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Detection of *Rickettsia japonica* in *Haemaphysalis longicornis* Ticks by Restriction Fragment Length Polymorphism of PCR Product

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PCR was applied to the detection of *Rickettsia japonica*, the causative agent of Oriental spotted fever (OSF), in ticks collected at two sites of the Muroto area on Shikoku Island, a major area in Japan where OSF is endemic. Primer pair *Rr*190.70p and *Rr*190.602n of the *R. rickettsii* 190-kDa antigen gene sequence of Regnery and others (R. L. Regnery, C. L. Spruill, and B. D. Plikaytis, J. Bacteriol. 173:1576–1589, 1991) primed the DNA extracted from *Haemaphysalis longicornis* ticks but not those extracted from *Haemaphysalis formosensis*, *Haemaphysalis flava*, *Haemaphysalis hystricis*, or *Amblyomma testudinarium* ticks. Digestion of the amplification product with the restriction endonucleases *Pst*I and *Alu*I produced the restriction fragment length polymorphism pattern specific to *R. japonica*. The *Hin*dIII and *Msp*I digests gave restriction fragment length polymorphism patterns identical to those of the PCR product from *R. japonica* DNA. Hemolymph preparations of *H. longicornis* ticks were demonstrated to contain rod-shaped organisms that were detected by immunofluorescence with the monoclonal antibody specific to *R. japonica* species. The primer pair did not amplify the DNA of a laboratory colony of *H. longicornis* ticks originally collected at an area where OSF is not endemic. Our results provided evidence that *H. longicornis* ticks might be an arthropod reservoir for *R. japonica* and a vector of OSF.

Rickettsia japonica is the etiologic agent of Oriental spotted fever (OSF), which is encountered in Japan (19, 22). The organism was isolated from patients' blood (20, 21) and was identified as a new species of spotted fever group (SFG) rickettsiae by its serologic specificity by using polyclonal and monoclonal antibodies (23, 24). The biologic and genomic characteristics of the organism were essentially the same as those of other SFG rickettsiae pathogenic for humans, i.e., *Rickettsia rickettsii* (Rocky Mountain spotted fever), *Rickettsia conorii* (boutonneuse fever), *Rickettsia sibirica* (North Asian tick typhus), *Rickettsia australis* (Queensland tick typhus), and *Rickettsia akari* (rickettsial pox) (22). Recently, it was demonstrated that *R. japonica* is differentiated from other SFG rickettsiae by restriction fragment length polymorphism (RFLP) with the products of PCR amplification (28, 29) with the *R. rickettsii* 190-kDa surface antigen gene primers described by Regnery et al. (16). The disease is found mainly in the southwestern part of Japan facing the Pacific Ocean (Fig. 1) (19). For the last 10 years, from 1983 to 1992, more than 100 cases of OSF have been documented, half of which were encountered in Muroto city, Kochi Prefecture, on Shikoku Island (19). The disease in the patients in the Muroto area had an incubation period of 4 to 7 days, with onset between April and October and with high incidence in July, August, and October; illness with fever and macular or maculopapular rash occurred in all patients, and headache occurred in some patients. The clinical study documented an eschar in 48% of patients (6, 19), indicating the existence of an arthropod vector. Until the present, however, no patients carrying ticks on their bodies have been found at Muroto Hospital. In addition, no confirmed evidence of the arthropod vector for *R. japonica* has been obtained.

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Recently, PCR assays have been applied to the detection of rickettsial DNA in arthropod vectors (1, 5, 7, 8, 13, 25). We attempted to find ticks infected with *R. japonica* by the PCR-RFLP technique. Here we report that *Haemaphysalis longicornis* ticks collected from the Muroto area carry the *R. japonica* organisms.

MATERIALS AND METHODS

Rickettsial strains. *R. japonica* type strain YH (ATCC VR-1363) was described previously (22). Since the organisms caused a persistent infection in Vero cells, carrier Vero cells were grown in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum and no antibiotics in a 5% CO_2–air incubator at 34°C for 4 to 6 days.

Collection of ticks. Ticks were collected by dragging a white cloth (1 by 1.2 m) across the vegetation on the grounds near the places where the latest infections of residents occurred. One site is the area near the meteorological station at the head of Muroto Promontory (site A in Fig. 2), and another is in a mountainous area (site B in Fig. 2). The species of ticks were identified by an entomologist (S.K.). In late October of 1992, nine ticks were collected; seven larval *H. longicornis* ticks were collected at site A and two nymphal *Amblyomma testudinarium* ticks were collected at site B. In early April of 1993, a total of 221 ticks were collected: 131 and 90 ticks at sites A and B, respectively. At site A, 54 nymphal *H. longicornis*, 36 nymphal and 17 adult *Haemaphysalis formosensis*, 12 nymphal and 7 adult *Haemaphysalis flava*, 1 adult *Haemaphysalis hystricis*, and 3 nymphal and 1 adult *A. testudinarium* were collected. At site B, 23 nymphal *H. longicornis*, 28 nymphal and 3 adult *H. formosensis*, and 32 nymphal and 4 adult *H. flava* were collected. Adult *H. longicornis* ticks from a laboratory colony were provided by K. Fujisaki; they were originally collected in an area on Honshu Island (Okayama Prefecture) where OSF is not endemic and were maintained at the National Institute of Animal Health, Tsukuba, Japan.

Tick processing. The classified ticks were kept in screw-cap glass bottles at ambient temperatures. All ticks collected in 1992 were used for the PCR test. The *H. longicornis*, *H. formosensis*, and *H. flava* ticks collected in 1993 were divided into two groups each; one for PCR analysis and another for detection of rickettsial organisms by tissue culture. *H. hystricis* and *A. testudinarium* ticks were subjected to the PCR analysis alone. For PCR analysis, the same species of ticks were pooled, washed in 70% ethanol and distilled water successively, and triturated by crushing them on sterile glass slides with a forceps. The triturate was suspended in 100 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM disodium EDTA. The triturate suspension was mixed with 7 volumes of digestion buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, 0.1 mg of proteinase K per ml) for incubation at 50°C for 1 h; this was followed by an additional incubation at 37°C for 48 h. DNA was extracted by

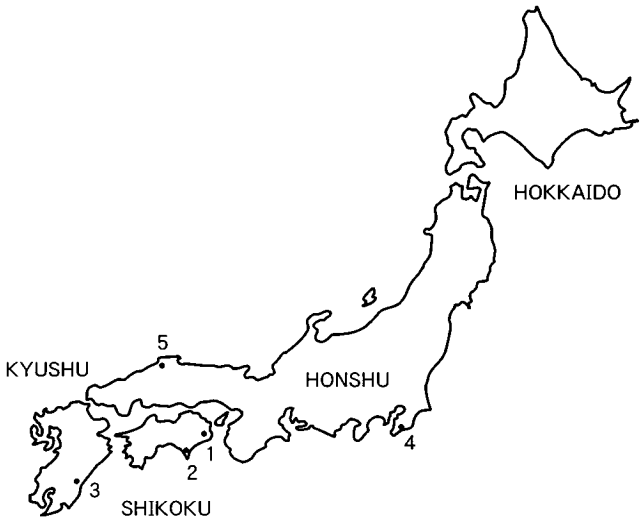

FIG. 1. Map of Japan. Numbers indicate the areas where the SFG rickettsioses were documented. 1, Anan, Tokushima Prefecture; 2, Muroto, Kochi Prefecture; 3, Miyazaki, Miyazaki Prefecture; 4, Amatsukominato, Chiba Prefecture; 5, Izumo, Shimane Prefecture. Patients with OSF were recognized on the smaller Awagi Island, located between Honshu and Shikoku islands.

the standard phenol-chloroform method (17). After precipitation with ethanol, the DNA was brought to dryness and was then dissolved in 50 μl of distilled water for storage at −85°C. The remaining ticks of *H. longicornis*, *H. formosensis*, and *H. flava* were used for the detection of rickettsial organisms.

PCR amplification. A pair of synthesized oligonucleotide primers derived from the gene sequence encoding the 190-kDa antigen of *R. rickettsii*, *Rr*190.70p and *Rr*190.602n, which was described by Regnery et al. (16), was used to prime the PCR amplification of *R. japonica* genomic DNA as described previously (28, 29). PCR amplification was performed in the 100-μl reaction mixture according to the protocol supplied with the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction mixture contained 10 μl of tick extracts. The condition of DNA amplification was 35 cycles of denaturation (24 s at 95°C), annealing (30 s at 48°C), and extension (2 min at 60°C) in a DNA thermal cycler. The PCR amplification of DNA was verified by 1.5% agarose gel electrophoresis (4 V/cm for 1 h). As a control, the genomic DNA of *R. japonica* was used after extraction from Percoll-purified rickettsiae by the standard phenol-chloroform method described previously (28).

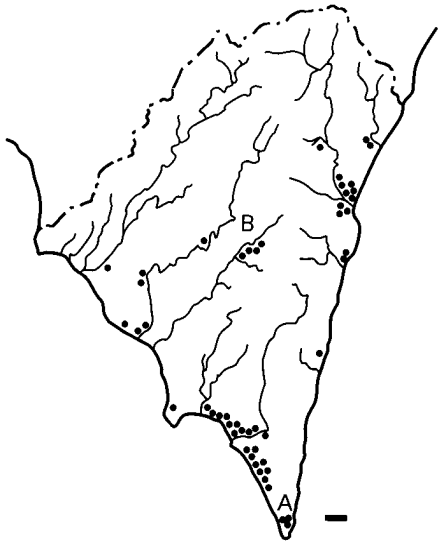

FIG. 2. Map of Muroto city. Site A, meteorological station; site B, Hinata Village. Cases of OSF encountered between 1983 and 1992 are shown as dots. Bar, 1 km.

TABLE 1. Tick species, life stages, sites of collection, and PCR results

Species	Stages	Collection site[a]	No. of ticks pooled	PCR result
H. longicornis	Larva[b]	A	5	+
		A	2	+
	Nymph	A	10	+
		A	8	+
	Nymph	B	8	+
H. formosensis	Nymph	A	10	−
		A	6	−
	Adult	A	3	−
		A	2	−
	Nymph	B	9	−
	Adult	B	1	−
H. flava	Nymph	A	4	−
	Adult	A	2	−
	Nymph	B	11	−
	Adult	B	1	−
H. hystricis	Adult	A	1	−
A. testudinarium	Nymph[b]	B	2	−
	Nymph	A	3	−
	Adult	A	1	−
H. longicornis	Adult	Colony	8	−
			3	−

[a] See Fig. 2.
[b] Collected in late October of 1992. Other ticks were collected in early April of 1993.

Restriction endonuclease digestion. The restriction endonucleases *Afa*I, *Alu*I, *Hin*dIII, *Msp*I, and *Pst*I were used to digest the PCR amplification products as described previously (28, 29). The digests were separated on 8% polyacrylamide gels run at 7 V/cm for 4 h and were visualized after staining with ethidium bromide.

Detection of rickettsial organisms. (i) Immunofluorescence tests of hemolymph preparations obtained from *H. longicornis* ticks were carried out. The hemolymph was collected on a slide as described previously (3). After the preparation was air dried and then fixed in acetone for 10 min at room temperature, the preparation was treated with a monoclonal antibody (3Y3B5) specific to *R. japonica* (24); this was followed by staining with fluorescein-labeled affinity-purified anti-mouse immunoglobulin G antibody (Cappel). (ii) Each species of the remaining ticks, including those used for hemolymph preparations, were pooled, kept at 37°C for 2 days for reactivation (9), and were then treated in povidone-iodine; this was followed by washing in 70% ethanol and sterile distilled water successively. Ticks were triturated aseptically and were suspended in sucrose-phosphate-glutamate buffer (2). The suspension was homogenized in a Dounce homogenizer as described previously (28). The homogenate was concentrated by centrifugation and was plated onto Vero cell monolayers in 25-cm^2 flasks for 1 h at 34°C; this was followed by incubation with Eagle's MEM containing 1% fetal calf serum in the presence of cefuzonam (semisynthetic cephalosporin antibiotic) at a concentration of 100 μg/ml, which permitted the growth of *R. japonica*. Incubation continued for 10 days in a 5% CO_2–air incubator at 34°C with three changes of the medium.

RESULTS

Detection of rickettsial DNA in ticks by PCR-RFLP. The locations where patients with OSF appeared between 1983 and 1992 in Muroto city are shown in Fig. 2. Ticks were collected in late October of 1992 and early April of 1993 at sites A and B (Fig. 2), where the latest infections occurred in the spring of 1992. Ticks were pooled separately and were triturated for PCR with the primer pair *Rr*190.70p and *Rr*190.602n (Table 1; see the footnote). Among ticks collected in 1992, the DNAs of only larval *H. longicornis* ticks were amplified. The amplified products were digested with the *Pst*I restriction endonuclease

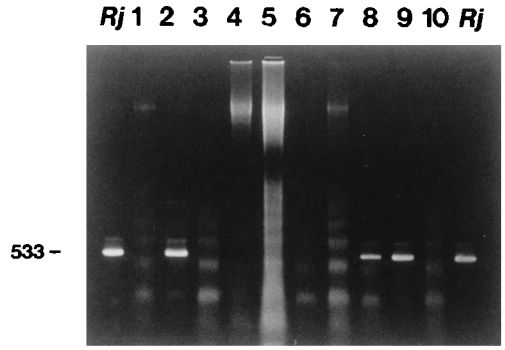

FIG. 3. Ethidium bromide-stained, agarose gel electrophoretogram of PCR-amplified DNAs primed with primer pair *Rr*190.70p and *Rr*190.602n of the *R. rickettsii* 190-kDa antigen gene. Lanes 1, 6, and 10, *H. flava*; lanes 2, 8, and 9, *H. longicornis*; lanes 3 and 7, *H. formosensis*; lane 4, *H. hystricis*; lane 5, *A. testudinarium*. Lanes 1 to 5, ticks at site A; lanes 6 to 10, ticks at site B. *Rj*, *R. japonica* DNA as a template. The number on the left indicates the molecular size (in base pairs) of the PCR product amplified from *R. japonica* DNA.

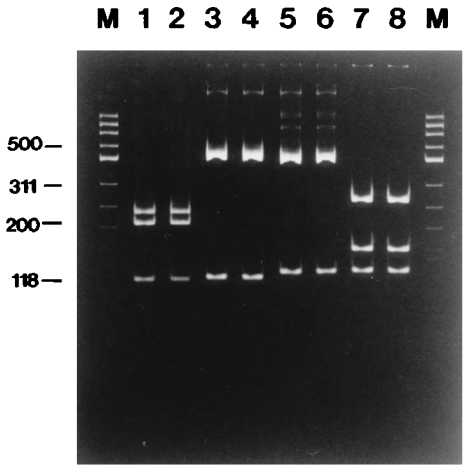

FIG. 4. Ethidium bromide-stained, polyacrylamide gel electrophoretogram of restriction endonuclease-digested, PCR-amplified DNAs. Lanes 1 and 2, *Alu*I; lanes 3 and 4, *Hin*dIII; lanes 5 and 6, *Msp*I; lanes 7 and 8, *Pst*I. Odd lane numbers, digests of PCR products of *H. longicornis*; even lane numbers, *R. japonica* reference strain. Lane M, molecular size marker (ϕX174 RF DNA cleaved with *Hin*fI); numbers indicate the molecular size (in base pairs) of the DNA fragments.

(data not shown). These results suggested that *H. longicornis* ticks might carry *R. japonica*. The extension of the studies was carried out in 1993. A total of 221 ticks were collected at the same sites; the majority of ticks were *H. longicornis*, *H. formosensis*, and *H. flava*. The DNAs of some of these ticks were tested for their abilities to be amplified by PCR (Table 1). The DNAs of only *H. longicornis* ticks collected at both sites A and B were amplified. The products from these tick samples migrated on agarose gel electrophoresis to the same position as that of the PCR fragment (533 bp) amplified from *R. japonica* genomic DNA (Fig. 3). No amplification was observed when *H. hystricis* and *A. testudinarium* ticks in the collection used for the present study were subjected to PCR (Fig. 3). Laboratory colony *H. longicornis* ticks originally collected in an area where OSF is not endemic were not amplified by the PCR (data not shown).

The PCR amplification products from three pools of nymphal *H. longicornis* ticks were subjected to restriction endonuclease digestions; the products demonstrated the same RFLP patterns. The profiles of one sample are shown in Fig. 4. The *Pst*I digests resulted in an RFLP pattern identical to that of *R. japonica* (lanes 7 and 8). The *Alu*I (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), and *Msp*I (lanes 5 and 6) digests produced RFLP patterns identical to those of *R. japonica*. The *Afa*I restriction endonuclease did not cleave the PCR products amplified from ticks like it did those from *R. japonica* (data not shown).

Detection of rickettsial organisms. Since *H. longicornis* ticks were found to carry the DNA that showed the PCR-RFLP pattern specific to *R. japonica*, hemolymph preparations were examined to demonstrate rickettsial organisms by an immunofluorescence test with a monoclonal antibody specific to *R. japonica*. Among 19 ticks collected at sites A and B, hemolymph preparations from 6 ticks were demonstrated to contain fluorescein-stained, rod-shaped organisms (Fig. 5).

The remaining ticks of *H. longicornis* together with those used for the immunofluorescence test were subjected to cultivation. A tissue culture method was used for that study. Vero cell monolayers were infected with homogenates of *H. longicornis* ticks in the presence of a cephalosporin antibiotic at a concentration permissive for the growth of *R. japonica* organisms. After 10 days of cultivation, no growth of intracellular organisms was observed in cultures (one-fifth of the cultures were contaminated). Although the DNAs of *H. formosensis* and *H. flava* ticks were not amplified in the PCR, these ticks were also subjected to cultivation, and no rickettsial organisms were detected.

DISCUSSION

Many rickettsial diseases are caused by arthropod-borne pathogens (4, 14, 15). *R. japonica*, the causative agent of OSF (19, 22), had been thought to be transmitted by an arthropod. However, no evidence to confirm this was demonstrated. Recently, Regnery et al. (16) described a PCR-RFLP rickettsial typing scheme that differentiated almost all recognized species of rickettsiae. With their primer pair *Rr*190.70p and *Rr*190.602n, the PCR-RFLP successfully differentiated *R. japonica* (28, 29). The present study intended to find a reservoir for *R. japonica* by the PCR-RFLP technique. The primer pair amplified the DNA extracted from larval and nymphal *H. longicornis* ticks that were collected in the Muroto area, a major area where OSF is endemic; in the present study adult ticks of this species could not be collected. The amplified products showed the same electrophoretic mobilities as that of

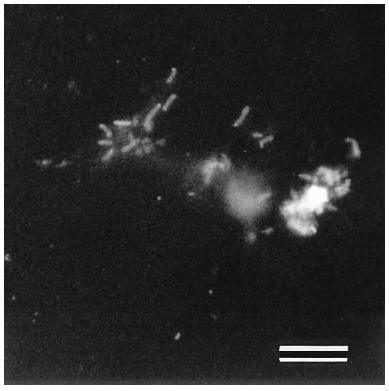

FIG. 5. Fluorescence microscopic photograph of rickettsial organisms in a hemolymph preparation obtained from *H. longicornis*. Some of the elongated organisms are at the stage prior to cell division. Bar, 10 μm.

the product from *R. japonica* genomic DNA. The *Pst*I restriction endonuclease cleaved the PCR products, showing the RFLP pattern to be identical to that of *R. japonica* (28). The identity of the DNA sequence carried by the *H. longicornis* tick with the *R. japonica* DNA was also provided by *Alu*I digestion, the RFLP pattern of which is unique to *R. japonica* (29). The *Hin*dIII and *Msp*I digests of the PCR product amplified from tick extracts gave RFLP patterns identical to those of *R. japonica*, indicating the same sequence between tick-carrying and *R. japonica* DNAs, although these RFLPs do not discriminate *R. japonica* from other species (29). The *Afa*I restriction endonuclease does not cleave the PCR product from tick samples because the *R. japonica* product is resistant to digestion by this enzyme. No PCR amplification of the extract from laboratory colony *H. longicornis* ticks originally collected in an area where OSF is not endemic indicated that *H. longicornis* ticks in the Muroto area have been infected with *R. japonica*. Examination of hemolymph preparations from *H. longicornis* by the immunofluorescence test with monoclonal antibody specific to *R. japonica* revealed the presence of fluorescein-stained, rod-shaped organisms. Although the organisms were not isolated from ticks, the present studies demonstrated the presence of *R. japonica* organisms in *H. longicornis*. Thus, *H. longicornis* ticks are thought to have the potential to be a vector for *R. japonica.*

H. longicornis ticks are distributed throughout Japan and the Far East (10, 11) and parasitize many animals (10, 11, 27). Bites of ticks of this species have been recorded on humans in Japan (26). Independent from the present study, it has been reported that among multiple species of ticks only *H. longicornis* ticks are active from May to October in the mountains in the central part of Honshu Island (12). It should be noted that the active period of this tick species corresponds to the seasons when OSF is prevalent (6, 19).

Concerning other ticks collected in the present study, the extracts of *H. formosensis* and *H. flava* could not be primed with the primers, although these ticks are predominant, like *H. longicornis*. Since a limited number of tick samples was tested in the present study, more work with these ticks is needed. The DNAs of *H. hystricis* and *A. testudinarium* ticks were also not amplified, but the size of the collection of these ticks was too small for the possibility of their infection with *R. japonica* to be discussed.

The PCR technique has been used for the detection of rickettsiae in arthropod vectors (1, 5, 7, 8, 13, 25). This technique is highly sensitive and specific for the detection of rickettsiae in arthropods without isolation of rickettsial organisms when the proper primers are available. The PCR-RFLP assays successfully demonstrated the presence of *R. japonica* genomic DNA. To obtain genomic DNA we used pooled ticks. Gage et al. (7) reported that saliva, hemolymph, and amputated legs from an individual tick can be used for PCR-RFLP assays without extraction by organic solvents. Their method is simple and useful for vector studies.

Recently, the isolation of an *R. japonica*-like organism from a larval tick of *Dermacentor taiwanensis* collected in Anan city, Tokushima Prefecture, was reported (18). This species of ticks has not been found in the Muroto area. That report describes that all stages of this tick species infrequently parasitize humans.

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