Detection of Japanese spotted fever rickettsiae DNA from ixodid ticks in Osumi Peninsula of Kagoshima Prefecture, Japan

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Abstract: We attempted to detect *Rickettsia japonica* DNA from ixodid ticks by the PCR analysis. Ixodid ticks were collected at Hami, Minamikata and Shintomi of Kimotsuki Town, Kagoshima Prefecture. Ixodid ticks were also collected at three popular camp sites, Sarugajou Gorge in Tarumizu City, Kamikawaotaki Park and Shouyojunomori in Kinkou Town, Kagoshima Prefecture. A total of 3,477 ixodid ticks was collected. They were identified as four genera and eight species as follows: *Haemaphysalis flava*, *H. longicornis*, *H. formosensis*, *H. hystricis*, *Ixodes nipponensis*, *I. ovatus*, *Amblyomma testudinarium* and *Dermacentor taiwanensis*. *Haemaphysalis formosensis* and *H. flava* seemed to be the dominant species in the survey areas. The nucleotide sequence of PCR products with a primer pair Rj5 and Rj10 was analyzed. The nucleotide sequences of four PCR products from *H. longicornis* (one sample), *H. formosensis* (one sample) and *H. hystricis* (two samples) were successfully decided, and their sequences accorded with *Rickettsia japonica* DNA.

Key words: Japanese spotted fever, *Rickettsia japonica*, tick, Kagoshima Prefecture

INTRODUCTION

Japanese spotted fever (JSF), caused by the rickettsia, *Rickettsia japonica*, is a tick-borne disease. In Kagoshima Prefecture, the annual incidence of JSF is currently about 10. JSF infection occurs from April to November in the Osumi Peninsula of Kagoshima Prefecture.

We observed the seasonal occurrence of ixodid ticks in the Osumi area, and attempted to detect *R. japonica* DNA from ixodid ticks by the PCR analysis (Noda et al., 2004). With a primer pair Rj5, Rj10, which amplifies the genomic DNA only from *R. japonica*, a 357 bp fragment was amplified from four samples of three sites; *Haemaphysalis flava* (three samples) and *H. hystricis* (one sample). *Haemaphysalis flava* and *H. hystricis* seem to be the impor-

tant vector ticks for Japanese spotted fever in Kagoshima Prefecture.

JSF infection occurs widely in the Osumi Peninsula of Kagoshima Prefecture, but the eastern area of the Osumi Peninsula seems to be the main endemic area of JSF. In the present study, we collected ixodid ticks in Kimotsuki Town, and attempted to detect *R. japonica* DNA by the PCR analysis. And, the nucleotide sequence of PCR products was analyzed. Ixodid ticks were also collected at three popular camp sites in the Osumi Peninsula.

MATERIALS AND METHODS

Collection of ixodid ticks

A survey of ixodid ticks by the flagging method on vegetation was conducted in the areas where patients were suspected

Fig. 1. Map showing survey sites in the Osumi Peninsula of Kagoshima Prefecture.

to have contracted JSF, and in popular camp sites from April 2003 to August 2005. Ixodid ticks were collected at Hami (16 sites), Minamikata (12 sites) and Shintomi (2 sites) in Kimotsuki Town, Kagoshima Prefecture. Ixodid ticks were also collected at three camp sites, Sarugajou Gorge in Tarumizu City, Kamikawaotaki Park and Shouyujunomori in Kinkou Town, Kagoshima Prefecture (Fig. 1). Adult and nymphal ticks collected were preserved in 70% ethanol until the species identification and PCR analysis. Larval ticks attached to the flag in the collection of summer were not used for PCR analysis.

Detection of Japanese spotted fever rickettsiae DNA

For PCR analysis, adult ticks were washed separately with distilled water. Nymphal ticks of the same species were separated into groups of ten or less, and each group of nymphs was washed with distilled water. The ticks were then cut by a heated knife, and were homogenized with 50 μ l distilled water in 1.5 ml microtubes. The suspension was mixed with

1/10 volume of 0.1 N NaOH for incubation at 37°C for 1 hr. After incubation, the suspension was mixed with 1/10 volume of 0.1 N HCl, and centrifuged at 2,000 g for 5 min (Katayama et al., 1996). The supernatant was used as the template DNA for PCR analysis.

Primers described by Katayama et al. (1996) and Furuya et al. (1995) were used for PCR analysis. The PCR amplification mixture (total volume, 50 μ l) contained 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 μ M each dNTPs, 1 μ M each primer, 1.25 units of *Taq* polymerase (TaKaRa Taq, Takara Shuzou), and 5 μ l of template DNA. The gelatin was removed from the PCR amplification mixture. The thermal cycle described by Katayama et al. (1996) was used for PCR analysis. Thermal cycling (on an Astec Program Temp Control System PC-800) was performed at the following setting: 2 min at 95°C and 30 thermal cycle with 30 sec at 94°C, 2 min at 57°C and 2 min at 70°C. The same primer pair R1, R2 was used for the first and second PCR. A primer pair R1, R2 was designed for amplifying the genomic DNA from the spotted fever

group rickettsiae and typhus group rickettsiae. The 5 μ l of the first PCR product was used for the second PCR. The 5 μ l of the second PCR product was used for the PCR analysis using the primer pair Rj5, Rj10. A primer pair Rj5, Rj10 was designed for amplifying the genomic DNA only from *R. japonica*. A 357 bp fragment was amplified from the positive sample. The PCR products were electrophoresed in 1.5% agarose gels.

The PCR products containing the 300–400 bp fragment were used for the analysis of nucleotide sequence. Template DNA was prepared by a Wizard[®] SV Gel and PCR Clean Up System (Promega). The nucleotide sequence of PCR products with a primer pair Rj5 and Rj10 was analyzed using a BigDye[®] Terminator v2.1 Cycle Sequencing Kit and ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems, USA). For the sequenced PCR, we used Rj5-2 (5'-ctaccactaggagctgtttct-3') primer. The reaction mixture was composed of 3.2 pmol primer, 1 μ l (10–40 ng) template DNA, 2 μ l BigDye Sequencing Buffer and 4 μ l Terminator Ready Reaction Mix in a final volume 20 μ l. Thermal cycling was performed at the following setting: 5 min at 95°C and 30 thermal cycle with 30 sec at 95°C, 10 sec at 50°C and 4 min at 60°C. Sequence similarity searches in GenBank were done with a BLAST program.

RESULTS

Collection of ixodid ticks

A total of 3,477 ixodid ticks was collected. They were identified as four genera and eight species as follows: *H. flava*, *H. longicornis*, *H. formosensis*, *H. hystricis*, *Ixodes nipponensis*, *I. ovatus*, *Amblyomma testudinarium* and *Dermacentor taiwanensis* (Table 1). *Haemaphysalis formosensis* was the most abundant in number (1,899), followed by *H. flava* (862) and *H. hystricis* (293). Therefore, *H. formosensis* and *H. flava* seemed to be the dominant species in the survey areas.

Detection of Japanese spotted fever rickettsiae DNA

All adult and nymphal ticks collected were used for PCR analysis, and the total tube number was 993. Twenty PCR products containing the 300–400 bp fragment were used for the analysis of nucleotide sequence. The nucleotide sequences of four PCR products were successfully decided, and their sequences accorded with *R. japonica* DNA. As shown in Table 1, the *R. japonica* DNA fragment was amplified from a sample of *H. longicornis* nymph collected at Hami, a sample of *H. formosensis* male adult collected at Hami, a sample of *H. hystricis* consisting of five nymphs collected at Hami and a sample of *H. hystricis* female adult collected at Minamikata.

DISCUSSION

At present, nine tick species, *D. taiwanensis*, *H. cornigera*, *H. flava*, *H. formosensis*, *H. hystricis*, *H. kitaokai*, *H. longicornis*, *H. megaspinosus* and *I. ovatus* have been confirmed to be arthropod reservoirs for *R. japonica* (Uchida et al., 1995; Katayama et al., 1996, 2001; Takada, 2003, 2005; Taketani et al., 2005). In our previous survey in the Osumi Peninsula, *R. japonica* DNA was detected from two species, *H. flava* and *H. hystricis* (Noda et al., 2004). Under the assumption that only one nymphal tick of the sample was infected with *R. japonica*, infection rates of *H. flava* and *H. hystricis* were 1.2% and 4.2%, respectively. In the present survey, *R. japonica* DNA was detected from three species, *H. longicornis*, *H. formosensis* and *H. hystricis*. Estimated infection rates of *H. longicornis*, *H. formosensis* and *H. hystricis* were 0.8%, 0.05% and 0.9%, respectively.

JSF infection occurs from April to November in the Osumi Peninsula of Kagoshima Prefecture. Seasonal occurrence of the ixodid population was surveyed at three sites of Osumi Peninsula (Noda et al., 2004). Nymphs of *H. flava* were collected throughout the year with a peak during

Table 1. Ixodid ticks collected by the flagging method in the Osumi area of Kagoshima Prefecture.

Species	Stage	Kimotsuki-cho			Tarumizu City	Kinko-cho		Total
		Hami	Minamikata	Shintomi	Sarugajo Gorge	Shouyou-junomori	Kamikawaotaki Park	
<i>Haemaphysalis flava</i>	Male	44	41	2			1	88
	Female	45	53	3				101
	Nymph	275	354	33		9	2	673
<i>Haemaphysalis longicornis</i>	Male							
	Female	2	4			12	4	22
	Nymph	13*	72		1	26	4	116
<i>Haemaphysalis formosensis</i>	Male	72*	22	1	53	2		150
	Female	45	29		67	2		143
	Nymph	879	675	33	16	3		1,606
<i>Haemaphysalis hystricis</i>	Male	37	18	3	8	2	10	78
	Female	29	30*	6	15	2	7	89
	Nymph	47*	70	2	4	2	1	126
<i>Ixodes nipponensis</i>	Male							
	Female							
	Nymph	14	2					16
<i>Ixodes ovatus</i>	Male	6	6	3				15
	Female	4	4					8
	Nymph							
<i>Amblyomma testudinarium</i>	Male	2						2
	Female		1					1
	Nymph	79	48	4	104	5		240
<i>Dermacentor taiwanensis</i>	Male	1						1
	Female		2					2
	Nymph							
Total		1,594	1,431	90	268	65	29	3,477

* Including each one sample which was positive for *R. japonica* DNA fragment.

the period between March and May. A large number of adults was collected from February to April but not in August and September. On the other hand, nymphs and/or adults of *H. longicornis* and *H. hystricis* were collected from April to September. The present data may suggest that *H. longicornis* and *H. hystricis* are important vector ticks in Kagoshima Prefecture.

A man and his wife were infected with JSF at the same time in July 2003 at a camp site, Sarugajou Gorge in Tarumizu City. Therefore, ixodid ticks were also collected at three popular camp sites containing Sarugajou Gorge, but *R. japonica* DNA was not detected from ixodid ticks. In the present survey, *R. japonica* DNA was detected from one female *H. hystricis* collected at the site of Minamikata. This site was located at the top of a hill, Kano-dake (87 m above sea level), which adjoins a residential area. Eight cottages were constructed on the hill, and families and young peoples enjoy outdoor amusements. There is a possibility that not only inhabitants near the camp sites but also visitors to the camp sites from non-endemic areas contact JSF. In the JSF cases of the man and wife, they lived in the northwestern area of Kagoshima Prefecture and visited a camp site of Sarugajou Gorge.

Haemaphysalis longicornis and *H. hystricis* seem to be important vectors of JSF in Kagoshima Prefecture. Of these tick species, the vectors for JSF rickettsia to humans may consist of a complex of dominant tick species parasitic to humans. Further studies on tick fauna and rickettsial agents are indispensable in determining the role played by each possible vector.

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