Survey of tick fauna possessing the ability to act as vectors of rickettsiosis in Toyama Prefecture, Japan

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Abstract: During 1991–2007 a total of 3,562 ixodid ticks were collected by flagging at altitudes from 20 to 1,470 m in Toyama Prefecture. Two genera and nine species were identified as follows: *Haemaphysalis flava*, *H. japonica*, *H. kitaokai*, *H. longicornis*, *H. megaspinosus*, *Ixodes monospinosus*, *I. nipponensis*, *I. ovatus*, and *I. persulcatus*. *Ixodes ovatus* was the most dominant species at elevations of 400 m and above, but was also distributed at the low altitudes. *Haemaphysalis flava* was predominant below 401 m above sea level. *Haemaphysalis kitaokai*, *H. megaspinosus*, and *I. monospinosus* were newly recorded in Toyama Prefecture. Rickettsial DNA detected from *I. monospinosus* was closely related to *Rickettsia helvetica*, indicating a potential threat of spotted fever group rickettsial infection in Toyama Prefecture.

Key words: Honshu, *Haemaphysalis*, *Ixodes*, Ixodidae, spotted fever group *Rickettsia*, vertical distribution

INTRODUCTION

Ticks transmit a greater variety of pathogenic microorganisms than any other arthropod vector groups, and are one of the most important vectors carrying diseases to humans, livestock, and companion animals (Jongejan and Uilenberg, 2004). In Japan, patients of tick-borne diseases including Japanese spotted fever (JSF), Lyme disease, tularemia, and tick-borne encephalitis have been reported (Takada, 2007). Recently, fatal cases of JSF caused by *Rickettsia japonica* were reported in Japan (Nomura et al., 2007), and concerns for tick-borne diseases are increasing. Because endemic

areas of spotted fever group (SFG) rickettsioses are rather latent, careful monitoring of SFG rickettsioses as an emerging infectious disease is required even in Toyama Prefecture where JSF patients have not yet been identified.

The occurrence of tick-borne disease is essentially correlated with the distribution of vector ticks. Therefore, it is important to survey tick fauna and detect pathogenic microorganisms in ticks. Although eight tick species have so far been recorded in Toyama Prefecture: *Amblyomma testudinarium*, *Haemaphysalis flava*, *H. japonica*, *H. longicornis*, *Ixodes acutitarsus*, *I. nipponensis*, *I. ovatus*, *I. persulcatus* (Ishikura et al., 1994, 2003; Kamimura and Kondo, 1977; Kishida,

Fig. 1. Map showing survey sites in Toyama Prefecture.

Table 1. A list of collection sites and altitudes.

No.	Site	Altitude (m a.s.l.)	No.	Site	Altitude (m a.s.l.)
1	Daira-takifuchi	240–300	18	Furudô-no-mori	110–140
2	Sôgatake-tozanguchi	520–618	19	Sarukurayama	345
3	Sôgatake-guchi	850	20	Shirakimine	750
4	Banbajima	900	21	Tochioretôge	520–600
5	Daikandai	1,470	22	Kitajima	445–500
6	Tateyama-bunadaira	1,200	23	Togamura-kamimomose	740
7	Bunasaka	1,100	24	Yamanokami	840
8	Tateyama-bijodaira	970–1,000	25	Tamukai	350
9	Hanakiri	600–700	26	Oku-daikanba	950
10	Kamegai-akasaka	600–610	27	Togamura-mizunashi	900–1,000
11	Tongariyama	300	28	Arayamatôge	350–386
12	Arimine-inonedani	1,200	29	Kakuma	150–200
13	Arimine-oritate	1,400	30	Iôzen	780–800
14	Arimine-tôgedani	1,200	31	Iôzen-guchi	250–280
15	Arimine-ôtawa	1,290	32	Ogaya	250
16	Jyôyama	100	33	Bunaotôge	980–990
17	Taikôyama	20			

1930; Kondo et al., 1976; Shimada et al., 2003), there is not enough information on tick distributions in the prefecture. As for

the rickettsial genes, several genotypes were detected in Toyama Prefecture until 2000 (Ishikura et al., 1994, 2003).

In the present study, we collected and classified ixodid ticks in Toyama Prefecture and identified rickettsial genes in the ticks by polymerase chain reaction (PCR). In this way, we tried to clarify the distribution of ticks as vectors of spotted fever group rickettsiae (SFGR) and assess the risk of SFGR infection from ticks.

MATERIALS AND METHODS

Collection of ixodid ticks

Ixodid ticks were collected by flagging at 33 sites (Fig. 1, Table 1) in Toyama Prefecture during 1991–2007. The survey sites ranged from 20 to 1,470 m above sea level (a. s. l.). Of these, three sites (No. 16–18) were located near residential areas and the remaining sites in mountainous areas. Field surveys by flagging were carried out 104 times. All of the adults and some of the nymphs attached to the white flannel flags were collected. Live ticks were identified by a microscope based on Yamaguti et al. (1971), Takada (1990), and Fujita and Takada (2007). After identification, some specimens were preserved under refrigeration or in 70% ethanol until PCR analysis.

Detection of SFGR DNA

From the ticks collected during 2006 and 2007, 184 adults and 59 nymphs (24 adults in 2006, 160 adults and 59 nymphs in 2007) were used for PCR analysis. The nymphs were separated into 16 groups containing five or less individuals. These samples were homogenized in PBS (–) and DNA was extracted with a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's procedures. To amplify the 17-kDa outer membrane antigen gene, PCR was carried out with puRe Taq Ready-To-Go PCR Beads (GE Healthcare) using primers R1 and R2 for genus *Rickettsia* and primers Rj5 and Rj10 for *R. japonica* that amplify 537–540 bp and 357 bp, respectively, as described by Furuya et al. (1995). The mixture containing 2.5 μ l of DNA was incubated at 95°C for 2 min

and 35 cycles at 94°C for 45 sec, 52°C for 30 sec and 72°C for 45 sec.

After purification of the amplicons, the sequences were determined with the BigDye Terminator v3.1 Cycle sequencing Kit and ABI 3130 sequencer (Applied Biosystems). The sequences of the 17-kDa genes were aligned using Clustal W (version 1.83). A phylogenetic tree was constructed by the neighbor-joining (NJ) method.

RESULTS

Collection of ixodid ticks

A total of 3,562 ixodid ticks was collected. They were morphologically identified as two genera and nine species (Table 2). *Ixodes ovatus* was the most abundant (49.6%), followed by *H. flava* (34.1%), *I. persulcatus* (9.5%), *H. japonica* (4.5%), and *H. longicornis* (1.9%). In contrast, four other species were captured in small numbers at limited sites: *H. kitaokai* was captured only at site No. 4; *H. megaspinosus* only at site No. 18; *I. monospinosus* only at sites No. 2 and 3; *I. nipponensis* only at sites No. 16 and 18 (Table 1, Fig. 1). At the sites near residential areas (No. 16–18), the following five species: *H. flava*, *H. longicornis*, *H. megaspinosus*, *I. nipponensis*, *I. ovatus* were collected. *Haemaphysalis kitaokai*, *H. megaspinosus*, and *I. monospinosus* were newly recorded from Toyama Prefecture.

Figure 2 shows the species constructions of adult ticks at different altitudes. *Haemaphysalis flava*, *H. japonica*, *I. ovatus*, and *I. persulcatus* were captured at all altitudes, but species constructions of them vary depending on the altitude. *Ixodes ovatus* was the most dominant species at elevations from 400 m and above, but was also distributed at low altitudes in the relative high abundance. *Haemaphysalis flava* was predominant below 401 m, diminished at higher altitudes and very few seen above 1,200 m. The abundance of *I. persulcatus* increased at higher altitudes, and was very low below 401 m.

Table 2. Number of individuals of ixodid ticks collected by the flagging method at 33 sites in Toyama Prefecture.

Tick		Collection site																																	Total	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33		
<i>Haemaphysalis flava</i>	♀	0	0	0	0	0	0	0	8	0	2	0	0	1	0	0	0	0	487	1	0	1	0	0	0	0	0	0	0	8	5	5	2	5	1	526
	♂	1	0	0	0	0	0	0	3	0	2	0	0	1	0	0	0	0	368	0	0	0	0	0	0	0	0	0	0	6	3	4	1	0	0	389
	Nymph	0	5	0	0	0	0	0	20	10	5	0	0	2	0	0	0	20	143	0	0	0	0	0	0	1	1	0	1	56	5	24	0	7	0	300
<i>H. japonica</i>	♀	2	2	0	3	3	0	1	8	0	1	1	0	11	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	35
	♂	3	3	0	2	0	0	0	10	1	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	28	
	Nymph	3	34	0	0	2	0	0	13	12	2	0	0	19	0	1	0	0	0	0	0	0	0	0	1	2	0	1	0	1	0	5	0	0	1	97
<i>H. kitaokai</i>	♀	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	♂	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
	Nymph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>H. longicornis</i>	♀	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	
	♂	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
	Nymph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	4	0	0	0	0	0	0	0	0	0	6	36	0	0	0	0	64	
<i>H. megaspinosa</i>	♀	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	♂	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
	Nymph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>Ixodes monospinosus</i>	♀	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	
	♂	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	
	Nymph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>I. nipponensis</i>	♀	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	
	♂	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	Nymph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
<i>I. ovatus</i>	♀	14	5	1	58	33	1	5	71	6	16	1	1	418	7	35	15	0	184	0	3	0	1	0	0	0	4	19	4	3	54	0	0	4	963	
	♂	18	5	1	41	22	2	2	77	4	13	3	0	298	3	32	8	0	155	0	1	0	3	1	4	0	1	14	3	0	69	0	0	10	790	
	Nymph	0	0	0	6	0	0	0	5	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	
<i>I. persulcatus</i>	♀	1	0	0	7	13	0	4	10	0	0	0	0	91	1	19	0	0	0	0	0	0	0	0	0	0	2	7	0	0	2	0	0	1	158	
	♂	1	0	0	6	10	0	2	8	0	0	0	0	94	3	22	0	0	0	0	1	0	0	0	0	0	3	8	0	0	0	0	0	4	162	
	Nymph	0	0	0	6	1	0	0	2	0	0	0	0	8	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	20	
Total		43	58	5	130	84	3	14	237	33	41	5	1	951	14	110	26	38	1,346	1	6	1	4	2	8	1	11	51	84	53	165	3	12	21	3,562

Fig. 2. Species compositions of adult ticks at different altitudes divided into four zones ranging from 0–400, 401–800, 801–1,200, and 1,201–1,600 m.

Table 3. Prevalence of rickettsial 17-kDa genes detected from ticks by PCR.

Tick species	No. positive/tested			Nymph*
	Adult			
	♀	♂	Total (%)	
<i>Haemaphysalis flava</i>	3/85	0/49	3/134 (2.2)	2/12
<i>H. japonica</i>	0/4	1/1	1/5 (20.0)	0/2
<i>H. longicornis</i>	0/1	—	0/1 (0.0)	0/2
<i>Ixodes monospinosus</i>	1/2	1/2	2/4 (50.0)	—
<i>I. nipponensis</i>	0/1	—	0/1 (0.0)	—
<i>I. ovatus</i>	0/13	0/7	0/20 (0.0)	—
<i>I. persulcatus</i>	0/6	0/13	0/19 (0.0)	—
Total	4/112	2/72	6/184 (3.3)	2/16

* Shown by pool number.

Haemaphysalis japonica was mostly collected within the range of 801–1,200 m, and very few occurred below 401 m.

Detection of SFGR DNA

Rickettsial genes were detected from *H. flava* (3 females and 2 nymphal groups), *H. japonica* (1 male), and *I. monospinosus* (1 male and 1 female) (Table 3). The prevalences of rickettsia in adult *H. flava*, *H. japonica* and *I. monospinosus* were 2.2%, 20.0% and 50.0%, respectively. The phylogenetic tree of detected rickettsial 17k-Da antigen genes is shown in Fig. 3. All rickettsial DNA samples were similar to the DNA already detected in Toyama

Prefecture (Ishikura et al., 2003). Four rickettsial DNA samples out of five detected from *H. flava* were closely related to Hf332. Rickettsial DNA No. T272 showed 99.0% nucleotide sequence homology with Hf332. A rickettsial DNA sample detected from *H. japonica* was closely related to Hj126. Two rickettsial DNA samples detected from *I. monospinosus* were closely related to Ip382 and differed in three nucleotides (99.3% identical) from that of *R. helvetica*. The *R. japonica* gene was not detected in the present study.

Fig. 3. Phylogenetic tree constructed based on the 17-kDa gene sequence of the spotted fever group rickettsiae from ticks. Samples are shown by No. and species. Reference strains are shaded and shown as "accession No./strain".

DISCUSSION

On the basis of the field surveys during 1991–2007, we recorded the tick fauna on vegetation below 1,470 m a.s.l. in Toyama Prefecture. Although nine tick species including three species newly recorded for the prefecture were collected, the following two species, *A. testudinarium* and *I. acutitarsus*, previously recorded in the prefecture were not found. Two species were recorded only from host animals in the prefecture: *A. testudinarium* was from human (Kamimura and Kondo, 1977; Kondo et al., 1976) and *I. acutitarsus* was from human and the Japanese serow *Capricornis crispus* (Kishida, 1930; Kondo

et al., 1976). Further tick collections from host animals may clarify these tick fauna in Toyama Prefecture.

Haemaphysalis flava, a causative agent of JSF in the endemic area (Mahara, 1997), was the most dominant species at low altitudes in the study area and is similar to other studies in Honshu (Fujimoto and Yamaguti, 1990; Fujita et al., 1981) and Kyushu (Nakao and Takada, 1997). However, the vertical distribution of *H. flava* can not be explained by its wide host range including birds (Yamaguti et al., 1971) and its high tolerance to low temperature (Fujimoto, 1993). More detailed studies on the ecology of *H. flava*, including interspecific competition, are necessary to clarify the factors limiting its ver-

tical distribution.

Although the vertical distribution of *H. japonica* has been poorly studied, known collecting areas of *H. japonica* were below 1,000 m (Fujita et al., 1981; Ishiguro et al., 1992). *Haemaphysalis japonica* at 1,470 m (site No. 5, see Table 1 and 2) is the highest altitude recorded for this species and suggests that *H. japonica* is tolerant to low temperatures.

Ixodes ovatus, was the most dominant species above 400 m in the present study, which is similarly to other studies in Honshu (Fujita et al., 1981; Fujimoto and Yamaguti, 1990; Ishiguro et al., 1992). The vertical distribution of *I. ovatus* appears to be because all developmental stages are less tolerant to high temperatures (Fujimoto, 1993).

Ixodes persulcatus, playing a significant role in the transmission of Lyme borreliosis spirochetes to humans in Japan (Miyamoto et al., 1991), are not distributed in the lower altitudes in the southern areas of central Honshu (Fujita et al., 1981; Fujimoto and Yamaguti, 1990; Ishiguro et al., 1992). The present findings are similar to these distributional patterns for *I. persulcatus*. A high tolerance to low temperatures in contrast to a low tolerance to high temperatures in *I. persulcatus* appears to be consistent with the vertical distribution of the species (Fujimoto, 1992, 1994).

High mountain ecosystems are considered sensitive to climate change. Recently, the vertical distribution limit of *I. ricinus* has obviously shifted to higher altitudes in the Krkonoše Mts., Czech Republic because of the changing climate (Materna et al., 2005; Danielová et al., 2008). In the present study, an obvious shift in vertical distribution of ticks was not observed because the distributional limit of ticks could not be precisely surveyed. However, shoot elongation of alpine dwarf pine *Pinus pumila* a principal component of vegetation in the alpine life zone has been affected by increasing summer temperatures during the last 25

years in Central Japan (Wada et al., 2005), so climate changes may affect the vertical distribution of ticks in Japan.

The rickettsial DNA detected from *I. monospinosus* was closely related to *R. helvetica* which is strongly considered to be the pathogen for SFGR infections in Fukui Prefecture (Noji et al., 2005; Takada et al., 2006; Ishiguro et al., 2008). Although *I. monospinosus* appears to be rare in Toyama Prefecture and was recorded only in the eastern mountain area (520–850 m a.s.l.), the prevalence of rickettsia in *I. monospinosus* is relatively high. Therefore, the present findings indicate a potential threat of SFGR infection from *I. monospinosus* in Toyama Prefecture.

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