High Prevalence of *Rickettsia raoultii* and Associated Pathogens in Canine Ticks, South Korea

Min-Goo Seo, Oh-Deog Kwon, Dongmi Kwak

Author affiliations: Animal and Plant Quarantine Agency, Gimcheon, South Korea (M.-G. Seo); Kyungpook National University, Daegu, South Korea (O.-D. Kwon, D. Kwak)

DOI: https://doi.org/10.3201/eid2610.191649

We studied the prevalence of tickborne pathogens in canine ticks, South Korea, during 2010–2015. Results revealed a high prevalence of the emerging pathogen *Rickettsia raoultii*. Dog ticks may be maintenance hosts for tickborne pathogens, suggesting the need to continually evaluate the potential public health threat posed by *R. raoultii*–infected ticks.

Ticks are responsible for mechanical damage to animal blood vessels and skin and are known to transmit a wide range of bacteria, viruses, and protozoa, causing severe infections in animals and humans (*1*). Most defined Rickettsiales are considered zoonotic emerging or reemerging pathogens; some can cause severe human illnesses, including anaplasmosis, rickettsioses, scrub typhus, and ehrlichiosis (*2*). Determining the ecology of local tick species and recognizing the tickborne pathogens they carry are of paramount public health importance. Our study assessed risk factors for and the prevalence and coinfectivity of several tickborne pathogens in ticks collected from dogs in South Korea.

Rickettsia spp. are emerging or reemerging pathogens with public health relevance; 1 species, *R. raoultii*, causes human tickborne lymphadenitis in many countries in Europe (*3*). Of note, *R. raoultii* had not been detected in humans, animals, or vectors in South Korea until recently, but it now appears to be endemic in ticks infesting dogs. We collected a total of 980 ticks in central (n = 442) and southern (n = 538) South Korea from 102 dogs during 2010–2015. We used both morphological and molecular methods (Appendix, https://wwwnc.cdc.gov/EID/article/26/10/19-1649-App1.pdf) to identify the tick species, which included *Haemaphysalis longicornis*, *H. flava*, and *Ixodes nipponensis*, then sorted them into 364 pools (1–7 ticks per pool) by dog, identified tick species, and developmental stage (larva, nymph, and adult).

Our findings are consistent with the results of a previous study from South Korea, in which *H. longicornis* ticks were found in 201 (48.9%), *Haemaphysalis* spp. ticks in 130 (31.6%), *H. flava* ticks in 71 (17.3%), and *I. nipponensis* ticks in 7 (1.7%) of 411 dogs (*4*). A previous study of *H. longicornis* tick prevalence proposed that, rather than rodents as previously thought, larger mammals, including dogs, might be the hosts for this tick species (*5*). Additional surveys are needed to assess the natural hosts of *H. longicornis* ticks.

Several tickborne pathogens were then screened by using primer sets specific to each pathogen (Appendix). The 16S rRNA genes of *R. raoultii* were found in 149 (40.9%), *R. monacensis* in 1 (0.3%), and *Candidatus* Rickettsia principis in 2 (0.6%) of 364 tick pools (Figure; Appendix Table 1). *R. raoultii* was detected in 100 nymph and 49 adult *H. longicornis* ticks in South Korea. *R. raoultii*–positive ticks were collected from 25 (24.5%) of 102 dogs, a relatively high proportion of those observed in this study.

R. monacensis causes spotted fever–like disease and has been found in multiple hard tick species in several European countries (*2*). It was detected in 16 (55.2%) of 29 pools of *I. nipponensis* ticks from small mammals in South Korea (*6*). In this study, however, *R. monacensis* was found in only 1 (0.3%) of 364 tick pools, in an adult *I. nipponensis* tick. One spotted fever group rickettsiae with *Candidatus* status was also identified in ticks in this study; *Candidatus* R. principis was identified in 2 (3.0%) of 67 *H. japonica douglasii* ticks in Russia in 2006 (*7*). In this study, *Candidatus* R. principis (0.6%) was detected in 1 *H. longicornis* nymph and 1 *H. flava* nymph. Additional tickborne pathogens were detected (Appendix Table 1, Figures 1, 2): the *E. canis* 16S rRNA gene was identified in 1 *H. longicornis* nymph (0.3%), and the *T. luwenshuni* 18S rRNA gene was identified in 20 *H. longicornis* nymphs (10.9%) and 24 *H. longicornis* adults (26.1%). No other tickborne pathogens were detected in this study.

Increased seasonal tick populations and activity in the summer and autumn impact the transmission of tickborne pathogens (*8*). In this study, we collected ticks from May to September, and found that tick abundance and distribution patterns were similar to those in a previous study in South Korea (*8*), which showed that both ticks and tickborne pathogens were more prevalent in southern regions and during the summer. South Korea is also steadily shifting to a subtropical climate due to global warming (*9*), which may influence this seasonal effect, as well. In another previous study in South Korea (*4*), ticks were collected from stray or pet dogs, but no ticks were found on military working dogs. These military dogs received routine veterinary care for preventive ectoparasite treatments. Therefore, tick prevention measures

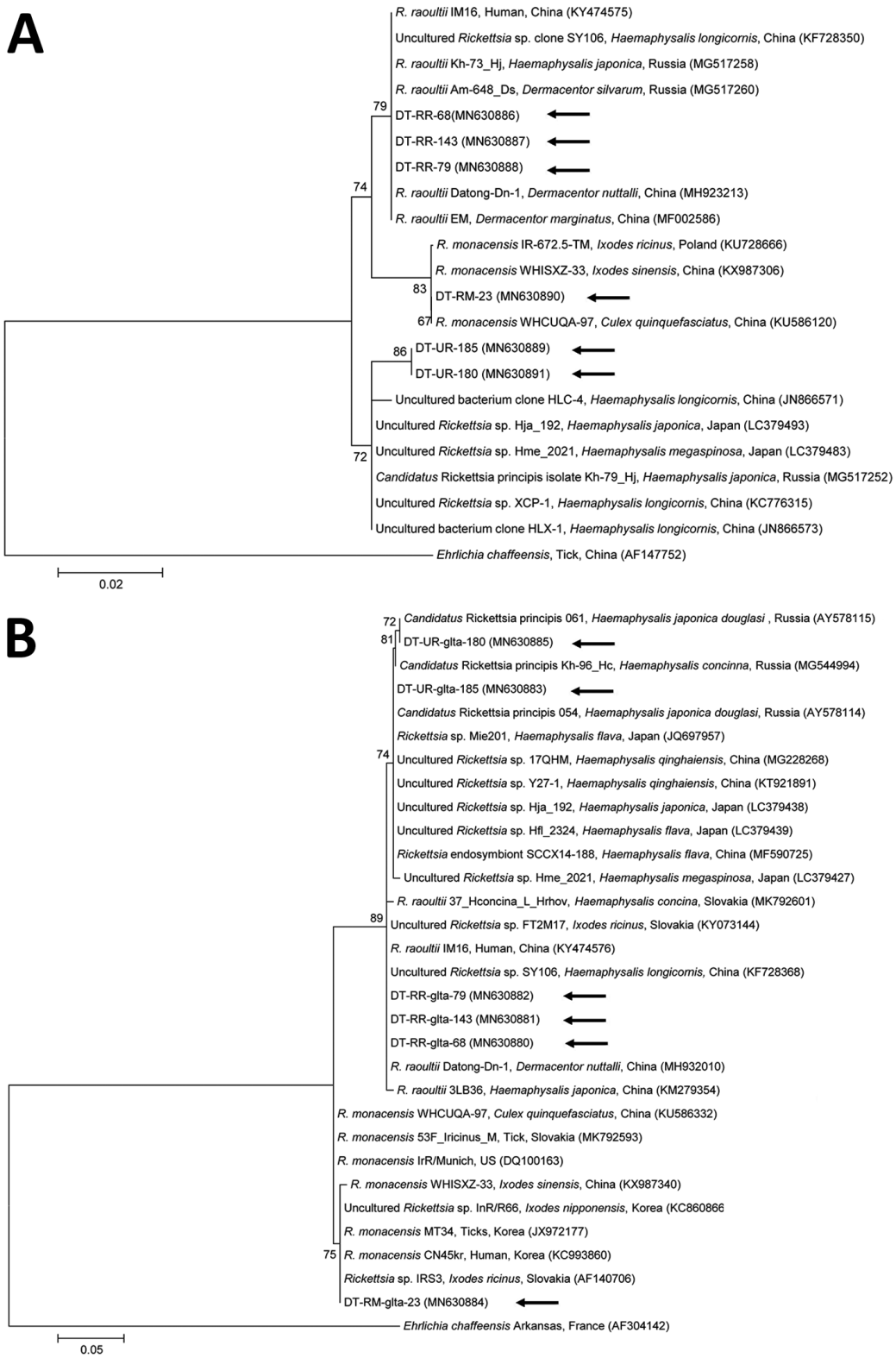


Figure. Phylogenetic trees constructed using the maximum-likelihood method based on nucleotide sequences of *Rickettsia* spp. from canine ticks, South Korea (black arrows), and reference sequences. A) 16S rRNA; (B) *glt*A. *Ehrlichia chaffeensis* sequences were used as outgroups. GenBank accession numbers for reference sequences are shown with the sequence name. Branch numbers indicate bootstrap support (1,000 replicates). Scale bar indicates phylogenetic distance.

should be effective in endemic areas with known tick seasons, when infestations are higher.

Our findings indicate the zoonotic potential of dog ticks in South Korea. Physicians and public health officers therefore need to be aware of the high potential and clinical complexity of infection with *R. raoultii* and other tickborne pathogens in order to confirm suitable testing and treatment needs in endemic areas (*10*). Therefore, we strongly recommend continuous evaluation of the potential public health threat posed by infected ticks to humans in South Korea. A better understanding of local tick species, including *H. longicornis*, and a more thorough characterization of TBP agents, such as *R. raoultii*, are critical.

This work was supported by a grant from the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education (grant number: NRF-2016R1D1A1B02015366).

The authors declare no conflict of interest.

About the Author

Dr. Seo received a PhD degree at Kyungpook National University, South Korea, and is currently working as a research scientist at the Animal and Plant Quarantine Agency, Gimcheon, South Korea. His primary research interests are vectors and vectorborne diseases.

References

1. Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 2012;28:437–46. [Erratum in: Trends Parasitol. 2013;29:516] https://doi.org/10.1016/j.pt.2012.07.003
2. Raoult D, Parola P, editors. Rickettsial diseases. New York: Informa Healthcare; 2007.
3. Oteo JA, Portillo A. Tick-borne rickettsioses in Europe. Ticks Tick Borne Dis. 2012;3:271–8. https://doi.org/10.1016/j.ttbdis.2012.10.035
4. Choe HC, Fudge M, Sames WJ, Robbins RG, InYong L, Chevalier NA, et al. Tick surveillance of dogs in the Republic of Korea. Systematic and Applied Acarology. 2011;16:215–22. https://doi.org/10.11158/saa.16.3.5
5. Zheng H, Yu Z, Zhou L, Yang X, Liu J. Seasonal abundance and activity of the hard tick *Haemaphysalis longicornis* (Acari: Ixodidae) in North China. Exp Appl Acarol. 2012;56:133–41. https://doi.org/10.1007/s10493-011-9505-x
6. Lee KM, Choi YJ, Shin SH, Choi MK, Song HJ, Kim HC, et al. Spotted fever group rickettsia closely related to *Rickettsia monacensis* isolated from ticks in South Jeolla province, Korea. Microbiol Immunol. 2013;57:487–95. https://doi.org/10.1111/1348-0421.12062
7. Mediannikov O, Sidelnikov Y, Ivanov L, Fournier PE, Tarasevich I, Raoult D. Far eastern tick-borne rickettsiosis: identification of two new cases and tick vector. Ann N Y Acad Sci. 2006;1078:80–8. https://doi.org/10.1196/annals.1374.010
8. Im JH, Baek J, Durey A, Kwon HY, Chung MH, Lee JS. Current status of tick-borne diseases in South Korea. Vector Borne Zoonotic Dis. 2019;19:225–33. https://doi.org/10.1089/vbz.2018.2298
9. Seo MG, Lee SH, VanBik D, Ouh IO, Yun SH, Choi E, et al. Detection and genotyping of *Coxiella burnetii* and *Coxiella*-like bacteria in horses in South Korea. PLoS One. 2016;11:e0156710. https://doi.org/10.1371/journal.pone.0156710
10. Li H, Zhang PH, Huang Y, Du J, Cui N, Yang ZD, et al. Isolation and identification of *Rickettsia raoultii* in human cases: a surveillance study in 3 medical centers in China. Clin Infect Dis. 2018;66:1109–15. https://doi.org/10.1093/cid/cix917

Address for correspondence: Dongmi Kwak, College of Veterinary Medicine, Kyungpook National University, 80 Daehakro, Bukgu, Daegu 41566, South Korea; email: dmkwak@knu.ac.kr

COMMENT LETTERS

Pulmonary Embolism and Increased Levels of D-Dimer in Patients with Coronavirus Disease

Kok Hoe Chan, Jihad Slim, Hamid S. Shaaban

Author affiliation: St. Michael's Medical Center, Newark, New Jersey, USA

DOI: https://doi.org/10.3201/eid2610.202127

To the Editor: We read with great interest the recent report by Griffin et. al. (*1*). Griffin et al. reported on 3 patients in whom pulmonary embolism developed after the cytokine storm phase of coronavirus disease (COVID-19); the patients were treated with steroids and tocilizumab. We have observed a transient elevation of D-dimer in patients after tocilizumab treatment, which leads to an interesting discussion about whether the pulmonary embolism observed in these COVID-19 patients was due to a persistent hypercoagulable state in the late phase of the disease or a transient one related to tocilizumab.

Tocilizumab is a humanized antihuman interleukin-6 (IL-6) receptor monoclonal antibody that inhibits IL-6 signaling. Use of tocilizumab in the COVID-19 pandemic has been growing. It

Article DOI: https://doi.org/10.3201/eid2610.191649

High Prevalence of *Rickettsia raoultii* and Associated Pathogens in Canine Ticks, South Korea

Appendix

Supplemental Methods

Tick Collection and Species Identification

A total of 980 ticks were collected from stray dogs in animal shelters located in central South Korea: Chungbuk (130 ticks from 14 dogs), Chungnam (145 ticks from 15 dogs), and Gyeongbuk (167 ticks from 17 dogs) and southern South Korea: Jeonbuk (165 ticks from 17 dogs), Jeonnam (180 ticks from 19 dogs), and Gyeongnam (193 ticks from 20 dogs). None of the infested dogs showed clinical symptoms of tickborne pathogens (TBPs). To note, several dogs had skin redness around the tick bites. Five to 10 ticks per dog were collected from a total of 102 dogs. We collected ticks from dogs from their head, neck, abdomen areas, and mouth parts with fine forceps and gently removed attached ticks. We then stored ticks in vials containing 70% ethanol. Ticks were first identified to the species level and classified morphologically into several developmental stages (*1*). Subsequently, different tick species were pooled as follows: per dog, identified species, and developmental stages (larva, nymph, and adult) into 364 tick pools, with 1 to 7 ticks per pool.

Tick and TBP Detection

Genomic DNA was extracted from each tick pool sample (n = 364 pools) using a commercial DNeasy Blood & Tissue Kit (Qiagen, https://www.qiagen.com/us) according to the manufacturer's instructions. Extracted DNA was then stored at −20°C until further use. The AccuPower HotStart PCR Premix kit (Bioneer, https://eng.bioneer.com) was used for PCR amplification. Molecular identification of tick species was conducted by amplifying the mitochondrial cytochrome c oxidase subunit I (COI) gene using specific primers (*2*). Several

TBPs were then screened by using specific primer sets for each pathogen. For example, rickettsial infections (*Anaplasma*, *Ehrlichia*, and *Rickettsia*) were initially assessed using a commercial AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer) to detect 16S rRNA. Positive samples were then amplified further for species identification. For *Rickettsia* spp., positive samples were confirmed by PCR targeting the citrate synthase gene (*glt*A) (*3*). The 5S (*rrf*)–23S (*rrl*) intergenic spacer gene of *Borrelia* spp. and the outer surface protein A gene fragment of *Borrelia burgdorferi* sensu lato was amplified by nested PCR (nPCR) (*4*). Multiple primer sets were used to amplify the *Coxiella* 16S rRNA gene, including *C. burnetii* and *Coxiella*-like bacteria (*5*). Piroplasm infections (*Babesia* and *Theileria*) were first screened using a commercial AccuPower Rickettsiales 2-Plex PCR Kit (Bioneer) to detect piroplasm 18S rRNA. The positive samples were then re-amplified by PCR using primers designed from the common sequence of the 18S rRNA genes of several *Babesia* species (*6*). Nested primer sets were used to amplify the internal transcribed spacer region sequence of *Bartonella* spp (*7*). The S segment of SFTSV was amplified by nPCR (*8*). Appendix Table 2 describes the primers and amplification conditions used for TBP detection in this study.

DNA Cloning

Amplicons of 364 pooled tick and 197 TBP-positive samples were purified using the QIAquick Gel Extraction Kit (Qiagen), ligated into pGEM-T Easy vectors (Promega, https://www.promega.com), transformed into *Escherichia coli* DH5α-competent cells (Thermo Fisher Scientific, https://www.thermofisher.com), and incubated at 37°C overnight. Plasmid DNA extraction was performed using a plasmid miniprep kit (Qiagen) according to the manufacturer's instructions.

DNA Sequencing and Phylogenetic Analysis

Recombinant clones were selected and sent to Macrogen (https://www.macrogen.com/en/main/index.php) for sequencing. The sequences were analyzed and aligned using the multiple sequence alignment program CLUSTAL Omega (v. 1.2.1), and the alignment was edited with BioEdit (v. 7.2.5). Phylogenetic analysis was performed using MEGA (v. 6.0) based on the maximum likelihood method with the Kimura 2-parameter distance

model. The aligned sequences were analyzed using a similarity matrix. The stability of the trees was estimated by bootstrap analysis using 1,000 replicates.

Statistical Analysis

GraphPad Prism (v. 5.04; GraphPad Software Inc., https://www.graphpad.com) was used for statistical analyses. The Chi-square test was performed to analyze significant differences among pathogens for each tick stage, and a value of $p < 0.05$ was considered statistically significant.

Supplemental Results

Tick Identification

Tick species were molecularly identified using primers for the COI gene (expected size 710 bp) to avoid potential mistakes in morphological identification. Three species were identified by morphological and molecular characteristics, and both methods showed congruent results. Furthermore, the nucleotide sequences from 8 representative ticks based on developmental stage and collected region were assessed for data analysis.

The 3 groups of ticks shared close genetic relationships with *H. longicornis* (98.0–100% nucleotide identity), *H. flava* (98.8–100% nucleotide identity), and *I. nipponensis* (98.9–100% nucleotide identity). A phylogenetic tree was assembled based on the COI genes of several ticks deposited in GenBank, and the ticks collected in this study were classified into 3 clades related to 3 species (Appendix Figure 3): *H. longicornis* (76.9%, 280/364 pools), *H. flava* (14.6%, 53/364 pools), and *I. nipponensis* (8.5%, 31/364 pools) (Appendix Table 1).

TBP Identification

In total, 69.6% (195/280) of *H. longicornis* ticks, including 66.7% (122/183) of nymphs and 79.3% (73/92) of adults, were PCR-positive for at least 1 TBP (Appendix Table 1). TBPs were significantly more abundant ($p = 0.0003$) in the adult stage compared to other developmental stages in *H. longicornis*. *R. raoultii* was the most abundant TBP in *H. longicornis*: 54.6% (100/183) of nymphs and 53.3% (49/92) of adults were PCR-positive. *T. luwenshuni* ($p = 0.0031$) was significantly more abundant in the adult stage compared to other stages in *H. longicornis*.

In *H. longicornis*, TBPs were detected in varying proportions in different geographical areas of South Korea: In the nymph stage, 53.8% (50/93) were detected in the central area and 80% (72/90) in the southern area; in the adult stage, 74.5% (35/47) were detected in the central area and 84.4% (38/45) in the southern area. TBPs were significantly more abundant in the adult stage of both central ($p = 0.0136$) and southern ($p = 0.002$) areas compared to the other stages in *H. longicornis*. *T. luwenshuni* from the southern area ($p = 0.0398$) was significantly more abundant in the adult stage compared to the other stages in *H. longicornis*. *R. raoultii* from the southern area ($p = 0.049$) was significantly more abundant in the nymph stage compared to the other stages in *H. longicornis*.

Among the positive samples, additional *glt*A gene analysis revealed that the ticks were positive for *R. raoultii* (43/364 pools, 11.8%), *R. monacensis* (1/364 pools, 0.3%), and *Candidatus* Rickettsia principis (2/364 pools, 0.6%). This was an expected result as comparisons of similarity values suggested that *glt*A is less conserved than the 16S rRNA gene in rickettsiae (*9*). The average rate of sequence change in *glt*A was quicker than the average rate of sequence change in the 16S rRNA gene. The *glt*A sequences may be valuable in uncovering close relationships.

R. raoultii-positive ticks were collected from dogs (24.5%, 25/102) from central South Korea: Chungbuk (2), Chungnam (3), and Gyeongbuk (6), and southern South Korea: (Jeonbuk (4), Jeonnam (4), and Gyeongnam (6). Eleven (3.0%) ticks were coinfected with *T. luwenshuni* and *R. raoultii*, while 1 (0.3%) tick was coinfected with *E. canis*, *T. luwenshuni*, and *R. raoultii*.

Molecular and Phylogenetic Analyses

H. longicornis COI gene sequences from this study showed 98.1–100% nucleotide identity with known *H. longicornis* COI gene sequences, consistent with the results of a phylogenetic analysis that classified *H. longicornis* into 2 groups with a neighborly relationship (Appendix Figure 3). *H. flava* COI gene sequences from this study showed 98.7–99.8% nucleotide identity and *I. nipponensis* COI gene sequences showed 98.5–99.5% nucleotide identity with known COI gene sequences (Appendix Figure 3).

Phylogenetic analyses showed that *E. canis* 16S rRNA nucleotide sequences (Appendix Figure 1) and *T. luwenshuni* 18S rRNA nucleotide sequences (Appendix Figure 2) were clustered with previously GenBank documented sequences.

The 1 *E. canis* sequence found in the present study shared 99.4–100% identity with the 16S rRNA gene in previously reported *E. canis* isolates. The 3 *T. luwenshuni* representative sequences from the present study shared 99.8–100% identity with the 18S rRNA gene. They also shared 96.6–99.8% identity with the 18S rRNA gene previously reported in *T. luwenshuni* isolates.

The 3 *R. raoultii* representative sequences from the present study shared 100% identity with 16S rRNA and 99.7–100% identity with *glt*A genes. They also shared 99.4–99.7% identity with 16S rRNA and 98.1–99.7% identity with *glt*A genes in previously reported *R. raoultii* isolates. The 1 *R. monacensis* sequence found in the present study shared 99.1–99.6% identity with 16S rRNA and 99.1–100% identity with *glt*A genes in previously reported *R. monacensis* isolates. The 2 sequences of *Candidatus* R. principis genes found in the present study shared 100% identity with 16S rRNA and 98.7% identity with *glt*A genes. They also shared 98.5–98.9% identity with 16S rRNA and 99.2–100% identity with the *glt*A genes in previously reported *Candidatus* R. principis isolates.

The representative sequences in this study were submitted to GenBank. Accession numbers are MN630872 (*I. nipponensis*), MN630873 (*H. flava*), MN630874–MN630879 (*H. longicornis*), MN630892 (*E. canis*), MN626388–MN626390 (*T. luwenshuni*), and MN630880–MN630891 (*Rickettsia* spp.).

References

1. Barker SC, Walker AR. Ticks of Australia. The species that infest domestic animals and humans. Zootaxa. 2014;3816:1–144. PubMed https://doi.org/10.11646/zootaxa.3816.1.1

2. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9. PubMed

3. Reis C, Cote M, Paul RE, Bonnet S. Questing ticks in suburban forest are infected by at least six tick-borne pathogens. Vector Borne Zoonotic Dis. 2011;11:907–16. PubMed https://doi.org/10.1089/vbz.2010.0103

4. VanBik D, Lee SH, Seo MG, Jeon BR, Goo YK, Park SJ, et al. *Borrelia* species detected in ticks feeding on wild Korean water deer (*Hydropotes inermis*) using molecular and genotypic analyses. J Med Entomol. 2017;54:1397–402. PubMed https://doi.org/10.1093/jme/tjx106

5. Seo MG, Lee SH, VanBik D, Ouh IO, Yun SH, Choi E, et al. Detection and genotyping of *Coxiella burnetii* and *Coxiella*-like bacteria in horses in South Korea. PLoS One. 2016;11:e0156710. PubMed https://doi.org/10.1371/journal.pone.0156710

6. Casati S, Sager H, Gern L, Piffaretti JC. Presence of potentially pathogenic *Babesia* sp. for human in *Ixodes ricinus* in Switzerland. Ann Agric Environ Med. 2006;13:65–70. PubMed

7. Ko S, Kim SJ, Kang JG, Won S, Lee H, Shin NS, et al. Molecular detection of *Bartonella grahamii* and *B. schoenbuchensis*-related species in Korean water deer (*Hydropotes inermis argyropus*). Vector Borne Zoonotic Dis. 2013;13:415–8. PubMed https://doi.org/10.1089/vbz.2012.1105

8. Yoshikawa T, Fukushi S, Tani H, Fukuma A, Taniguchi S, Toda S, et al. Sensitive and specific PCR systems for detection of both Chinese and Japanese severe fever with thrombocytopenia syndrome virus strains and prediction of patient survival based on viral load. J Clin Microbiol. 2014;52:3325–33. PubMed https://doi.org/10.1128/JCM.00742-14

9. Roux V, Rydkina E, Eremeeva M, Raoult D. Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. Int J Syst Bacteriol. 1997;47:252–61. PubMed https://doi.org/10.1099/00207713-47-2-252

Appendix Table 1. Prevalence of tickborne pathogens detected by PCR in ticks from dogs in South Korea, 2010–2015

Species	Region	Stage	No. of tick pools	No. positive (%)					
				E. canis (16S rRNA)	*T. luwenshuni* (18S rRNA)	*R. raoultii* (16S rRNA)	*R. monacensis* (16S rRNA)	*Candidatus* Rickettsia principis (16S rRNA)	Total
Haemaphysalis longicornis	Central	Larva	2	0	0	0	0	0	0
		Nymph	93	0	9 (9.7)	41 (44.1)	0	0	50 (53.8)
		Adult	47	0	11 (23.4)	24 (51.1)	0	0	35 (74.5)*
	Southern	Larva	3	0	0	0	0	0	0
		Nymph	90	1 (1.1)	11 (12.2)	59 (65.6)*	0	1 (1.1)	72 (80.0)
		Adult	45	0	13 (28.9)*	25 (55.6)	0	0	38 (84.4)*
	Subtotal	Larva	5	0	0	0	0	0	0
		Nymph	183	1 (0.6)	20 (10.9)	100 (54.6)	0	1 (0.6)	122 (66.7)
		Adult	92	0	24 (26.1)*	49 (53.3)	0	0	73 (79.3)*
Haemaphysalis flava	Central	Nymph	18	0	0	0	0	0	0
		Adult	10	0	0	0	0	0	0
	Southern	Nymph	12	0	0	0	0	1 (8.3)	1 (8.3)
		Adult	13	0	0	0	0	0	0
	Subtotal	Nymph	30	0	0	0	0	1 (3.3)	1 (3.3)
		Adult	23	0	0	0	0	0	0
Ixodes nipponensis	Central	Nymph	8	0	0	0	0	0	0
		Adult	6	0	0	0	0	0	0
	Southern	Nymph	10	0	0	0	0	0	0
		Adult	7	0	0	0	1 (14.3)	0	1 (14.3)
	Subtotal	Nymph	18	0	0	0	0	0	0
		Adult	13	0	0	0	1 (7.7)	0	1 (7.7)
		Total	364	1 (0.3)	44 (12.1)	149 (40.9)	1 (0.3)	2 (0.6)	197 (54.1)

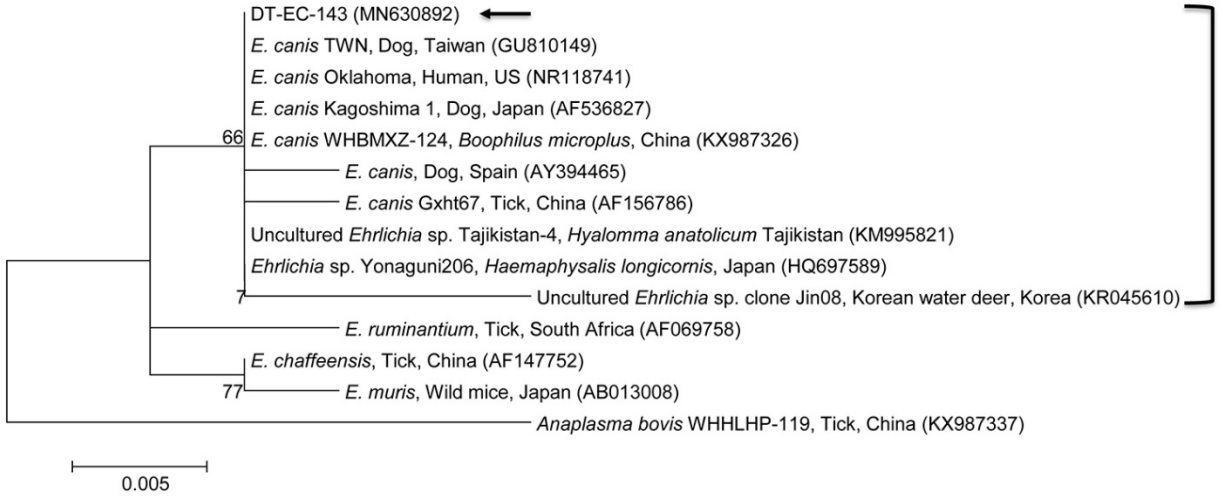
*Designates significant differences in prevalence ($p < 0.05$) among the different stages.

Appendix Table 2. Primers used for the detection of tickborne pathogens in ticks from dogs.

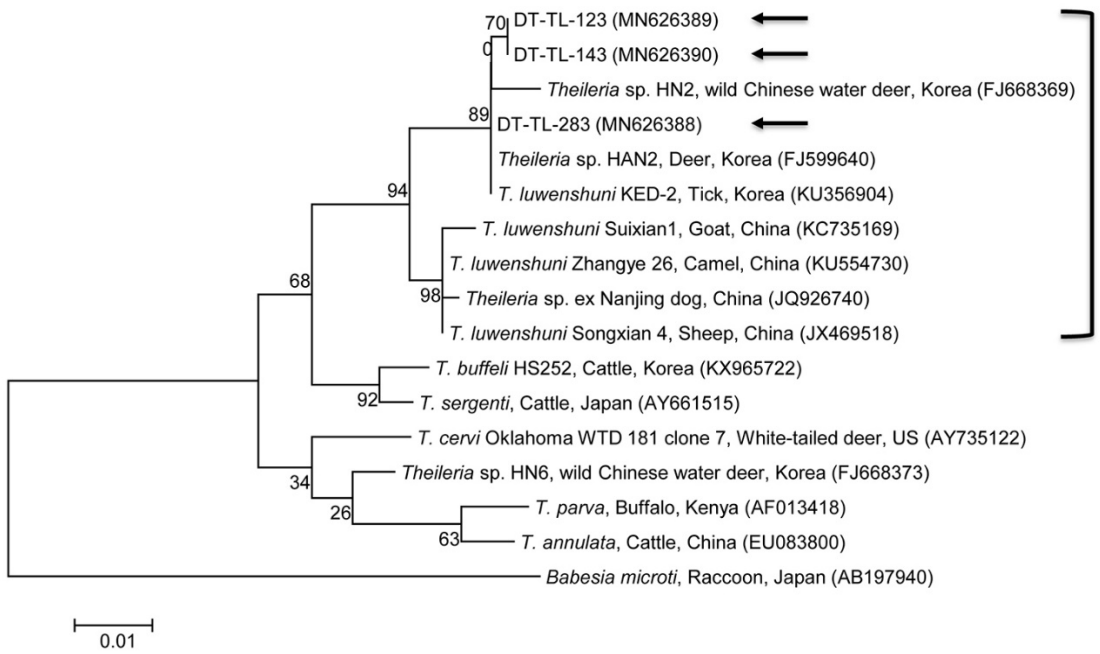
Organism	Gene	Primer	Sequence 5' to 3'	Size (bp)	Amplification condition	Reference
Invertebrate	Mitochondrial cytochrome c oxidase subunit I	LCO1490	GGTCAACAAATCATAAAGATATTGG	710	95°C/5 min; 35 cycles: 95°C/60 s, 40°C/60 s, 72°C/30 s; 72°C/10 min	(*2*)
		HC02198	TAAACTTCAGGGTGACCAAAAAATCA			
Anaplasma spp.	16S rRNA	†		429	95°C/5 min; 45 cycles: 95°C/30 s, 59°C/30 s, 72°C/30 s; 72°C/10 min	Commercial AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer)
Ehrlichia spp.	16S rRNA	†		340	95°C/5 min; 45 cycles: 95°C/30 s, 59°C/30 s, 72°C/30 s; 72°C/10 min	Commercial AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer)
Rickettsia spp.	16S rRNA	†		252	95°C/5 min; 45 cycles: 95°C/30 s, 59°C/30 s, 72°C/30 s; 72°C/10 min	Commercial AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer)
Rickettsia spp.	*glt*A	Rsfg877	GGGGGCCTGCTCACGGCGG	380	95°C/10 min; 35 cycles: 95°C/60 s, 51°C/60 s, 72°C/60 s; 72°C/10 min	(*3*)
		Rsfg1258	ATTGCAAAAAGTACAGTGAACA			
Borrelia spp.	5S–23S rRNA	N1	GAGCTTAAAGGAACTTCTGATAA	561	95°C/5 min; 35 cycles: 95°C/45 s, 54°C/45 s, 72°C/60 s; 72°C/5 min	(*4*)
		C1c	TTTGTACTGTTATTGTGTCTT			
		N2	ATGGWTCTGGAAYRCTYGAAG	533	95°C/5 min; 35 cycles: 95°C/45 s, 50°C/45 s, 72°C/60 s; 72°C/5 min	
		C2c	CTTARAGTAACWGTTCCTTCT			
Coxiella spp.	16S rRNA	Cox16SF1	CGTAGGAATCTACCTTRTAGWGG	1321–1429	93°C/3 min; 30 cycles: 93°C/30 s, 56°C/30 s, 72°C/60 s; 72°C/5 min	(*5*)
		Cox16SR2	GCCTACCCGCTTCTGGTACAATT			
		Cox16SF2	TGAGAACTAGCTGTTGGRRAGT	624–627	93°C/3 min; 30 cycles: 93°C/30 s, 56°C/30 s, 72°C/60 s; 72°C/5 min	
		Cox16SR2	GCCTACCCGCTTCTGGTACAATT			
Bartonella spp.	ITS-1	QHVE-OF	TTCAGATGATGATCCCAAGC	736	94°C/10 min; 35 cycles: 94°C/60 s, 55°C/60 s, 72°C/120 s; 72°C/5 min	(*7*)
		QHVE-OR	AACATGTCTGAATATATCTTC			
		QHVE-IF	CCGGAGGGCTTGTAGCTCAG	484		

Organism	Gene	Primer	Sequence 5' to 3'	Size (bp)	Amplification condition	Reference
		QHVE-IR	CACAATTTCAATAGAAC			
SFTVS	S segment	NP-2F	CATCATTGTCTTTGCCCTGA	461	50°C/30 min; 95°C/15 min; 40 cycles: 95°C/20 s, 52°C/40 s, 72°C/30 s; 72°C/5 min	(*8*)
		NP-2R	AGAAGACAGAGTTCACAGCA			
		N2F	AAYAAGATCGTCAAGGCATCA	346	25 cycles: 94°C/20 s, 55°C/40 s, 72°C/30 s	
		N2R	TAGTCTTGGTGAAGGCATCTT			
Babesia spp. and *Theileria* spp.	18S rRNA	†		676	95°C/5 min; 35 cycles: 95°C/30 s, 59°C/30 s, 72°C/30 s; 72°C/5 min	Commercial AccuPower *Babesia* and *Theileria* PCR Kit (Bioneer)
Babesia spp. and *Theileria* spp.	18S rRNA	BJ1	GTCTTGTAATTGGAATGATGG	452	95°C/5 min; 40 cycles: 94°C/30 s, 54°C/30 s, 72°C/40 s; 72°C/5 min	(*6*)
		BN2	TAGTTTATGGTTAGGACTACG			

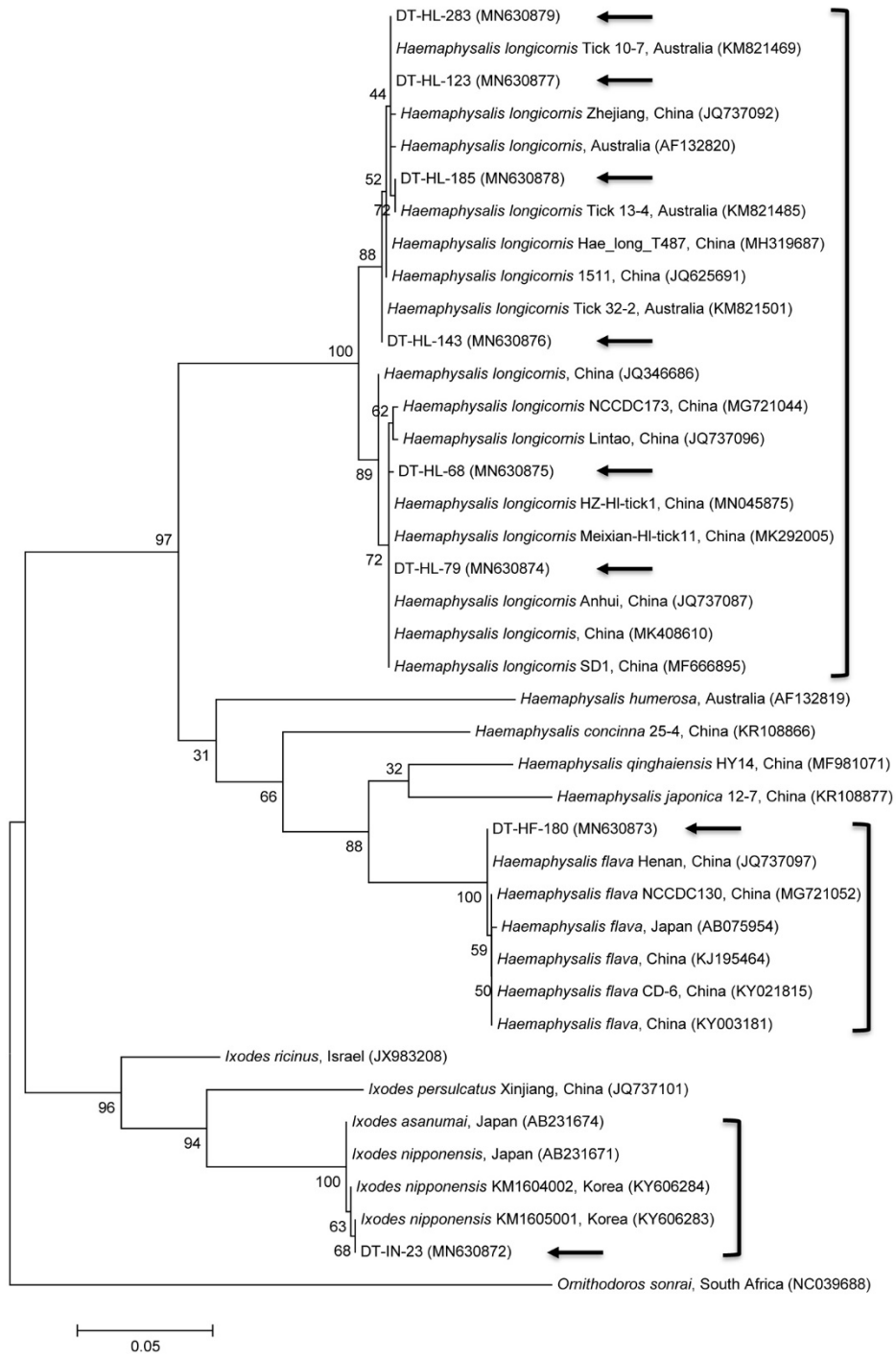
*SFTVS, Severe fever with thrombocytopenia syndrome virus
*Commercial PCR kits were used for the detection of these pathogens.



Appendix Figure 1. Phylogenetic tree constructed using the maximum likelihood method and based on *Ehrlichia canis* 16S rRNA nucleotide sequences. *Anaplasma bovis* was used as the outgroup. Black arrows indicate sequences analyzed in this study. GenBank accession numbers for other sequences are shown with the sequence name. Branch numbers indicate bootstrap support (1,000 replicates). Scale bar indicates phylogenetic distance.



Appendix Figure 2. Phylogenetic tree constructed using the maximum likelihood method and based on *Theileria luwenshuni* 18S rRNA nucleotide sequences. *Babesia microti* was used as the outgroup. Black arrows indicate sequences analyzed in this study. GenBank accession numbers for other sequences are shown with the sequence name. Branch numbers indicate bootstrap support (1,000 replicates). Scale bar indicates phylogenetic distance.



Appendix Figure 3. Molecular identification of ticks based on phylogenetic analysis using the maximum likelihood method with the mitochondrial cytochrome c oxidase subunit I gene. *Ornithodoros sonrai* was used as the outgroup. Black arrows indicate sequences analyzed in this study. GenBank accession numbers for other sequences are shown with the sequence name. Branch numbers indicate bootstrap support (1,000 replicates). Scale bar indicates phylogenetic distance.