Table 1. Clinical characteristics of 14 dogs positive for *Anaplasma bovis*

ID	Prefecture	Breed	Age (yr)	Sex	Tick infestation	History of diseases
J18	Aomori	Setter	7	M	NR	Filariasis
N4	Chiba	Mix	10	F	–	None
N34	Chiba	Border collie	3	M	NR	None
O10	Tokyo	NR	NR	NR	NR	NR
H28	Wakayama	Mix	11	M	NR	None
97	Tottori	Siberian Husky	NR	NR	NR	Heart failure
Z27	Hiroshima	Mix	8	F	+	None
Z37	Hiroshima	Plott hound	5	F	+	None
36	Yamaguchi	Kishu	2	M	NR	Pemphigus
136	Tokushima	Mix	7	M	+	None
138	Tokushima	L.Retriever	8	M	+	None
139	Tokushima	S.Sheep Dog	8	M	+	None
D27	Fukuoka	Shiba	3	M	NR	None
D37	Fukuoka	Mix	5	M	NR	None
8	Kagoshima	NR	NR	NR	NR	NR

NR, not recorded; F, female; M, male; L.Retriever, Labrador retriever; S.Sheep Dog, Shetland sheepdog.

were positive for *A. bovis*. Five of the resulting 15 amplicons were selected at random and their nucleotide sequences determined to confirm the nested PCR results. Sequences of all five samples were identical and showed 100% identity with *A. bovis*. The clinical characteristics of these 15 dogs are shown in Table 1. Their geographical distribution dogs ranged from Aomori Prefecture in the north to Kagoshima Prefecture in the south, although 10 of the 15 dogs lived in western Japan. Tick infestation was reported for five dogs. *Hyalomma* spp., *Rhipicephalus appendiculatus*, and *Amblyomma variegatum* are known vectors of *A. bovis* (20–22), although these particular ticks have not been detected in Japan. However, *A. bovis* DNA has been detected in *H. longicornis* ticks collected in Japan and Korea (11,23). This latter tick species has a geographical distribution ranging from northern to southern Japan and targets a wide variety of host animals (24). Unfortunately, we were unable to identify the tick species found on the dogs in the present study. As there is no evidence for transmission of the pathogen to dogs by ticks, further epidemiological studies are required to clarify the relationship between *A. bovis* infection in dogs and ticks.

A dog (J18) in Aomori Prefecture tested positive for both *A. bovis* and *Wolbachia* sp., although the effect of this dual infection on pathogenicity could not be determined. Furthermore, although one of the dogs which tested positive for *A. bovis* had a history of heart failure, the pathogenicity of *A. bovis* for dogs remains unknown. Veterinarians should be alert to the possible health risks posed by this agent in dogs.

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Conflict of interest None to declare.

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ORIGINAL RESEARCH

Molecular Survey of *Anaplasma* and *Ehrlichia* Infections of Feral Raccoons (*Procyon lotor*) in Hokkaido, Japan

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Abstract

Infection by *Anaplasma* and *Ehrlichia* in feral raccoons (*Procyon lotor*) in Hokkaido, Japan, was examined by molecular methods. A polymerase chain reaction (PCR) screen for Anaplasmataceae, based on 16S rRNA, showed that 38 (5.4%) of 699 raccoons examined were positive. These 38 positive samples were examined for *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Ehrlichia chaffeensis*, and *Ehrlichia canis* infection by species-specific nested PCR. Nested PCR results indicated that 36 of the 38 samples were positive for *A. bovis*. All 38 samples were PCR negative for *A. phagocytophilum*, *E. chaffeensis*, and *E. canis*. This is the first report of the detection of *A. bovis* in the peripheral blood of raccoons. A total of 124 raccoons were infested with ticks, including *Ixodes ovatus*, *Ixodes persulcatus*, and *Haemaphysalis* spp. The rate of *A. bovis* infection in raccoons infested with *Haemaphysalis* spp. (46.7%, 7/15) was significantly higher than that in raccoons without *Haemaphysalis* spp. infestation (3.7%, 4/109, $p < 0.001$). No significant differences were observed in *A. bovis* infection rates between raccoons infested with *I. ovatus* or *I. persulcatus* and those not so infested. A total of four ticks (two males and two nymphs) and one larval pools from four raccoons showed positive for *A. bovis*–specific nested PCR. This results support the correlation between the *A. bovis* infection of raccoons and *Haemaphysalis* infestation. In conclusion, raccoons could be possible reservoir animals for *A. bovis*, and *A. bovis* infection in raccoons may be related to infestation with *Haemaphysalis* spp.

Key Words: *Anaplasma*—*Ehrlichia*—Japan—Raccoon.

Introduction

Members of the family Anaplasmataceae belong to the order Rickettsiales and comprise the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Aegyptianella*, *Wolbachia* and *Candidatus* Neoehrlichia. Members of the Anaplasmataceae family are obligate intracellular, Gram-negative bacteria (Dumler et al. 2001, 2005). Infections with Anaplasmataceae pathogens were previously known only as diseases important to veterinary medicine. However, within the last 2 decades, several new species or strains have been detected as the causative pathogens of emerging infectious diseases in both humans and animals. Especially, *Anaplasma* and *Ehrlichia*, members of this family, are important emerging tick-borne pathogens (Dugan et al. 2005). In nature, they are maintained between ticks and wild mammals, including deer and rodents (Kawahara et al. 2006). Raccoons (*Procyon lotor*) are some of the reservoir animals for *Anaplasma phagocytophilum*, and *Ehrlichia chaffeensis* and *Ehrlichia canis* infection in raccoons has been reported in the United States (Comer et al. 2000, Levin et al. 2002, Dugan et al. 2005, Yabsley et al. 2008). Raccoons are medium-sized carnivores native to North America. Due to the influence of the cartoon "Rascal Raccoon" on television in 1977, raccoons became popular as pet animals in Japan and a large number of raccoons were imported from North America (Yanagihara-Agetsuma 2004). However, the wild nature is eventually manifested in raccoons as they mature (Yanagihara-Agetsuma 2004). As a result of the intentional release or escape of pet raccoons, large numbers of raccoons have naturalized in most parts of Japan (Ikeda 2008).

Anaplasma and *Ehrlichia*, both originally found in the United States (McQuiston et al. 1999, Rikihisa 1991), might have been introduced into Japan by raccoons. DNA fragments of *A. phagocytophilum* and *Anaplasma bovis* have recently been detected from Sika deer (*Cervus nippon*), cattle, and several tick species in Japan (Ohashi et al. 2005, Kawahara et al. 2006, Ooshiro et al. 2008, Jilintai et al. 2009, Wuritu et al. 2009, Yoshimoto et al. 2010). *E. chaffeensis* DNA has also been

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detected from Sika deer in Japan (Kawahara et al. 2009). We therefore attempted to detect *A. phagocytophilum*, *A. bovis*, *E. chaffeensis*, and *E. canis* pathogens of both human and veterinary importance, by molecular analysis of peripheral blood samples obtained from feral raccoons in Japan. The objective of this study was also to clarify the epidemiologic role of raccoons for these pathogens in Japan.

Materials and Methods

Raccoon blood and tick samples

A total of 699 raccoons were captured between May and October 2007 and between March and October 2008, as part of raccoon population control programs implemented by the Hokkaido Government and the Ministry of the Environment in west-central Hokkaido, Japan (Fig. 1). Raccoons were anesthetized with an intramuscular injection of ketamine hydrochloride. Blood samples from the heart were collected in ethylenediaminetetraacetic acid tubes and centrifuged at 1000 *g* for 10 min; plasma was removed and the remaining blood components were frozen at −20°C for polymerase chain reaction (PCR) assays.

Raccoons were also examined for tick infestation. When tick infestation was observed, as many ticks were collected as possible and stored in 70% ethanol for later morphological identification.

DNA extraction and PCR

DNA was extracted from blood or tick samples using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA samples were stored at −20°C in 200 μL of TE buffer until further use.

Screening PCR was performed using the group-specific primer pair, EHR16SD and EHR16SR, which amplifies the 16S rRNA gene of the family Anaplasmataceae (Parola et al. 2000).

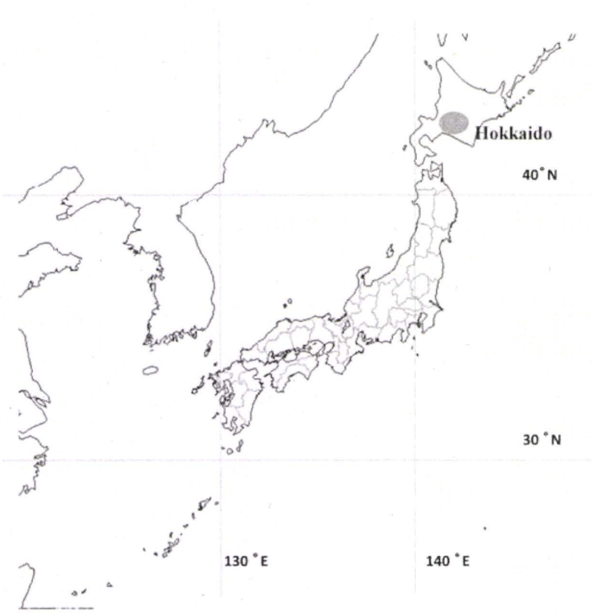

FIG. 1. Shading shows the location of the study area.

Samples that are positive for the screening PCR were examined by species-specific nested PCR. We used the primer pair EC9 and EC12A for the first amplification of *A. phagocytophilum* and *A. bovis* DNA. The resulting PCR products were then used as templates for the species-specific second amplification (Kawahara et al. 2006) with primers AB1f and AB1r for *A. bovis* (Kawahara et al. 2006), and newly designed primers AP-f1 and AP-r1 for *A. phagocytophilum*. The second PCR for *A. phagocytophilum* was performed under the following conditions: 40 cycles of denaturation (95°C, 60 s), annealing (58°C, 60 s), and extension (72°C, 60 s). For *E. chaffeensis*, primers NS16SCH1F and NS16SCH1R were used for the first amplification and NS16SCH2F and NS16SCH2R for the second (Kawahara et al. 2009). For *E. canis*, primers fD1 and EHR16SR were used for the first PCR, and *E. canis*–specific primers CANIS and GA1UR were used for the second PCR (Warner and Dawson 1996). To prevent contamination in nested PCR, we have performed each reaction setup in separate chamber using exclusive pipets and tips. Nucleotide sequences of all primers used here are listed in Table 1. Specificity and sensitivity of newly designed *A.phagocytophilum*–specific nested PCR were examined by using DNA of *A. phagocytophilum*, *A. bovis*, *Anaplasma marginale*, *Anaplasma central*, *Anaplasma platys*, *E. canis*, *Ehrlichia muris*, *Wolbachia pipientis*, *Neorickettsia risticii*, and *R. japonica* DNA. The sensitivity of the nested PCR was also examined by using diluted DNA extracted from *A. phagocytophilum* infected culture cells.

In the nested PCR, DNA samples extracted from the *A. phagocytophilum* strain Webster, *E. chaffeensis* strain Arkansas, and *E. canis* strain Israel were used as positive controls, and distilled water was used as the negative control. No positive controls were used for the *A. bovis*–specific PCR to prevent cross contamination.

Sequencing and phylogenetic analysis

To determine nearly full-length sequences of the 16S rRNA genes amplified by species-specific nested PCR, additional PCR amplifications were performed using primer sets fD1 and EHR16SR, and EHR16SD and Rp2, respectively (Weisburg et al. 1991). When a strong band was detected after PCR, products were purified using the Qiaquick PCR purification kit (QIAGEN GmbH). Direct sequencing of the PCR products and analysis of sequences obtained were performed as described previously (Inokuma et al. 2007b, 2008). Homology searches based on the sequences of the PCR products were performed using BLAST (National Center for Biotechnology Information). Phylogenetic relationships of the obtained sequences to other sequences registered in GenBank were determined using the neighbor-joining method.

Statistical analysis

Chi-squared tests were performed to compare rates of species-specific nested PCR amplification in raccoons infested with each tick species against those from noninfested raccoons. StatMate IV Version 4.01 was used for the analysis, where a *p*-value of <0.05 was considered significant.

Results

In the initial screening PCR, 38 (5.4%) of 699 raccoons examined were positive for Anaplasmataceae. Species-specific

Table 1. Sequences of Primers Used in This Study

Primer name	*Sequence (5′–3′)*	*PCR product size (bp)*	*Reference*
Screening PCR primers for 16S rRNA gene of Anaplasmataceae			
EHR16SD	5′-GGTACCYACAGAAGAAGTCC-3′	345	Parola et al. (2000)
EHR16SR	5′-TAGACATCATCGTTTACAGC-3′		
Universal primers for 16S rRNA gene			
fD1	5′-AGAGTTTGATCCTGGCTCAG-3′	1450	Weisburg et al. (1991)
Rp2	5′-ACGGCTACCTTGTTACGACTT-3′		
EC9	5′-TACCTTGTTACGACTT-3′	1462	Kawahara et al. (2006)
EC12A	5′-TGATCCTGGCTCAGAACGAACG-3′		
Species-specific primers			
CANIS	5′-CAATTATTTATAGCCTCTGGCTATAGGA-3′	410	Warner and Dawson (1996)
GA1UR	5′-GAGTTTGCCGGGACTTCTTCT-3′		
AP-f1	5′-CATGCAAGTCGAACGGTTA-3′	770	Present study
AP-r1	5′-CATCAACACGGAGATAAATTATC-3′		
AB1f	5′-CTCGTAGCTTGCTATGAGAAC-3′	550	Kawahara et al. (2006)
AB1r	5′-TCTCCCGGACTCCAGTCTG-3′		
NS16SCH1F	5′-ACGGACAATTGCTTATAGCCTT-3′	1195	Kawahara et al. (2009)
NS16SCH1R	5′-ACAACTTTTATGGATTAGCTAAAT-3′		
NS16SCH2F	5′-GGGCACGTAGGTGGACTAG-3′	443	Kawahara et al. (2009)
NS16SCH2R	5′-CCTGTTAGGAGGGATACGAC-3′		

PCR, polymerase chain reaction.

nested PCRs on these 38 positive samples showed that 36 were positive for *A. bovis*, whereas all were PCR negative for *A. phagocytophilum*, *E. chaffeensis*, and *E. canis*. Randomly selected 7 positive amplicons among 36 positives for *A. bovis*–specific nested PCR were analyzed for nucleotide sequences to confirm the results. All seven sequences were identical and showed 99.8% nucleotide identity (510/511 bp) to the 16s rRNA gene of *A. bovis* detected from South Africa (U03775). To confirm the result of rest of 2 among 38 positive in the initial screening PCR, the 2 amplicons of the screening PCR were analyzed by direct sequence method; however, they cannot be determined.

In the specificity test for the newly designed *A. phagocytophilum*–specific nested PCR, only *A. phagocytophilum* DNA was positive. The sensitivity test of the nested PCR revealed that it can detect DNA extracted from one infected cell in 1 μL (data not shown).

A total of 13 samples were successfully sequenced over ~1400 bp of the 16S rRNA gene, excluding the primer region. These sequences have been deposited in GenBank under the accession numbers GU937011 to GU937023. The 13 sequences were identical and showed 99.7% nucleotide identity to the 16s rRNA gene of *A. bovis* detected from South Africa (U03775). This sequence was also 99.5% identical to an *A. bovis* 16s rRNA sequence detected from Sika deer in Shimane, Japan (AB211163). In the 16S rRNA gene-based phylogenetic tree, these 13 sequences clustered in the same clade as *A. bovis* (Fig. 2). F2

A total of 672 ticks were collected from 124 of the 699 raccoons. *Ixodes ovatus* was the predominant tick species, followed by *Haemaphysalis* spp. and *Ixodes persulcatus* (Table 2). T2 It was difficult to identify the *Haemaphysalis* specimens at the species level because most of ticks were fully engorged or semi-engorged. The rate of *A. bovis* infection in raccoons infested with *Haemaphysalis* spp. (46.7%, 7/15) was significantly higher than that in raccoons without *Haemaphysalis* spp. infestation (3.7%, 4/109, $p < 0.001$). No significant differences were observed in *A. bovis* infection rates between raccoons infested with *I. ovatus* or *I. persulcatus* and those not so infested (Table 3). T3

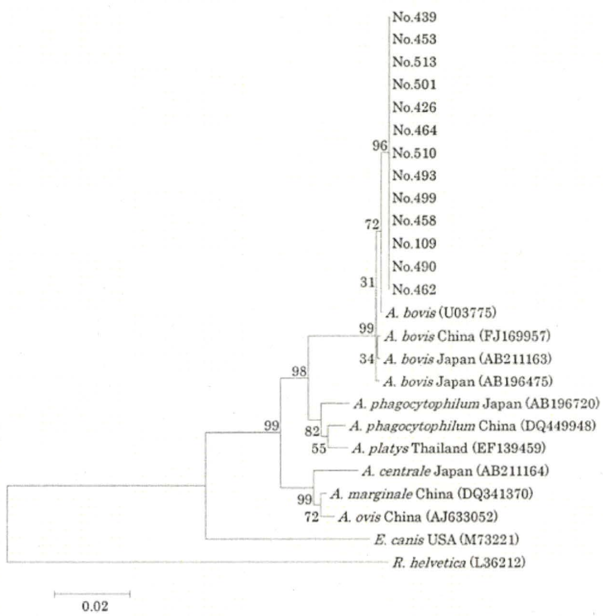

FIG. 2. Phylogenetic relationships among various *Anaplasma* spp. based on the nucleotide sequences of the 16S rRNA gene. Clustal W software and the neighbor-joining method were used to construct the phylogenetic tree. Scale bar represents 2% divergence. Numbers at the nodes are percentages of bootstrap re-samplings supporting the topology shown. Numbers 109, 426, 439, 453, 458, 462, 464, 490, 493, 499, 501, 510, and 513 are identification numbers of raccoons from which DNA was isolated.

TABLE 2. NUMBER OF TICKS RECOVERED FROM 124 RACCOONS IN HOKKAIDO, JAPAN

	Female	*Male*	*Nymph*	*Larvae*	*Total*
Ixodes ovatus	277	64	0	0	341
Ixodes persulcatus	101	20	22	2	145
Haemaphysalis sp.	1	5	43	137	186
Total	379	89	65	139	672

A total of 57 *Haemaphysalis* spp. (4 males, 18 nymphs, and 35 larvae) were collected from 7 raccoons among 36 positives for *A. bovis*–specific nested PCR. DNA was extracted from each adult and nymphal tick. Five larvae were pooled into one tube and DNA was also extracted from each tube. A total of 29 tick samples (4 males, 18 nymphs, and 7 larval pools) were analyzed to detect *A. bovis* by the species-specific nested PCR. As a result, 5 among 29 samples from 4 raccoons were positive (2/4 males, 2/18 nymphs, and 1/7 pools of larvae) (Table 4).

TABLE 4. RESULTS OF *ANAPLASMA BOVIS*–SPECIFIC NESTED POLYMERASE CHAIN REACTION OF *HAEMAPHYSALIS* SPP COLLECTED FROM *ANAPLASMA BOVIS*-POSITIVE RACCOONS

		Number of ticks/pools	
ID of Raccoon	*Stage*	*Examined*	*Positive*
BRA-08-02	N	2	0
BRA-08-08	M	1	0
BRA-08-10	M	1	0
BRA-08-21	M	2	2
BRA-08-42	N	2	1
BRA-08-47	N	7	1
	L	1	0
BRA-08-53	N	7	0
BRA-08-53	L	6	1
Total		29	5

N, nymph; M, male; L, larval pool.

Discussion

In a screening PCR based on 16S rRNA for Anaplasmataceae, 38 of 699 raccoons tested were positive. These 38 positive samples were examined for *A. phagocytophilum*, *A. bovis*, *E. chaffeensis*, and *E. canis* infection by species-specific nested PCR. These samples included 36 that were positive for *A. bovis* and two that were negative in all the nested PCRs. It is possible that those two raccoons were infected with other species in the Anaplasmataceae family. This is the first report of the detection of *A. bovis* from the peripheral blood of raccoons; these results suggest that raccoons could be possible reservoir animals for *A. bovis*.

Although the known vectors of *A. bovis* are *Hyalomma* spp., *Rhipicephalus appendiculatus*, and *Ammblyomma variegatum* (Donatien and Lestoquard 1936, Matson 1967, Rioche 1967), those particular ticks have not been detected in Japan. *A. bovis* DNA has been detected from *Haemaphysalis longicornis* and *Haemaphysalis megaspinosa* collected from Honshu and Hokkaido, respectively (Kawahara et al. 2006, Yoshimoto et al. 2010), and these ticks are possible vectors of *A. bovis* in Japan. In our study, *A. bovis* infection rates in raccoons infested with *Haemaphysalis* spp. were significantly higher than those in raccoons without *Haemaphysalis* spp. infestation. No significant differences in *A. bovis* infection rates were observed between raccoons infested with *I. ovatus* or *I. persulcatus* and those not infested. *A. bovis* infection in raccoons may be related to infestation with *Haemaphysalis* spp. To confirm the correlation between the positive raccoons for *A. bovis*–specific nested PCR and the infestation with *Haemaphysalis* spp., *Haemaphysalis* ticks collected from seven raccoons that showed positive for *A. bovis*–specific nested PCR. As a result, four ticks (two males and two nymphs) and one larval pools from four raccoons showed positive for *A. bovis*–specific nested PCR. This results support the correlation between the *A. bovis* infection of raccoons and *Haemaphysalis* infestation. Negative ticks might not intake enough amount of blood to be infected from the host animals. Further studies are required to confirm the role of *Haemaphysalis* spp. in *A. bovis* infection.

A. bovis causes an economically devastating disease in livestock; its principal symptoms include fever, anorexia, diarrhea, and, infrequently, involvement of the central nervous system (Matson 1967). Because raccoons frequently come near areas where humans and domestic animals live, it is possible that *A. bovis* infection is spread widely among domestic animals by raccoons.

All 38 samples subjected to nested PCR were negative for *A. phagocytophilum*, *E. chaffeensis*, and *E. canis*. *A. phagocytophilum* causes granulocytic anaplasmosis in humans, dogs, and horses, and pasture fever in ruminants (Chen et al. 1994, Rikihisa 2006, Inokuma 2007). Recently, *A. phagocytophilum* DNA has been detected from *I. persulcatus*, *I. ovatus*, Sika deer, and cattle in Japan, including Hokkaido (Ohashi et al. 2005, Kawahara et al. 2006, Ooshiro et al. 2008, Jilintai et al. 2009, Wuritu et al. 2009, Yoshimoto et al. 2010). *E. chaffeensis* is an agent that causes monocytotropic ehrlichiosis in humans and dogs (Paddock and Childs 2003, Inokuma 2007), and its DNA was recently detected from Sika deer in Japan (Kawahara et al.

TABLE 3. NUMBER OF RACCOONS INFESTED WITH TICKS IN HOKKAIDO, JAPAN

		Haemaphysalis *sp.*		I. ovatus		I. persulcatus	
		Infestation	*No infestation*	*Infestation*	*No infestation*	*Infestation*	*No infestation*
PCR	Positive[a]	7 (46.7%)[b]	4 (3.7%)[b]	6 (6.6%)	5 (15.2%)	2 (4.0%)	9 (12.2%)
	Negative[c]	8	105	85	28	48	65
Total		15	109	91	33	50	74

[a]Number of raccoons species-specific nested PCR positive for *Anaplasma bovis*.
[b]$p < 0.001$.
[c]Number of raccoons species-specific nested PCR negative for *A. bovis*.

2009). Although raccoons are some of the important reservoir animals of *A. phagocytophilum* and *E. chaffeensis* in the United States (Comer et al. 2000, Levin et al. 2002, Dugan et al. 2005, Yabsley et al. 2008), neither of these pathogens were detected from raccoons in this study.

E. canis is an important pathogen that causes canine ehrlichiosis (Inokuma et al. 2003). Although there have been reports of raccoons infected with *E. canis* and seropositive for *E. canis* in the United States and Japan, respectively (Dugan et al. 2005, Inokuma et al. 2007a), *E. canis* DNA was not detected from raccoons in this study. It is possible that *E. canis* was not introduced into Japan by raccoons.

This study suggests that raccoons could be possible reservoir animals for *A. bovis* and that they play an important role in the maintenance of *A. bovis* in nature. More epidemiologic studies are required to confirm the epidemiologic role of raccoons in *A. bovis* infection.

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Disclosure Statement

No competing financial interests exist.

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AUTHOR QUERY FOR VBZ-2010-0052-SASHIKA_1P

AU1: Please expand TE.
AU2: Please expand the genus name in "*R. japonica.*"
AU3: Disclosure Statement accurate? If not, please amend as needed.
AU4: In Ref. "Dumler et al. 2005" please mention the editor's name.
AU5: Please confirm the corresponding author's name and address.

短　報

日本紅斑熱発生地域および近隣の発生が少ない地域における知識および受診行動

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序　文

日本紅斑熱は治療が遅れると重症化することがあるため，早期診断と適切な抗菌薬による早期治療が必要な疾患であり，地域住民や医師への啓発が重要といわれている[1)2)]．1984年に我が国で初めて症例が報告され[1)]，兵庫県では1988年に淡路島で県内初の患者が確認され，毎年1～4例の患者が発生し，2008年に初めて淡路島外から1例が報告された（兵庫県淡路県民局洲本健康福祉事務所，以下洲本保健所）．淡路島では以前より，洲本保健所が一般市民に対し積極的に啓発活動を行っている[3)]．今回，兵庫県の発生・啓発状況の異なる2地域で日本紅斑熱の知識，情報入手手段と受診行動の実態を調査し，関連を検討した．

方　法

2009年5月～7月の期間に，自記式質問紙票調査（別紙1）による横断研究を行った．まず，淡路島内の日本紅斑熱多発地帯に位置するA校を選び，さらに近隣市（非多発地帯）で進学・就職率等で類似したB校を比較対象に選んだ．A校とB校の所在地を発生地とともに示す（Fig. 1）[1)]．それらの生徒の保護者（A校：299名，B校：469名）を対象に自記式質問票への回答を依頼した．調査項目は回答者の属性，疾患知識，受診行動とした（Fig. 2）．分析方法は学校別に疾患知識および疾患知識と受診行動に関連する要因を解析（Fisher法，P-value 0.05未満を有意とした）した．回答者のうち，医療福祉関係者は解析から除き，率を算出する際は分母から無回答を除いた．

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平成23年3月20日

結果および考察

A校は保護者246名から有効回答が得られ（有効回答率82%），そのうち医療・福祉関係者を除くと187名であった．B校は保護者283名から有効回答があり（有効回答率60%），そのうち医療・福祉関係者を除くと244名であった．性別は両校とも女性が多かった（A校：87%，B校89%）．年齢はA校では年齢の記入のある183名のうち142名（78%）が40代で，平均値44.9歳（標準偏差4.34），中央値は45歳で，B校では235名のうち179名（76%）が40代で，平均値45.1歳（標準偏差6.57）中央値は45歳であった．両校間で，年齢の平均値に有意差はなかった（Welchの検定，p=0.66）．疾患知識について比較すると（Table 1），2校間で有意差が認められたのは，「病名を知っている」（p-value=0.029）と「症状を知っている」（p-value=0.039）であった．知識の有無と受診行動との関連については（Table 2），A校では有意差は認められなかった．B校は，「感染経路を知っている」群（p-value=0.028）と「症状を知っている」群（p-value=0.0009）で疾患知識がない群に比べ有意に受診率が高かった．

疾患知識全般について，日本紅斑熱の報告の多い地域のA校のほうが，これまで報告がない地域のB校と比較して，「病名を知っている」と「症状を知っている」率が有意に高かった．これは保健所等による啓蒙活動による[3)]ところが大きいと推察される．疾患知識と受診行動の関係をみると，B校において，症状と感染経路に関する知識との正の関連があった．つつが虫病に関しては松井らがその関連を示したが[5)]，日本紅斑熱に関しては本研究が初めて示した知見である．今回の調査では医療福祉関係者が比較的多いのは，両

Fig. 1 School A and B sites
Prefectures where Japanese spotted fever was reported are in black (2006-2009)[1]

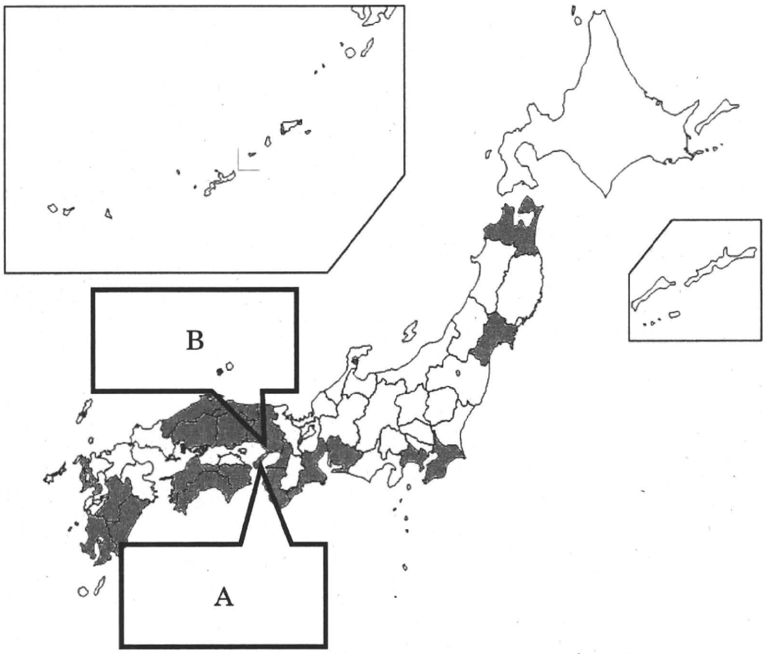

Table 1 Knowledge of Japanese spotted fever (JSF)

	School	Name	Infection route	Symptoms	Infection site	Mortality
JSF	A (n=187)	*58 (31%)*]*	13 (7%)	*30 (16%)*]*	3 (2%)	14 (8%)
	B (n=244)	*52 (21%)*	12 (5%)	*22 (9%)*	1 (0.4%)	10 (4%)

School A is on Awaji island, where many JSF cases were reported. School B is in Kobe, where few cases were reported.
* $p<0.05$
JSF: name ($p=0.029$), symptoms ($p=0.039$)

Table 2 Knowledge of Japanese spotted fever (JSF)

		With knowledge			Without knowledge				
		consult	Not consult	Consultation	Consult	Not consult	Consultation	Odds	p
A+B	name	77	33	70% (77/110)	227	88	72% (227/315)	0.91	0.77
	infection route	20	5	80% (20/ 25)	280	113	71% (280/393)	1.61	0.49
	symptoms	43	9	82% (43/ 52)	257	109	70% (257/366)	2.03	0.08
	infection site	4	0	100% (4/ 4)	296	118	71% (296/414)	3.60	0.37
	mortality	21	3	88% (21/ 24)	278	115	70% (278/393)	2.90	0.11
A	name	35	23	60% (35/ 58)	93	33	74% (93/126)	0.54	0.097
	infection route	8	5	62% (8/ 13)	119	49	71% (119/168)	0.66	0.67
	symptoms	21	9	70% (21/ 30)	106	45	70% (106/151)	0.99	>0.99
	infection site	3	0	100% (3/ 3)	124	54	70% (124/178)	3.06	0.47
	mortality	11	3	79% (11/ 14)	116	51	69% (116/167)	1.62	0.70
B	name	42	10	81% (42/ 52)	134	55	71% (134/189)	1.72	0.21
	infection route	12	0	100% (12/ 12)	161	64	72% (161/225)	9.99	0.028
	symptoms	22	0	100% (22/ 22)	151	64	70% (151/215)	19.16	0.0009
	infection site	1	0	100% (1/ 1)	172	64	73% (172/236)	1.12	>0.99
	mortality	10	0	100% (10/ 10)	162	64	72% (162/226)	8.34	0.055

School A is on Awaji island, where many JSF cases were reported. School B is in Kobe, where few cases were reported.

Fig. 2 Questionnaire used in this study

別紙1 質問票 (1枚目)

「このアンケートの趣旨に同意して下さるかどうか」に関してお伺いします。

質問1	このアンケートの趣旨に同意してくださいますか？	（同意する ・ 同意しない）

このアンケートの趣旨に同意下さいました方は以降の質問にお答えください。

「ある症状が出た時に受診するかどうか」に関してお伺いします。

質問2	もしあなたが2日～30日前に山野や畑に行き、発熱、発疹(ほっしん)があり、ダニのさし口がある(またはダニにかまれたかもしれない)時、医師の診察を受けますか？	(はい・いいえ)

「つつが虫病」に関してお伺いします。

質問3-(1)	「つつが虫病」という病名を聞いたことがありますか？	(はい・いいえ)
質問3-(2)	質問3-(1)で「はい」と答えた方に質問します。「つつが虫病」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )
	以降の質問はすべての方にお伺いします。	
質問4	「つつが虫病」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問5	「つつが虫病」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問6	兵庫県内で「つつが虫病」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問7	「つつが虫病」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問8	これまでの質問を答えた後で「つつが虫病」について情報を得たいと思いましたか？	(はい・いいえ)
質問9	今後、もしあなたが「つつが虫病」について情報を得るとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

「日本紅斑熱(にほんこうはんねつ)」に関してお伺いします。

質問10-(1)	「日本紅斑熱」という病名を聞いたことがありますか？	(はい・いいえ)
質問10-(2)	質問10-(1)で「はい」と答えた方に質問します。「日本紅斑熱」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

別紙1 質問票 (2枚目)

	以降の質問はすべての方にお伺いします。	
質問11	「日本紅斑熱」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問12	「日本紅斑熱」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問13	兵庫県内で「日本紅斑熱」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問14	「日本紅斑熱」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問15	これまでの質問を答えた後で「日本紅斑熱」について情報を得たいと思いましたか？	(はい・いいえ)
質問16	今後、もしあなたが「日本紅斑熱」について情報を得るとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他( )

「回答いただいているご自身」についてお伺いします。

質問17	年齢	(歳)
質問18	性別	(男 ・ 女)
質問19	学校に通われております生徒様との続柄	1.祖父 2.祖母 3.父親 4.母親 5.兄 6.姉 7.その他( )
質問20	居住されている市	(市)
質問21	御職業	1.農業 2.林業 3.医療・福祉 4.建築業 5.無職 6.その他( )
質問22	仕事中に草木や土に触れる機会はありますか？	(はい・いいえ)
質問23	仕事以外の趣味(キャンプや登山等)や生活(山菜とり等)を目的として山にどの程度入りますか？	1.ほぼ毎日 2.ほぼ毎週 3.ほぼ毎月 4.半年に1回ぐらい 5.年1回ぐらい 6.入らない
質問24	以前あなたまたは周囲の人が「つつが虫病」にかかったことがありますか？	(はい・いいえ)
質問25	以前あなたまたは周囲の人が「日本紅斑熱」にかかったことがありますか？	(はい・いいえ)

以上でアンケートは終了です。ご協力ありがとうございました。

校の医学部進学率がいずれも10% 程度と高い（医師は子を医学部に入れる傾向が見られる）ことによる可能性も考えられ，医療関係者は一般市民の調査を目的としたため解析からは除いた．今後同様の調査をする場合は対象の代表性確保が重要である．今回は知識があるかどうかという質問項目のみであったため，よりよい啓発を行うためには，より詳細な知識とリスク認知，およびそれらの受診行動への影響を今後も調査していく必要がある．その際には，各々の知識の正確さなども含めることが肝要と思われた．

なお，この研究の一部は平成21年度厚生労働科学研究費補助金リケッチアを中心としたダニ媒介性細菌感染症の総合的対策に関する研究（主任研究者：岸本壽男）により実施された．調査にご協力下さいました，関係各位に深謝致します．

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Knowledge and Attitude in Medical Behavior in Japanese Spotted Fever in Endemic and Non-endemic Areas

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Fig. 2　Questionnaire used in this study

別紙1　質問票 (1枚目)

「このアンケートの趣旨に同意して下さるかどうか」に関してお伺いします。

質問1	このアンケートの趣旨に同意してくださいますか？	（同意する ・ 同意しない）

このアンケートの趣旨に同意下さいました方は以降の質問にお答えください。

「ある症状が出た時に受診するかどうか」に関してお伺いします。

質問2	もしあなたが2日～30日前に山野や畑に行き、発熱、発疹(ほっしん)があり、ダニのさし口がある(またはダニにかまれたかもしれない)時、医師の診察を受けますか？	(はい・いいえ)

「つつが虫病」に関してお伺いします。

質問3-(1)	「つつが虫病」という病名を聞いたことがありますか？	(はい・いいえ)
質問3-(2)	質問3-(1)で「はい」と答えた方に質問します。 「つつが虫病」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他(　　　)
	以降の質問はすべての方にお伺いします。	
質問4	「つつが虫病」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問5	「つつが虫病」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問6	兵庫県内で「つつが虫病」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問7	「つつが虫病」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問8	これまでの質問を答えた後で「つつが虫病」について情報を得たいと思いましたか？	(はい・いいえ)
質問9	今後、もしあなたが「つつが虫病」について情報を得るとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他(　　　)

「日本紅斑熱(にほんこうはんねつ)」に関してお伺いします。

質問10-(1)	「日本紅斑熱」という病名を聞いたことがありますか？	(はい・いいえ)
質問10-(2)	質問10-(1)で「はい」と答えた方に質問します。 「日本紅斑熱」という病名は、どういうところで聞きましたか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他(　　　)

別紙1　質問票 (2枚目)

	以降の質問はすべての方にお伺いします。	
質問11	「日本紅斑熱」は屋外でダニにかまれることにより感染するということを知っていましたか？	(はい・いいえ)
質問12	「日本紅斑熱」は発熱と発疹を起こす病気ですが、このことを知っていましたか？	(はい・いいえ)
質問13	兵庫県内で「日本紅斑熱」に感染する可能性がある地域があることを知っていましたか？	(はい・いいえ)
質問14	「日本紅斑熱」は場合によっては命にかかわる病気であることを知っていましたか？	(はい・いいえ)
質問15	これまでの質問を答えた後で「日本紅斑熱」について情報を得たいと思いましたか？	(はい・いいえ)
質問16	今後、もしあなたが「日本紅斑熱」について情報を得ようとした場合どのような手段で情報を得たいですか？ (あてはまるもの、すべてに○をつけて下さい)	1.家族・知人 2.新聞 3.テレビ 4.医学書・医学雑誌 5.医学書以外の本・雑誌 6.保健所 7.講演 8.病院 9.インターネット 10.その他(　　　)

「回答いただいているご自身」についてお伺いします。

質問17	年齢	(　　　歳)
質問18	性別	(男 ・ 女)
質問19	学校に通われております生徒様との続柄	1.祖父 2.祖母 3.父親 4.母親 5.兄 6.姉 7.その他(　　　)
質問20	居住されている市	(　　　市)
質問21	御職業	1.農業 2.林業 3.医療・福祉 4.建築業 5.無職 6.その他(　　　)
質問22	仕事中に草木や土に触れる機会はありますか？	(はい・いいえ)
質問23	仕事以外の趣味(キャンプや登山等)や生活(山菜とり等)を目的として山にどの程度入りますか？	1.ほぼ毎日 2.ほぼ毎週 3.ほぼ毎月 4.半年に1回ぐらい 5.年1回ぐらい 6.入らない
質問24	以前あなたまたは周囲の人が「つつが虫病」にかかったことがありますか？	(はい・いいえ)
質問25	以前あなたまたは周囲の人が「日本紅斑熱」にかかったことがありますか？	(はい・いいえ)

以上でアンケートは終了です。ご協力ありがとうございました。

Public Health

Note

High incidence of rickettsiosis correlated to prevalence of *Rickettsia japonica* among *Haemaphysalis longicornis* tick.

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Running head

SURVEILLANCE OF ENDEMIC SFG RICKETTSIOSIS

Abstract

Endemic spotted fever group rickettsiosis was reported in Shimane prefecture, Japan. From an analysis of 14 clinical cases found in the endemic area, the infectious agent of spotted fever group rickettsiosis was identified as *Rickettsia japonica*. In this study, we also found that *Rickettsia japonica* was highly infected with the vector tick, *Haemaphysalis longicornis*, in the endemic area. These findings suggest that the high incidence of rickettsiosis in Shimane prefecture can be explained by the high prevalence of *Rickettsia japonica* among *Haemaphysalis longicornis* ticks.

Key words

Ixodid ticks, Japanese deer, Japanese Spotted Fever, *Rickettsia japonica*, Shimane Peninsula

Spotted fever group (SFG) rickettsiosis is known as an important arthropod-borne infectious disease throughout the world. In Japan, Japanese spotted fever (JSF), which is caused by infection of *Rickettsia japonica* (*R.japonica*), is known as the tick-borne SFG rickettsiosis. Since the first case of JSF was reported in 1984 [6], 800 or more cases of SFG rickettsiosis have been reported, mainly in the western part of Japan and Pacific coastal areas [8]. In Shimane Prefecture, 110 cases of SFG rickettsiosis were serologically confirmed between 1987 and 2009 [4,9,10,12], of which 102 cases were found in the Misen mountains in the western area of Shimane Peninsula (Figure). However, it is unclear why an endemic focus of SFG rickettsiosis was found in Shimane prefecture.

In order to solve this problem, we attempted to identify the endemic *Rickettsia* species infecting the cases of SFG rickettsiosis cases reported in a disease accumulation locus. Furthermore, numerous ixodid ticks collected in Shimane Prefecture were provided for genetic study to identify the most competent vector species of the pathogenic *Rickettsia*.

We obtained 24 specimens from 14 patients confirmed serologically with SFG rickettsiosis as follows: 14 blood specimens (6 blood clots and 8 whole blood samples), 8 eschars, and skin samples from one patient with a biting tick. The biting tick was morphologically identified as a female *Haemaphysalis longicornis* (*H.longicornis*) tick. These samples were collected in 2008 or 2009, and all were obtained from patients infected in the Misen mountains.

We extracted DNA using the Generation Capture column Kit (QIAGEN, Germantown, MD, USA) according to manufacture's instruction. Blood clot was homogenized in PBS, then 200 μl of supernatant of homogenized sample was used for DNA extraction. Eschar and skin samples were pretreated with protease before DNA extraction. Whole blood was used directory for DNA extraction.

The *Rickettsia* 17-kDa genus-common antigen gene and citrate synthesis gene (g*ltA*) were amplified using previously published primer pairs, R1 (5'- TCAATTCACAACTTGCCATT-3') and R2 (5'- TTTACAAAATTCTAAAAACC-3') [1], and RpCS877p (5'-GGGGGCCTGCTCACGGCGG-3') and RpCS1258n (5'-ATTGCAAAAAGTACAGTGAACA-3') [3], respectively. Following PCR amplification, DNA fragments were separated by agarose gel electrophoresis and extracted using the Qiaex Gel Extraction Kit (QIAGEN). DNA sequencing was performed using an ABI PRISM® BigDye™ Terminator v1.1 Kit (Life Technologies, Carlsbad, CA, USA) using an ABI Prism 310 Genetic Analyzer (Life Technologies). The nucleotide sequences were compared with the corresponding sequences deposited in GenBank using BLAST (http://blast.ddbj.nig.ac.jp/top-j.html).

We succeeded in detecting both the *Rickettsia* 17-kDa genus-common antigen gene and the *gltA* gene from 4 samples of whole blood, 8 eschars, and both a skin sample with a biting tick and the tick itself (Table 1). All nucleotide sequences of the *Rickettsia* 17-kDa genus-common antigen gene and the *gltA* gene were found to be 100% identical to *R. japonica* (Accession nos. D16515, and DQ909073) and also distinguishable from other *Riskettsia* species. These results indicate that *R. japonica* is the causative agent of the SFG rickettsiosis in the Misen mountains, suggesting that JSF is endemic in this area.

From 1999 to 2009, we captured 2,099 adult ticks by the flagging method in the Misen mountaines (Area A: ca. 10km wide), in the area east of the Misen mountains (Area B:ca.10 km wide neighboring the Misen mountains), in the eastern half of Shimane Peninsula (Area C: ca.40 km wide) and in the Chugoku mountains in the southern part of Shimane Prefecture (Area D) (Figure). Ticks were morphologically identified. We also extracted DNA from 2,099 ixodid ticks individually as same as above method, and then used for PCR examination. The prevalence of *Rickettsia* in ticks was compared across the surveyed area by Fisher's exact test. P-values less than 0.05 were considered significant.

Of 2,099 ticks, 1,149 were collected in the Misen mountains (Area A), and 584, 294, and 117 ticks were collected in Area B, C and D, respectively (Figure and Table 2). These ticks were morphologically identified and were found to include 2 genera and 8 species (Table 2). These collection sizes were mostly associated with the incidence of SFG rickettsiosis cases in the 4 areas, and Area A, as the most endemic area, should be surveyed in detail.

The *R. japonica* DNA was detected from 15 *H. longicornis* (4.19%) and 1 *Ixodes ovatus* (*I.ovatus*) (5.26%) in Area A, and one each of *H. longicornis* (0.55%) and *I. ovatus* (1.20%) in Area B (Table 2). The DNA sequences of the ticks were identical to those of *R. japonica* (Accession nos. D16515 and DQ909073) and DNA from clinical specimens. Statistical analysis revealed that the prevalence of *R. japonica* in ticks collected in Area A was significantly higher than that in Areas B－D (Fisher's exact test of Area A to Areas B－D: P-value = 0.0032). In particular, the frequency of *R. japonica* was higher among *H. longicornis* than among other ticks in Area A (Fisher's exact test of *H. longicornis* to sum of others in Area A: P-value = 2.36e-07) (Table 2). These analyses suggested that *R. japonica* is highly prevalent among the population of *H. longicornis* ticks inhabiting the Misen mountains (Area A) in Shimane Prefecture.

Rickettsia DNA was also detected from *I. ovatus* collected in Areas A and B. The tick was previously expected as a potential transmission vector of *R. japonica* in Japan. However, it was still unclear whether the tick correlates with disease accumulation in Misen mountains, because