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RESEARCH ARTICLE

Tick-borne pathogens isolated from ticks, rodents, and a shrew in Gangwon and Gyeonggi provinces in the Republic of Korea

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ABSTRACT

The prevalence of tick-borne pathogens (TBP), *Orientia tsutsugamushi, Rickettsia* and *Borrelia* spp. in wild small animals, namely wild rodents, is now widely investigated. This study is to present the prevalence and distribution of *O. tsutsugamushi*, *Rickettsia* and *Borrelia* spp. in wild small animals and ticks collected from Gyeonggi and Gangwon provinces, Republic of Korea (ROK) in 2014. A total of 131 wild small animals, rodents and shrews, and 2,954 ticks were collected from Gyeonggi and Gangwon provinces from May to November 2014. The wild small animals (KR1-9) and ticks (K1-17) were grouped in accordance with capture dates and locations. Among the wild small animals, a total of 393 tissues and blood samples were extracted from six selected small animal series (KR1–3, KR6–8). Also, each date and location-grouped ticks were identified for its species and pooled according to the stage of development. Molecular identification for *Rickettsia*, *Orientia*, and *Borrelia* species was performed using polymerase chain reaction (PCR). To detect TBPs among wild small animals and ticks, primer sets targeting the 56 kDa protein encoding gene of *Orientia* spp., outer membrane protein B gene (*OmpB*) of *Rickettsia* spp., and 5S-23S intergenic spacer region (*IGS*) gene of *Borrelia* spp. were used. Of the 393 wild small animals' blood and tissue samples, 199 (50.6%) were positive for *Orientia* spp., 158 (40.2%) were positive for *Borrelia* spp., and 55 (14.0%) were positive for *Rickettsia* spp. Moreover, a total of 14 tick pools (n = 377) was positive for *Rickettsia* spp. (n=128, 34.0%) and *Borrelia* spp. (n=33, 8.8%). High prevalence of *Orientia* spp. and *Rickettsia* spp. in rodents and shrews were observed. This study presents significant insights by presenting data collected in 2014 that the prevalence of TBP was already high in mid 2010s. This study highlights the sustainable routine surveillance model for TBP.

Keywords: *Borrelia*; *Orientia*; *Rickettsia*; ticks; rodents.

INTRODUCTION

Ticks and wild small animals, including rodent and shrew, are vectors and reservoirs. Undoubtedly, these zoonotic pathogens are known to be distributed throughout the ROK (Cho *et al*., 1998; Kim *et al*., 2006; Lee *et al*., 2014). The geographical distribution of reservoir populations has a close relationship with the seasonal distribution and prevalence of vector-borne diseases (Chong *et al*., 2013a). Increasing attention has been given to ticks and TBP as the annual number of tick- and mite-borne diseases among ROK populations has increased due to climate change (Im *et al*., 2019). In addition, tick species associated with small mammals, including rodents and shrews, are vectors of certain pathogenic bacteria (Hoogstraal, 1967; Stanek *et al*., 2012).

Among the tick-borne diseases (TBD), there are 3 main pathogens causing TBD in Korea, which are *Borrelia* spp., *Rickettsia* spp. and *O. tsutsugamushi*. The Lyme disease caused by spirochetes of the *B. burgdorferi* sensu lato (s.l.) complex is documented throughout the world (Rizzoli *et al*., 2011). Of 36 known species of *Borrelia*, 12 are causative agents of Lyme disease or borreliosis, including *B. burgdorferi*, *B. afzelii*, and *B. garinii* (Steere *et al*., 2004). In Korea, *Borrelia* spp. were previously detected only in ticks, rodents, and shrews however, *Borrelia* spp.-infection cases have been reported in human recently (Lee *et al*., 2011b; Moon *et al*., 2013; Ahn *et al*., 2020; Kim *et al*., 2021a).

Pathogenic *Rickettsia* spp. are distributed worldwide and are carried by parasitic haematophagous arthropods, such as lice, ticks, and fleas (Parola & Raoult, 2001; Parola *et al*., 2013; Loong *et al*.,

2020; Hirunkanokpun *et al.*, 2022; Ruh *et al.*, 2022). The causative agent of scrub typhus, *O. tsutsugamushi*, is a gram-negative obligate intracellular bacterium that causes an acute febrile infectious disease (Lee *et al*., 2011a). An earlier study has shown that the incidence of scrub typhus is directly correlated with outdoor activity and fieldwork, but also is increasing in urban areas (Sul & Kim, 2017). An incidental scrub typhus infection is observed on humans by trombiculidae larval mites. Wild small animals, that are hosts to both mites and ticks, are also reservoirs of *O. tsutsugamushi*.

Wild small animals harbor tick- and mite-borne pathogens. To determine the prevalence of pathogens associated with wild small animals, tissues (blood, liver, heart, kidney, and spleen) were assayed for the presence of *Orientia*, *Rickettsia*, and *Borrelia* spp. Blood and tissues are routinely used for the detection of *Orientia* spp. in humans and small animals using PCR (Cosson *et al*., 2015; Razzauti *et al*., 2015).

As climate change is becoming severe, more attention on ticks and related infection is now highlighted with the increasing trend of clinical cases on TBDs. However, interest in this area was only gained relatively recently hence, data on the prevalence of TBPs isolated from vectors, ticks and small mammals, is, especially in mid 2010s when cases on TBDs started to rise, is relatively insufficient (Sul & Kim, 2017). Here we present our data collected in 2014 as a key stone bridging previous and current prevalence of TBDs for clear understanding of phenomena. In this study, we have determined the presence and distribution of *Orientia, Rickettsia,* and *Borrelia* species isolated from wild small animals and ticks collected from Gyeonggi and Gangwon provinces, ROK, in 2014.

MATERIAL AND METHODS

Sample collections

From May to November 2014, a total of 384 Sherman traps (Collapsible live-capture Sherman® traps, 7.7 × 9 × 23cm - H.B. Sherman, Tallahassee, FL) were used to collect small animals throughout five counties (Paju, Gimpo, Hwaseong, Pyeongtaek, and Gapyeong), Gyeonggi province, and three counties (Cherwon, Chuncheon, and Pyeongchang), Gangwon province, ROK. Ticks were collected by dragging methods as described previously (Chong *et al*., 2013a, 2013b) in five counties (Pocheon, Yangju, Paju, Pyeongtaek, and Gapyeong), Gyeonggi province, and four counties (Cherwon, Chuncheon, Pyeongchang, and Hoengseong), Gangwon province. Survey sites included residential areas, low-lying grasses and herbaceous vegetation and deciduous, conifer, and mixed forested hillsides. After sample collection, total number of 131 small mammals, including *Apodemus agrarius* (n=124), *Crocidura lasiura* (n=4), and *Microtus fortis* (n=3), and 2,954 ticks, *Haemaphysalis flava* (n=1,449), *H. longicornis* (n=1,371), *Ixodes nipponensis* (n=89), *I. persulcatus* (n=36), and *H. japonica* (n=9), were collected.

Identification and DNA extraction

Live captured wild small animals were carried to Korea University where they were euthanized and species, sex, and weight determined. Blood was collected by cardiac puncture and then kidney and spleen were removed and placed in sterile 2 ml cryovials. Ticks were identified for its species and then placed in sterile 2 ml cryovials in pools according to stage of development (adult, 1; nymphs, 1-5; and larvae, 1-10), collection site and date of collection and then returned to a -70°C freezer until used. Individuals and pools of ticks were washed with 70% ethanol (EtOH) and rinsed with distilled phosphate buffered saline (PBS) solution. After trituration of the tick and mouse tissues, total nucleic acids were extracted by using Chelex-100 method as described previously (Chong *et al*., 2013a).

Oligonucleotide primers

Each primer was selected to determine to identify *O. tsutsugamushi* and *Rickettsia* and *Borrelia* spp. DNA was extracted from the blood and the tissue of the small animals. The 5S-23S intergenic spacer region was selected as a biomarker for *Borrelia* spp. and a primer set that amplifies the 56 kDa surface membrane protein gene was used for the detection of *O. tsutsugamushi*. In addition, primers for the outer membrane protein B (*Omp B*) gene were designed to identify *Rickettsia* spp. as listed in Table 1.

Nested polymerase chain reaction

For DNA amplification using outer primer sets, the final reaction volume was set to 20 µl containing 0.5 U Taq polymerase, 250 mM of dNTPs, 50 mM of Tris-HCl (pH 8.3), 40 mM of KCl, 1.5 mM of $MgCl_2$, gel loading dye, and 10 pmol of specific primer. PCR reactions were performed on a Veriti 96 well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). After the first round of PCR, a subsequent nested PCR or semi-nested PCR reaction was performed using outer primer and inner primer sets (Table 1). The inner reaction products were visualized by gel electrophoresis using 1.2% agarose gel containing 0.5 µg/ml of ethidium bromide and the band size compared to a standard molecular weight marker (100 bp DNA Ladder, iNtRON Biotechnology, Seongnam, ROK).

Table 1. Molecular detection of *Orientia tsutsugamushi* and *Borrelia* and *Rickettsia* spp. from wild small mammals and ticks using nested PCR

Bacteria	Target gene	Primer name	Nucleotide sequence (5'→3')	Base pair	Denaturation	PCR condition (°C)		
						Annealing	Extension	Cycles
Borrelia	*5S–23S*	23SN1 23Sa	ACCATAGACTCTTATTACTTTGAC TAAGCTGACTAATACTAATTACCC	373	94	50	72	30–35
	IGS	23SN2 5SCB	ACCATAGACTCTTATTACTTTGACCA GAGAGTAGGTTATTGCCAGGG	227	94	52	72	30–35
Orientia tsutsugamushi	*56 kDa*	tslf tslr	CCAGGATTTAGAGCAGAG CGCTAGGTTTATTAGCAT	509	94	45	72	30–35
		OT bor IF* OT bor IR*	CCTCAGCCTACTATRAKKCC AGCATTTGATAATGCAGCAAGACC	350	94	52	72	30–35
Rickettsia	*OmpB*	rompB OF# rompB OR#	GTCAGCGTTACTTCTTCGAKKC CCRTACTCCATCTTAGCATCAG	474	94	52–54	72	30–35
		rompB SFG IF* rompB SFG IR*	GTTTAATACGTGCTGCTAACCAA AAGATCCTTCTGATGTTGCAACA	265	94	54–56	72	30–35

*IF: Inner forward, IR: Inner reverse, #OF: Outer forward, OR: Outer reverse.

Analysis of PCR products and sequencing

PCR products for the 56 kDa-target protein encoding gene, *OmpB*, and *5S-23S IGS* gene were loaded on a 1.5% agarose gel and analyzed by UV illumination. Amplified products were purified using a QIAquick Gel Extraction Kit (Qiagen) and sequenced by Bioneer (Daejeon, ROK) using corresponding specific primers. The sequences were then compared to previously published sequences deposited at GenBank using BLAST program from the National Center for Biotechnology Information (NCBI). Multiple sequence alignment was conducted using Clustal X, version 1.83 (http://www.bio-soft.net/fomat.html). Phylogenetic analyses were performed using MEGA software (version 6.1).

Ethics statement

Trapping of small animals was approved by US Forces Korea (USFK) in accordance with USFK Regulation 40–1 "Prevention, Surveillance, and Treatment of Hemorrhagic Fever with Renal Syndrome". Wild small animals were euthanized by cardiac puncture and tissues were dissected under isoflurane anesthesia. All procedures and handling of small animals were conducted under an approved protocol by the Korea University Institutional Animal Care and Use Committee (KU-IACUC, #2010–212).

RESULTS

Nested PCR and sequence analysis of wild small animals

Of the 131 small mammals, 124 (94.7%) were *A. agrarius*, 4 (3.1%) were *C. lasiura*, and 3 (2.3%) were *M. fortis* (Table 2). Molecular detection was able to determine *O. tsutsugamushi* (targeting the 350 bp of 56 kDa protein encoding gene fragment), *Borrelia* spp. (targeting the 227 bp fragment of 5S-23S IGS), and *Rickettsia* spp. (targeting the 407 bp OmpB encoding gene fragment). Molecular detection on blood from small mammals showed that 37 (28.2%) were positive for *O. tsutsugamushi*, 3 (2.3%) were positive for *Rickettsia* spp., 6 (4.6%) were positive for *Borrelia* spp., whereas assay of spleen tissue showed that 61 *O. tsutsugamushi* (46.6%), 15 *Rickettsia* spp. (11.5%), and 45 *Borrelia* spp. (34.4%) were positive by PCR.

For *Orientia* spp., the nested PCR identified a 350 bp fragment from 199 (50.6%) blood, heart, kidney, and spleen DNA samples. In order to avoid sequencing duplicated tissue samples, only one *O. tsutsugamushi* was selected per small mammal. Thus, the total number of 17 DNA PCR products were selected for sequencing. Among the *O. tsutsugamushi* positive samples, there were seven positive samples for Boryong, one UAP1 (*O. tsutsugamushi* Pa-joo-like), and one Young-worl strain shared the highest sequence identities from 98-100% based on GenBank references (Figure 1).

Table 2. Distribution of wild small mammals and Ticks spp. in Gyeonggi and Gangwon provinces, Republic of Korea

Classification	Species	No of species n(%)	Province n(%)	
			Gyeonggi	Gangwon
Small mammal	*A. agrarius*	124 (94.7)	91 (93.8)	33 (97.1)
Small mammal	*C. lasiura*	4 (3.1)	3 (3.1)	1 (2.9)
Small mammal	*M. fortis*	3 (2.3)	3 (3.1)	0 (0.00)
Total no. of small mammals		131 (100.0)	97 (100.0)	34 (100.0)
Tick	*H. flava*	1,449 (49.1)	932 (49.7)	517 (48.0)
Tick	*H. longicornis*	1,371 (46.4)	865 (46.1)	506 (47.0)
Tick	*I. nipponensis*	89 (3.0)	79 (4.2)	10 (1.0)
Tick	*I. persulcatus*	36 (1.2)	0 (0.00)	36 (3.3)
Tick	*H. japonica*	9 (0.3)	0 (0.00)	9 (0.8)
Total no. of ticks		2,954 (100.0)	1,876 (100.0)	1,078 (100.0)

For *Rickettsia*, duplex nested PCR was performed to identify spotted-fever group (SFG) and typhus group (TG) in wild small mammals. Among the 15 *Rickettsia* PCR positive samples, DNA sequences of six positive spleen-DNA samples were selected for sequencing, including four (66.7%) *R. monacensis*, one (16.7%) *R. felis*, and one (16.7%) *R. parkeri* that shared high sequence identities of 98-99, 99%, and 100%, respectively (Figure 2).

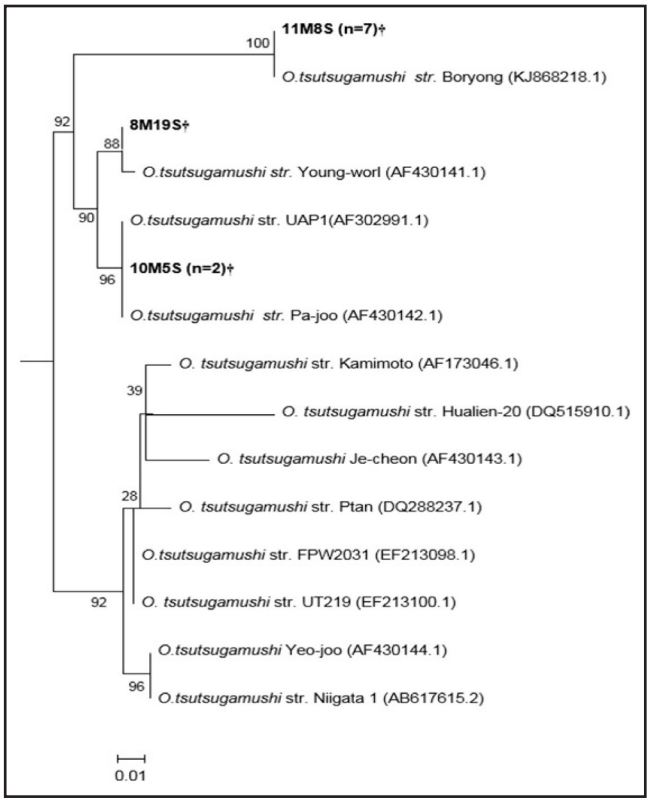

Figure 1. Phylogenetic tree showing the position of *Orientia* 56 kDa encoding genes from wild small mammal collected in Gyeonggi and Gangwon provinces, Republic of Korea. The tree was made using MEGA 6.1 software after alignment of GenBank published *Orientia* 56 kDa encoding gene sequences and sequenced by the Clustal X program. The tree was constructed by a Maximum-Likelihood (ML) method. Bootstrap values are indicated at each node (1000 replicates). The scale bar indicates 5% nucleotide divergence. (†, Rodent numbers, and Spleen numbers).

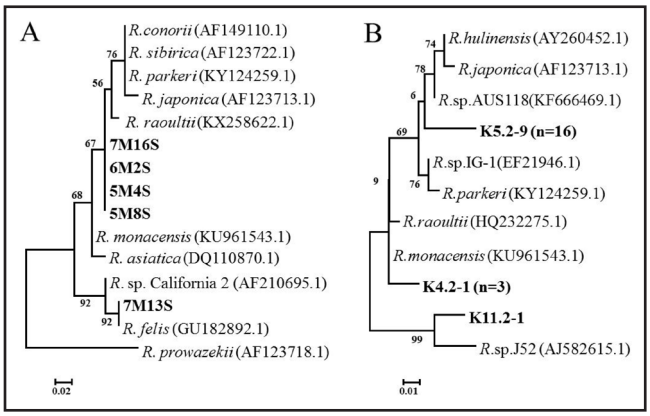

Figure 2. Phylogenetic tree showing the position of *Rickettsia OmpB* genes from the sequences of (A) wild small mammals; and (B) ticks collected in Gyeonggi and Gangwon provinces, Republic of Korea. The trees were constructed by a ML method. Bootstrap values are indicated at each node (1000 replicates). The scale bar represents (a) 2%; and (b) 1% nucleotide divergence.

Borrelia specific semi-nested PCR identified the presence of 227 bp fragment from 158 (40.2%) of 393 blood, heart, kidney, and spleen wild small mammal samples. A total of 45 spleen samples positive for *Borrelia* were subjected to sequencing analyses. Sequencing of 5S–23S IGS gene fragment showed similarities with *B. afzelii* (97-99%), *B. garinii* (97-99%), *B. valaisiana* (99%), and *B. burgdorferi* (98-99%). Of 37 positive *Borrelia* spp., these were genotyped further into *B. afzelii* (n=19, 51.4%), *B. valaisiana* (n=9, 24.3%), *B. garinii* (n=5, 13.5%), and *B. burgdorferi* (n=4, 10.8%) (Figure 3).

Nested PCR and sequence analysis of selected ticks

A total of 2,954 ticks belonging to five species, *H. flava* (n=1,449, 49.1%), *H. longicornis* (n=1,371, 46.4%), *I. nipponensis* (n=89, 3.0%), *I. persulcatus* (n=36, 1.2%), and *H. japonica* (n=9, 0.3%) (Table 2). Of a total of 377 pools of ticks tested, 128 (34.0%) were positive for *Rickettsia* spp. (Table 4). Twenty samples were selected and PCR products subjected to sequencing. Of these 20 samples, 16 (80.0%), 3 (15.0%), and 1 (5.0%) shared 98-99, 99-100, and 97% sequence identities with *Rickettsia sp.* AUS118, *R. monacensis*, and *Rickettsia* sp. J52, respectively. Among 33 *Borrelia* positives, nine were subjected to DNA sequencing of which 4 (44.5%), 3 (33.3%), and 2 (22.2%) shared 98-100, 98-99, and 97-99% sequence identities with *B. burgdorferi*, *B. afzelii*, and *B. garinii*, respectively (Table 3).

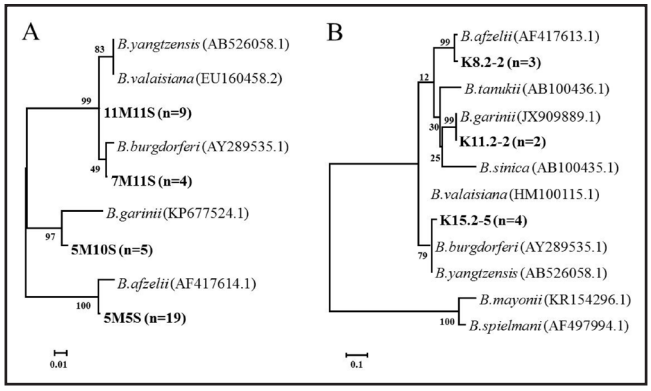

Figure 3. Phylogenetic tree constructed from partial sequences of *Borrelia* 5S-23S IGS from (A) wild small mammals; and (B) ticks. The trees were constructed by a ML method. Bootstrap values are indicated at each node (1000 replicates). The scale bar represents (A) 1%; and (B) 10% nucleotide divergence.

Table 3. Results of nested PCR and sequencing for the detection of *Orientia tsutsugamushi* and *Rickettsia* and *Borrelia* spp. in wild small mammals (a) & ticks (b) collected from Gyeonggi and Gangwon provinces, Republic of Korea

(a) Target gene	Source of isolation	Organ	No. of positives n(%)	No. of sequences n(%)	Sequence results	Similarity in percentile
Orientia 56kDa	*A. agranius* *M. fortis*	Blood Heart Kidney	37 (28.2) 58 (44.3) 43 (32.8)	2/2 (100.0)	*O. tsutsugamushi* Boryong	99–100
	A. agranius *M. fortis*	Spleen	61 (46.6)	8/15 (53.3)	*O. tsutsugamushi* Boryong *O. tsutsugamushi str.* UAP1 *O. tsutsugamushi* Young-worl	98–100 98–99 98–99
Rickettsia OmpB	*A. agranius* *C. laisura*	Blood Heart Kidney	3 (2.3) 18 (13.7) 19 (14.5)			
	A. agranius	Spleen	15 (11.5)	6/6 (100.0)	*R. monacensis* *R. felis* *R. parkeri*	98–99 99 100
Borrelia 5S-23S IGS	*A. agranius* *M. fortis* *C. laisura*	Blood Heart Kidney	6 (4.6) 48 (36.6) 59 (45.0)			
	A. agranius *M. fortis*	Spleen	45 (34.4)	37/45 (82.2)	*B. afzelii* *B. valaisiana* *B. garinii* *B. burgdorferi*	97–99 99 97–99 98–99
(b) Target gene	Source of isolation		No. of positives n(%)	No. of sequences sequences n(%)	Sequence results	Similarity in percentile
*Rickettsia OmpB**	*H. flava* *H. longicornis* *H. japonica* *I. nipponensis* *I. persculcatus*		128 (34.0)	20/20 (100.0)	*R.* sp. AUS118 *R. monacensis* *R.* sp. J52	98–99 99–100 97
*Borrelia 5S-23S IGS**	*H. flava* *H. longicornis* *I. nipponensis* *I. persculatus*		33 (8.8)	9/9 (100.0)	*B. burgdorferi* *B. afzelii* *B. garinii*	98–100 98–99 97–99

Figures in parentheses indicate percentages.

Table 4. Distribution of *Rickettsia* and *Borrelia* spp. positive ticks collected from May-November, 2014 based on PCR results

Date	Sample	Province	Total no.	PCR Results	
				SFG_ *OmpB*[a]	Bor_*5S-23S*[b]
30/May/2014	K1	Gyeonggi	25	15 (60)	–
30/May/2014	K2	Gyeonggi	20	18 (90)	1 (5)
28/Jun/2014	K3	Gangwon	18	3 (5.5)	1 (6.3)
28/Jun/2014	K4	Gyeonggi	7	2 (29)	–
1/Aug/2014	K5	Gyeonggi	23	7 (30)	–
23/Aug/2014	K6	Gangwon	90	28 (30)	–
23/Aug/2014	K7	Gyeonggi	16	5 (30)	6 (37)
24/Aug/2014	K8	Gyeonggi	24	20 (83)	5 (20)
13/Sep/2014	K9	Gyeonggi	30	3 (20)	5 (16.6)
14/Sep/2014	K10	Gyeonggi	30	2 (13)	4 (13.3)
27/Sep/14	K11	Gangwon	14	6 (42)	–
27/Sep/14	K12	Gangwon	20	2 (10)	–
10/Oct/2014	K13	Gangwon	11	2 (18)	1 (9)
19/Oct/2014	K14	Gyeonggi	21	8 (38)	6 (28)
19/Oct/2014	K15	Gyeonggi	8	3 (12)	1 (12)
19/Oct/2014	K16	Gyeonggi	16	3 (12)	3 (18)
19/Nov/2014	K17	Gyeonggi	4	1 (25)	–
Total (K1-K17)		377	128 (34.0)	33 (8.8)	

[a]SFG_*ompB:* Spotted fever group *OmpB* gene; [b]Bor_*5S-23S IGS: Borrelia 5S-23S IGS* gene; Numbers in parentheses indicate percentages.

DISCUSSION

This molecular and epidemiological survey was conducted whereby a total of 131 wild small mammals and 377 pools of ticks were collected for the detection of *O. tsutsugamushi*, *Rickettsia* spp. and *Borrelia* spp. *O. tsutsugamushi* Boryong strain, *R. monacensis*, *and B. afzelii* were the primary species detected in wild small mammals, whereas *B. burgdorferi* and *Rickettsia sp.* AUS118 were the primary species detected in pools of ticks collected in Gyeonggi and Gangwon provinces, ROK. The two dominant species, *H. longicornis*, known for reservoir of severe fever with thrombocytopenia syndrome (SFTS) virus, and *H. flava*, a reservoir of multiple TBPs, showed the highest positive rates in collected during August (46.4 and 46.8%, respectively) (Table 3). The distribution of these two dominant species was similar between Gyeonggi and Gangwon provinces. However, in Gangwon province, two other species (*H. japonica* and *I. persulcatus*) also were observed. Their habitat is known to be dense forest environment however their emergence was observed in several sites, probably due to geographical and environmental change, indicating enlarged areas of their habitat. The number of hard tick species is increasing in trend as the total number of reported hard tick species is 14, including *Amblyomma testudinarium*, *H. concinna*, *H. flava*, *H. formosensis*, *H. japonica*, *H. longicornis*, *H. ornithophila*, *I. nipponensis*, *I. persulcatus*, *I. pomerantzevi*, *I. simplex*, *I. turdus*, *I. vespertilionis*, and *Rhicepicephalus sanguineus* sensu lato (St John *et al*., 2021).

Grasses and herbaceous vegetation are the primary habitat for *A. agrarius* and trombiculid mites, the reservoirs and vectors of *O. tsutsugamushi*, (Kong *et al*., 2007). *O. tsutsugamushi* Boryong was the primary strain (70%), while one sample obtained from rodent spleen was similar to the UAP1 strain. In 2001, only two samples were reported to be positive for UAP1 strain (*O. tsutsugamushi* Pa-joo-like) (Tamura *et al*., 2001). Another study has identified UAP7 strain (Genbank accession number: AF302995.1) in two human samples in Gyeonggi province, ROK (Park *et al*., 2010). This indicates that different types of UAP strains might be geographically present.

R. monacensis was present at rates of 80% and 17% in wild small mammals and ticks respectively. However, *R*. AUS118 and *Rickettsia* sp. J52 were also identified from ticks and 16 (80%) of 20 *Rickettsia* spp. were identified as *R*. AUS118 that demonstrates a close phylogenetic relationship with *R. hulinensis* and *R. japonica*. According to NCBI, *R*. AUS118 from Australia (KF666469.1) and *Rickettsia* sp. J52 from England (AJ582615.1) are reported as new *Rickettsia* species. This indicates that a new type of *Rickettsia* might have already spread to Gyeonggi and Gangwon provinces in the ROK. Further, a recent study reveals that *Rickettsia* spp. are becoming more diverse than other species of *Rickettsia*, such as Ca. *R. longicornii*, Ca. *R. jingxinensis*, Ca. *R. tasmanensis* strain T152, *R. lusitaniae*, *R. tamurae*, and *R. raoultii* (Kim *et al*., 2021b; Park *et al*., 2021; Seo *et al*., 2021; Truong *et al*., 2022).

The seasonal occurrence of ticks varies by developmental stages, with dense populations of larval ticks collected from July through September, while higher numbers of nymphs are collected from May-October and higher numbers of adults collected in August and September prior to large numbers of larval ticks. These findings are consistent with the previous studies (Chong *et al*., 2013a, 2013b). Chong *et al*. (2013a) have investigated the monthly occurrence of ticks and reported that ticks were most active from April to October. In this study, *H. flava* nymphs and *I. nipponensis* adults were collected until mid-November. The prolonged activities of ticks were, possibly, due to the increased temperatures (>10°C) during November. Further, changes in regional and/or environmental factors may lead to changes in the seasonal activity of ticks (Lindgren & Gustafson, 2001; Burtis *et al*., 2016).

Due to recent climate changes and the migration of migratory birds, arthropods and associated pathogens may potentially be introduced from overseas into domestic regions (Lindgren & Gustafson, 2001; Parola *et al*., 2013; Choi *et al*., 2014; Burtis *et al*., 2016). Recently, there has been an increase in the number of patients bitten by ticks during outdoor activities at nearby parks and suburbs. This has led to significant increases in the number of cases of TBDs (Sul & Kim, 2017). Therefore, there is a need to

collect reservoir hosts and vectors over extended periods of time to determine the distribution and density of *Ixodida* spp. Gajda *et al.* (2017) suggested that the role of small mammals, including rodents and shrews, and their relationship between *Rickettsia* spp. in agricultural and unmanaged habitats is unclear, emphasizing that additional studies should be done. In this study, the prevalence and distribution of ticks and TBPs in short period of time were analyzed and determined. This study provides insight to understand the trend in the prevalence and distribution of ticks and TBPs by presenting data collected in mid 2010s when the prevalence of ticks and TBPs started to surge.

The study has some limitations. First, only partial genes (*56 kDa*, *OmpB*, and *5S-23S IGS* genes) were analyzed. In addition, the samples were also collected for about seven months of the year. Overall, we confirmed PCR positive rates. Sequencing results of each gene showed a high species separation rate, although there were differences depending on the species. Therefore, further investigation is required to compensate for the insufficient sample number and period of time. In addition, wild small animals and ticks were collected only from two provinces: Gangwon and Gyeonggi. The frequency of the survey should be carefully done for the entire ROK Peninsula, including an array of different reservoir host and tick habitats.

Although, reported co-infection rate is not high, ticks may harbor more than one pathogen, potentially infecting humans with multiple bacterial species. The results demonstrated high prevalence of *O. tsutsugamushi* and *Borrelia* and *Rickettsia* spp. in small mammals and ticks. Additionally, recreational outdoor and farming activities are gaining popularity recently in ROK. For this reason, there should be sustainable routine surveillance of small mammals and associated tick species in habitats frequented by humans. Additionally, a study design based on sampling within gene specific areas could provide important insights about species-specific taxonomic determinations and epidemiological cycles.

In conclusion, the results of this study indicate that the high prevalence of *O. tsutsugamushi*, *Borrelia* spp. and *Rickettsia* spp. was observed in wild small mammals and ticks in mid 2010s. This indicates that in mid 2010s, the prevalence of TBPs was already surging at the time when the investigation on the prevalence of TBPs was seriously taken into account. Hence, This study highlights for sustainable routine surveillance model for TBPs.

Conflict of Interests

None to declare.

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