequencing. Culture isolates were stored at -70^{o}C in frozen medium [a total of 100 ml; M199 tissue culture medium with glutamine and Earle's salts (GIBCO, USA), 1 ml of ×100 glutamine (GIBCO, USA), 1 ml of ×100 sodium pyruvate (GIBCO, USA), 20% bovine fetal calf serum (heat inactivated), and 3 ml sodium bicarbonate (7.5% solution) (GIBCO, USA), 10% DMSO, pH: 7.1-7.4] for later use.

Results

A total of 1,618 ticks from two genera and three species [570 *H. longicornis*, 306 *H. flava* and 742 *Ixodes* (*I.*) *nipponensis*] was collected from grass vegetation and forest leaf litter (933 ticks) and small mammals (685 ticks) from 2004 to 2005 near or at US military installations and training sites in northern Gyeonggi-do near the DMZ, Korea (Fig. 1, Table 1). *H. longicornis* ticks were the most frequently collected species from the grass fields. Except for one *H. flava*, all ticks taken from captured small mammals were *I. nipponensis* larvae and nymphs (Table 1).

Species-specific PCR assays were performed using DNA samples from 420 individuals and pools of ticks, and DNA samples from spleens of 403 small mammals. Five of the ten tick-borne pathogens examined in this study were detected in ticks [*A. phagocytophilum* (16, 1.0%), *A. platys* (16, 1.0%), *E. chaffeensis* (63, 3.9%), *B. burgdorferi* (16, 1.0%), and *Rickettsia* spp. (198, 12.2%)] (Table 1). At least fifty-one ticks had a mixed infection with two pathogens: *E. chaffeensis* and *Rickettsia* spp. (32 samples), *A. phagocytophilum* and *E. chaffeensis* (3 samples), *A. phagocytophilum* and *Rickettsia* spp. (4 samples), *Rickettsia* spp. and *A. platys* (3 samples), *B. burgdorferi* and *Rickettsia* spp. (6 samples), *E. chaffeensis* and *B. burgdorferi* (2 samples), and *A. phagocytophilum* and *B. burgdorferi* (1 sample) (Table 2). At least eight ticks had mixed infections with three pathogens: *A. platys*, *E. chaffeensis* and *Rickettsia* spp. (5 samples), *B. burgdorferi*, *Rickettsia* spp. and *A. phagocytophilum* (2 samples), and *A. phagocytophilum*, *A. platys* and *Rickettsia* spp. (1 sample) (Table 2).

A total of 403 small mammals were collected from US military installations and training sites in northern Gyeonggi-do near the DMZ, and these included five rodents, *Apodemus agrarius* (358), *Rattus norvegicus* (6), *Tscherskia triton* (2), *Mus musculus* (2), *Myodes regulus* (1) and a shrew, *Crocidura lasiura* (34) (Table 3). Four of the ten tick-borne pathogens examined in this study were detected by PCR in the small mammals [*A. phagocytophilum* (20, 5.0%), *A. platys* (34, 8.4%), *E. chaffeensis* (127, 31.5%) and *Bartonella* spp. (24, 6.0%)] (Table 3). *Apodemus agrarius* was PCR positive for *A. phagocytophilum*, *A. platys*, *E. chaffeensis* and *Bartonella* spp., while *Mus musculus* was only positive for *E. chaffeensis*. *Crocidura lasiura* was positive only for *A. platys* and *E. chaffeensis* (Table 3).

A total of 376 small mammals had single infections with rickettsial pathogens, while 26 *Apodemus agrarius* had mixed infections of two (23 samples), or three (3 samples) pathogens and a single *Crocidura lasiura* was positive for two pathogens (Table 4).

The frozen and homogenized samples of spleens of *Apodemus agrarius* were cultured and grew as a non-hemolytic gram-negative organism after 14 days, with only a few small white colonies. PCR amplification from the 10 isolates using *glt*A primers produced a 356 bp fragment and sequencing results were strongly suggestive of *Bartonella elizabethae* by phylogenetic analysis [17].

Table 3. Tick-borne pathogens identified by PCR from the spleens of small mammals

Species	No. of DNA	No. of PCR positive (%)									
		A. phagocytophilum	*A. platys*	*E. canis*	*E. chaffeensis*	*E. ewingii*	*E. muris*	*Bartonella* spp.	*Borrelia burgdorferi*	*Rickettsia japonica*	*Rickettsia* spp.*
Apodemus agrarius	358	20 (5.6)	22 (6.1)	0	126 (35.2)	0	0	24 (6.7)	0	0	0
Rattus norvegicus	6	0	0	0	0	0	0	0	0	0	0
Tscherskia triton	2	0	0	0	0	0	0	0	0	0	0
Mus musculus	2	0	0	0	1 (50.0)	0	0	0	0	0	0
Myodes regulus	1	0	0	0	0	0	0	0	0	0	0
Crocidura lasiura	34	0	12 (35.3)	0	9	0	0	0	0	0	0
Total (%)	403	20 (5.0)	34 (8.4)	0	127 (31.5)	0	0	24 (6.0)	0	0	0

*Spotted fever group of Rickettsia.

Table 4. The number of mixed infections observed in small mammals

Small mammals	Species	Number of mixed pathogen	Number of samples
Apodemus agrarius	*A. phagocytophilum / Bartonella* sp. / *E. chaffeensis*	3	1
	A. phagocytophilum / A. platys / Bartonella sp.		1
	A. platys / Bartonella sp. / *E. chaffeensis*		1
	Subtotal		3
	A. phagocytophilum / E. chaffeensis	2	5
	A. phagocytophilum / Bartonella sp.		1
	A. platys / E. chaffeensis		1
	A. platys / Bartonella sp.		3
	A. phagocytophilum / A. platys		2
	Bartonella sp. / E. chaffeensis		11
	Subtotal		23
Crocidura lasiura	*A. phagocytophilum/A. platys*	2	1
	Total		27

Discussion

An analysis of ticks and small mammal tissues demonstrated a high rate of infection of tick-borne pathogens in northern Gyeonggi-do near the DMZ. Most *Ehrlichia* and *Anaplasma* spp. tick-borne infections occur in *Ixodes* spp. in the US and Europe [1,31]. In Asia, *Ehrlichia* spp. was previously identified from *Haemaphysalis* spp. as well as *Ixodes* spp. [13,17,18]. *H. longicornis* are widespread throughout Korea, and especially around the pastures for grazing cattle or where deer congregate.

I. nipponensis are two-host ticks with larvae and nymphs found on rodents and a shrew. Infection rates of *Rickettsia* spp. (56.5%) and *B. burgdorferi* (25.8%) were relatively high among the selected rodents and a shrew tested. Ticks collected from grass vegetation and forest leaf litter were negative for *B. burgdorferi*, which may be a result of the small sample size of *I. nipponensis* from the "collected vegetation". In experimentally infected mice, *B. burgdorferi* DNA can be detected from the foot and lymph nodes by PCR until 55 days post-inoculation [25]. In that study, *B. burgdorferi* DNA was detected from the spleen tissues 15 days post inoculation, but not at 55 days post inoculation. Persistent infections have also been reported in the skin, blood, CSF and synovial fluid of human patients [2,25]. In the present study, *B. burgdorferi* DNA was not detected from the spleens of rodents and a shrew or the ticks, but was identified from the *I. nipponensis* removed from the small mammals. This suggests that wild rodents are a natural reservoir of *B. burgdorferi* in Korea, with *I. nipponensis* as an important vector for the larger animal hosts.

In this study, there was a very high prevalence of *Rickettsia* spp. in *H. longicornis*, *H. flava* and *I. nipponensis* ticks, but not in rodents and a shrew. Our previous studies during 2001 through 2003 detected *Rickettsia* spp. only from *H. longicornis* and *Apodemus agrarius* [18]. The PCR primer set in the previous studies targeted the *R. rickettsii rOmp*A gene [30], and we were able to sequence the amplicons. The resultant phylogenetic tree showed that Korean rickettsias were closely related to the *Rickettsia* spp. strain FUJ98 in China [18].

Additionally, these results showed that only one *Ixodes* spp. tick collected from vegetation was found infected with *A. phagocytophilum* (0.1%) [18]. In the present study, the *A. phagocytophilum* infection rate observed in rodents and a shrew tissues (5.6%) was similar to the rate of infection for *I. nipponensis* ticks collected from rodents and a shrew (5.2%), while only 1.8% of *I. nipponensis* collected from vegetation were positive for *A. phagocytophilum*.

Specific DNA of *E. canis, E. ewingii, E. muris* and *R. japonica* was not amplified in this study. There have been previous reports of the spotted fever group rickettsiosis, including *R. japonicus*, in Korean patients and ticks [15,28].

Our results demonstrate that ticks and rodents and a shrew captured near the DMZ of Korea were infected with *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, and *Rickettsia* spp. Although infections with *Ehrlichia* and *Anaplasma* spp. have generally been considered to be observed only in a defined range of hosts, including rodents and some large mammals, our studies suggest that several *Ehrlichia* and *Anaplasma* spp. can be transmitted to a variety of hosts in nature. Therefore, additional efforts to define the spectrum of host susceptibility in domestic and wild animals are needed.

H. longicornis, *H. flava* and *I. nipponensis* should be considered as potential vectors of *A. phagocytophilum*, *A. platys*, *E. chaffeensis* and *Rickettsia* spp., while *Apodemus agrarius*, *Crocidura lasiura* and *Mus musculus* may be reservoir hosts of selected tick-borne pathogens in Korea.

Until now, there have not been reports of clinical cases for *A. phagocytophilum*, *E. chaffeensis* and *B. elizabethae* in humans and animals in the Korea, as compared with the numerous reports throughout the world. For some diseases, such as rabies and malaria, there have been reported outbreaks along the DMZ [12,27]. Therefore, in the future, it will become important to perform surveillance for pathogens, including *Anaplasma*, *Ehrlichia*, *Bartonella*, *Borrelia*, and *Rickettsia* spp., in vectors and wild animals, as well as in civilian and military populations that reside or train near the DMZ. It is imperative to continue the efforts to identify additional tick-borne pathogens to further disclose the extent and possible public health significance of these agents.

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