

Detection of Rickettsial DNA in Ixodid Ticks Recovered from Dogs and Cats in Japan

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(Received 27 April 2005/Accepted 19 August 2005)

ABSTRACT. DNA from ticks recovered from 1137 dogs and 133 cats from all over Japan were examined for *Rickettsia* infection by citrate synthase gene (*gltA*)-based PCR and partial nucleotide sequencing. A total of 91 dog tick samples and 18 cat tick samples showed a single band of the appropriate size in the nested PCR. Sequence analysis was successfully performed on 102 samples. DNA of *Rickettsia japonica* or closely related *Rickettsia* spp. strains were detected from 38 ticks in 16 prefectures mainly in western Japan. The other 33, detected from 13 prefectures including Hokkaido and Okinawa, were found to be *Rickettsia helvetica* or closely related strains. A total of 29 DNA that showed highest homology with *Rickettsia akari* or closely related strains were detected in 19 prefectures, widespread throughout Japan. *Rickettsia canada*-like DNA was detected from *Haemaphysalis* sp. removed from a dog in Fukuoka, and 'Candidatus *Rickettsia tarasevichiae*'-like DNA was from *Ixodes* sp. removed from a dog in Hokkaido.

KEY WORDS: canine, epidemiology, feline, *Rickettsia*, tick.

J. Vet. Med. Sci. 67(12): 1217–1222, 2005

Humans and animals are often exposed to a large number of tick species, depending on the distribution of these arthropod vectors in the environment. Recently, interest in ticks of domestic animals has been increasing, because of emerging and re-emerging tick-borne diseases and their zoonotic nature, including rickettsial, bacterial and protozoal pathogens. Rickettsiae belong to the order *Rickettsiales* and are obligate intracellular, gram-negative bacteria. Several species cause disease in humans and other animals and have a worldwide distribution. This genus was subdivided into three groups, the typhus group (TG), the ancestral group and the spotted fever group (SFG) on the basis of phenotypic criteria [2].

In Japan, *Rickettsia japonica*, classified into the SFG *Rickettsia*, was discovered to be the causative agent of Japanese spotted fever (JSF) [7]. The JSF patients have been mainly identified in the western part of Japan since 1984, when the first patient was reported in Tokushima prefecture. Recently, many *Rickettsia* spp., including *Rickettsia helvetica* and *Rickettsia felis*, have been detected from ticks in Japan [2, 6]. However, this wide variety of *Rickettsia* species in Japan has been examined only in limited localities and the overall distribution of *Rickettsia* in Japan has not yet been determined. In this study, the detection and analysis of *Rickettsia* species from ticks recovered from domestic dogs and cats all over Japan was attempted using molecular methods including PCR and sequence analysis of the citrate synthase gene (*gltA*).

MATERIALS AND METHODS

Ticks and extraction of DNA: A total of 4122 ticks were recovered from 1137 domestic dogs and a total of 287 ticks

were recovered from 133 domestic cats from 47 prefectures in Japan and stored in 70% ethanol for morphological identification [12, 13]. After identification, one tick per dog or cat was selected for screening analysis. All the ticks selected were semi- or fully-engorged adult females or nymphs. Total DNA was extracted with a method described previously [4].

Amplification and sequencing of rickettsial *gltA* gene: A nested PCR was used to detect rickettsial *gltA* gene fragments from the ticks. The first PCR amplification was performed in a 25- μ l reaction mixture containing 5 μ l of each DNA template with a genus specific primer set, RpCS.877p and RpCS.1273r [11]. A 1:100 dilution of the first PCR product with DW, was used as a template for the second PCR, using an inner primer set; RpCS.896f (GGC TAA TGA AGC GGT AAT AA), PpCS.1258n (ATT GCA AAA AGT ACA GTG AAC), that was designed based on the common sequence of the *gltA* of the genus *Rickettsia*. The first and second PCR were carried out under the following conditions: 35 cycles of denaturation (94 C, 60 sec), annealing (54 C, 60 sec) and extension (72 C, 90 sec). The second PCR product was electrophoresed at 100V in 2% agarose gel (Wako Chemicals Ind) for 30 min, stained with ethidium bromide, and verified by UV illumination. The 363bp PCR product with a positive reaction was purified using the QIA PCR purification kit (QIAGEN) for direct sequence analysis with a Perkin-Elmer ABI Prism 377 automated DNA sequencer at the DNA Core Facility of the Center for Gene Research, Yamaguchi University.

Sequence analysis: The sequence data of the PCR products was analyzed using the BLAST 2.0 program (National Center for Biotechnology Information) for homology search. The determined sequences were then analyzed for phylogenetic relationships with other sequences registered in GenBank. Multiple alignment analysis, the determination

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of pair-wise percent identities of the sequences, distance matrix calculations and the construction of phylogenetic trees were all performed with the ClustalW program version 1.8 in the DNA data bank of Japan. Tree figures were generated using the Tree View program version 1.6.6. The GenBank accession numbers of the *gltA* gene sequences of other species used to analyze the data are as follows: *R. prowazekii*, M17149; *R. japonica*, U59724; *R. akari*, U41752; *R. felis*, U33922; *R. slovaca*, U59725; *R. conorii*, U59730; *R. cadada*, U59713; *R. honei*, AF022817; *R. helvetica*, U59723; *R. australis*, U59718; *R. montana*, U74756; *R. massiliae*, U59719; ‘*Candidatus* *Rickettsia tarasevichiae*’, AF503167; *Rickettsia* sp. AT1, AB114796; *Rickettsia* sp. In56, AB114819; *Rickettsia* sp. IO1, AB114797; *Rickettsia* sp. IP2, AB114801; *Rickettsia* sp. Hf151, AB114815; *Rickettsia* sp. IM1, AF394900; *Rickettsia* sp. FLA1, AF394898; *Rickettsia* sp. DT1, AF394897.

RESULTS

DNA was successfully extracted from a total of 1137 dog ticks and 133 cat ticks. A total of 91 dog tick samples and 18 cat tick samples showed a single band of the appropriate

size in the nested PCR. The dogs and cats infested with these particular ticks did not show any clinical signs of rickettsial infection such as fever, rash and arthritis. The peripheral blood of these dogs and cats was not available for analysis.

Approximately 322 bp of the rickettsial *gltA* sequences excluding the primer region were determined. A total of 102 PCR products were successfully analyzed for the partial nucleotide sequence (GenBank accession numbers; AB204416 to AB204517). The determined sequences were divided into 4 groups based on their homologies, obtained through BLAST and phylogenetic analyses (Fig. 1). A total of 38 ticks were classified into the same group as *R. japonica* and related species, including strains FLA1, DT1 and Hf151 which have been recently detected from ticks in Japan [5, 6] (Group1). These rickettsial DNA fragments showed highest homology of 99.1 to 100% with *R. japonica* or one of the related strains FLA1, DT1 and Hf151. The other 33 ticks that showed 99.3 to 100% nucleotide sequence homology with *R. helvetica*, or closely related strains IM1 and IP2, and IO1 detected from *Ixodes* ticks in Japan, were clustered into Group 2. A total of 29 ticks were clustered into Group 3. This cluster contains 1 tick that

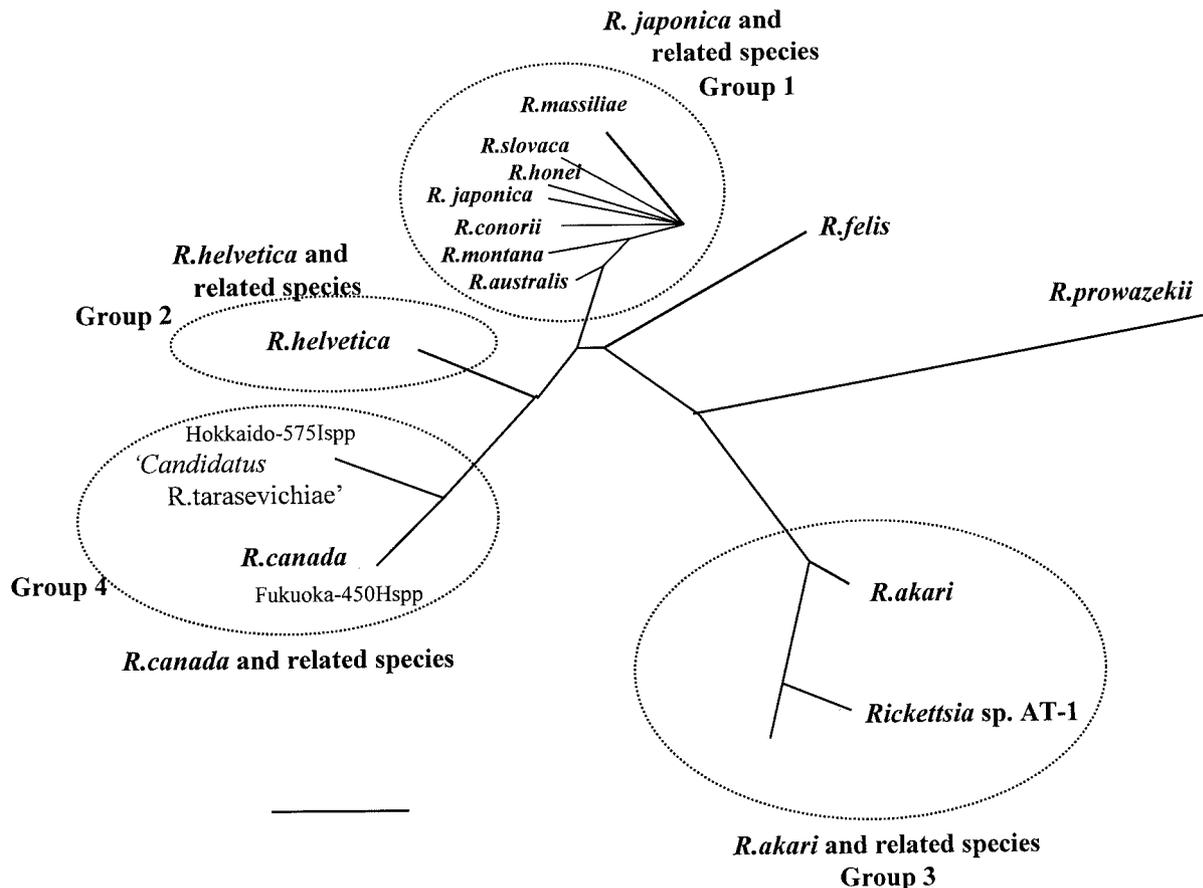


Fig. 1. Phylogenetic relationship of various *Rickettsia* spp. registered in GenBank and 102 partial sequences determined in this study based on the nucleotide sequences of citrate synthase gene. The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 1% divergence.

showed 100% homology with *Rickettsia* sp. AT1, closely related to *R. akari* detected in Japan [6], and 28 ticks that showed 99.1 to 100% homology with *Rickettsia* sp. In56 [5]. One of the remaining 2 ticks (Group4) showed highest homology of 99.1% with *R. canada*, and another showed 99.8% homology with ‘*C. R. tarasevichiae*’ [14].

The distribution of the rickettsial genes detected in this study is summarized in Table 1 and Fig. 2. The rickettsial DNA in Group1 was detected from 15 prefectures mainly in western Japan. Group 2 rickettsial DNA was detected from 13 prefectures from Hokkaido to Okinawa prefectures. Group 3 rickettsial DNA was detected from 19 prefectures

Table 1. Prefectures, species, stages and host animals of positive ticks for Rickettsial PCR

Group	Prefecture	Tick No.	Species	Stage	Host	Accession No.	Group	Prefecture	Tick No.	Species	Stage	Host	Accession No.
1	Ishikawa	205	I	F	dog	AB204416	2	Nagasaki	1052	HL	F	dog	AB204479
1	Kyoto	281	H	L	dog	AB204418	2	Nagasaki	1054	HF	F	dog	AB204480
1	Kyoto	838	HL	N	dog	AB204454	2	Nagasaki	1055	HL	N	dog	AB204481
1	Kyoto	847	HL	N	dog	AB204455	2	Nagasaki	1056	HL	N	dog	AB204482
1	Kyoto	848	HL	N	dog	AB204456	2	Oita	1058	HL	F	dog	AB204484
1	Nara	297	HL	N	dog	AB204419	2	Oita	1059	H	N	dog	AB204485
1	Nara	871	HL	F	dog	AB204459	2	Oita	1060	H	F	dog	AB204486
1	Wakayama	307	HF	F	dog	AB204421	2	Oita	1061	H	N	dog	AB204487
1	Wakayama	308	HF	M	dog	AB204422	2	Oita	1062	H	F	dog	AB204488
1	Wakayama	314	HL	N	dog	AB204423	2	Oita	1064	H	N	dog	AB204489
1	Wakayama	318	HF	M	dog	AB204424	2	Kumamoto	1067	H	N	dog	AB204490
1	Wakayama	880	HL	M	dog	AB204461	2	Kumamoto	1068	H	N	dog	AB204491
1	Hyogo	328	HL	N	dog	AB204425	2	Kumamoto	1069	HL	F	dog	AB204492
1	Tottori	912	HF	F	dog	AB204463	2	Kumamoto	1071	HL	F	dog	AB204493
1	Tottori	921	HF	F	dog	AB204464	2	Kumamoto	1073	HC	F	dog	AB204474
1	Tottori	926	H	F	dog	AB204465	2	Kumamoto	1074	HL	F	dog	AB204494
1	Tottori	928	HF	F	dog	AB204467	2	Kumamoto	1076	HL	N	dog	AB204495
1	Tottori	951	HL	F	dog	AB204468	2	Kagoshima	1118	H	F	dog	AB204498
1	Shimane	361	H	N	dog	AB204427	2	Okinawa	1124	RS	N	dog	AB204500
1	Okayama	343	HL	F	dog	AB204426	2	Okinawa	1125	RS	F	dog	AB204501
1	Hiroshima	377	H	F	dog	AB204428	3	Hokkaido	1	IO	F	cat	AB204502
1	Hiroshima	386	HF	N	dog	AB204429	3	Fukushima	112	IN	F	cat	AB204512
1	Hiroshima	967	HL	F	dog	AB204469	3	Fukushima	634	HF	N	dog	AB204445
1	Kochi	1109	I	F	dog	AB204497	3	Saitama	667	IN	F	dog	AB204446
1	Tokushima	415	HL	F	dog	AB204430	3	Saitama	672	IN	F	dog	AB204447
1	Saga	458	HF	N	dog	AB204433	3	Tokyo	689	HF	F	dog	AB204448
1	Saga	462	HL	N	dog	AB204434	3	Yamanashi	27	IN	F	cat	AB204504
1	Saga	1038	HL	M	dog	AB204475	3	Aichi	788	IN	F	dog	AB204451
1	Saga	1039	HL	N	dog	AB204476	3	Aichi	795	HL	F	dog	AB204452
1	Shiga	237	HL	N	dog	AB204417	3	Aichi	798	I	F	dog	AB204453
1	Nagasaki	116	HL	M	cat	AB204513	3	Ishikawa	47	IN	F	cat	AB204505
1	Nagasaki	1041	HL	N	dog	AB204477	3	Ishikawa	751	IN	F	dog	AB204450
1	Nagasaki	1043	HL	F	dog	AB204478	3	Kyoto	63	IN	F	cat	AB204506
1	Nagasaki	1057	HL	F	dog	AB204483	3	Osaka	865	IN	F	dog	AB204458
1	Miyazaki	128	HL	N	cat	AB204515	3	Nara	303	HL	F	dog	AB204420
1	Miyazaki	513	H	N	dog	AB204435	3	Wakayama	878	IN	F	dog	AB204460
1	Miyazaki	1093	HC	F	dog	AB204496	3	Wakayama	893	I	L	dog	AB204462
1	Kagoshima	519	HL	N	dog	AB204436	3	Tottori	86	H	F	cat	AB204507
2	Hokkaido	2	IP	F	cat	AB204503	3	Tottori	88	IO	F	cat	AB204508
2	Hokkaido	559	I	F	dog	AB204437	3	Tottori	90	HF	F	cat	AB204509
2	Hokkaido	562	I	F	dog	AB204438	3	Okayama	97	IN	F	cat	AB204510
2	Hokkaido	567	I	F	dog	AB204439	3	Hiroshima	100	IO	F	cat	AB204511
2	Hokkaido	569	I	F	dog	AB204440	3	Hiroshima	983	IO	F	dog	AB204470
2	Hokkaido	572	I	F	dog	AB204441	3	Ehime	444	H	F	dog	AB204431
2	Akita	617	HL	F	dog	AB204443	3	Kochi	1014	IN	F	dog	AB204472
2	Fukushima	625	HL	N	dog	AB204444	3	Nagasaki	120	HL	F	cat	AB204514
2	Ishikawa	745	IO	F	dog	AB204449	3	Miyazaki	129	IN	F	cat	AB204516
2	Osaka	860	H	F	dog	AB204457	3	Kagoshima	130	IN	F	cat	AB204517
2	Tottori	927	HF	F	dog	AB204466	3	Kagoshima	1107	HF	F	dog	AB204499
2	Hiroshima	1025	IO	F	dog	AB204473	4	Hokkaido	575	I	F	dog	AB204442
2	Tokushima	1008	HC	F	dog	AB204471	4	Fukuoka	450	H	F	dog	AB204432

HL: *Haemaphysalis longicornis*, HF: *H. flava*, HC: *H. campanulata*, H: *Haemaphysalis* sp., IP: *Ixodes persulcatus*, IO: *I. ovatus*, IN: *I. nipponensis*, I: *Ixodes* sp., RS: *Rhipicephalus sanguineus*, F: Femals, M: Male, N: Nymph, L: Larva. Accession numbers are registered sequences in GenBank.

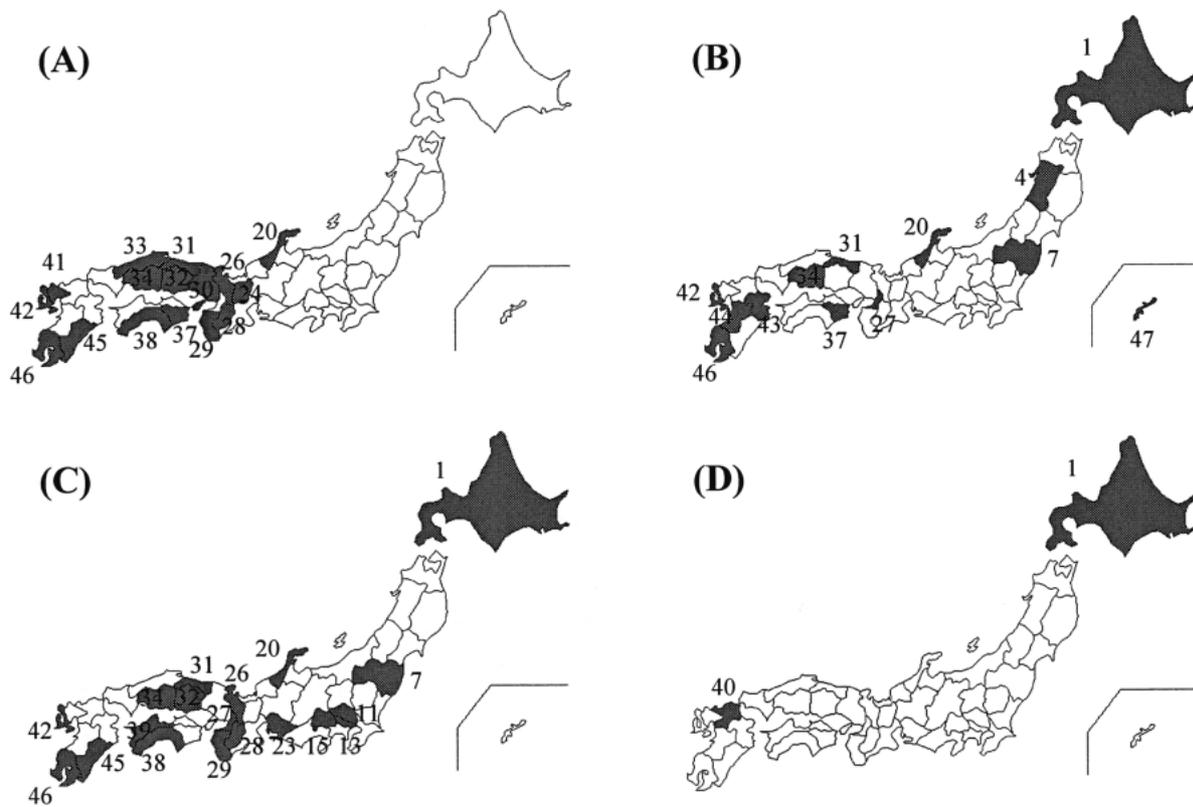


Fig. 2. The distribution of rickettsial DNA detected from ticks removed from dogs and cats in Japan. Prefectures that showed positive samples are in black in (A) *R. japonica* and related species, (B) *R. helvetica* and related species, (C) *R. akari* and related species, and (D) *R. canada* and related species. Each number represents a prefecture. 1: Hokkaido, 2: Aomori, 3: Iwate, 4: Akita, 5: Yamagata, 6: Miyagi, 7: Fukushima, 8: Tochigi, 9: Gunma, 10: Ibaraki, 11: Saitama, 12: Chiba, 13: Tokyo, 14: Kanagawa, 15: Yamanashi, 16: Nagano, 17: Shizuoka, 18: Niigata, 19: Toyama, 20: Ishikawa, 21: Fukui, 22: Gifu, 23: Aichi, 24: Shiga, 25: Mie, 26: Kyoto, 27: Osaka, 28: Nara, 29: Wakayama, 30: Hyogo, 31: Tottori, 32: Okayama, 33: Shimane, 34: Hiroshima, 35: Yamaguchi, 36: Kagawa, 37: Tokushima, 38: Kochi, 39: Ehime, 40: Fukuoka, 41: Saga, 42: Nagasaki, 43: Oita, 44: Kumamoto, 45: Miyazaki, 46: Kagoshima, 47: Okinawa.

from Hokkaido to Kagoshima. In Group 4, *R. canada*-like rickettsial DNA was detected in Fukuoka Prefecture, and 'C. R. tarasevichiae'-like DNA was from Hokkaido.

The information on tick stages and host animals for the positive tick samples are summarized in Table 1. Rickettsial DNAs in Group 1 were detected from 22 *H. longicornis* (7 females, 3 males and 12 nymphs), 8 *H. flava* (4 females, 2 males and 2 nymphs), 1 female of *H. campanulata*, 5 *Haemaphysalis* sp. (2 females, 2 nymphs and 1 larva) and 1 female of *Ixodes* sp. Most of them were recovered from dogs and only two *H. longicornis* were from cats. Group 2 rickettsial DNAs were from 10 *H. longicornis* (6 females and 4 nymphs), 2 females of *H. flava*, 2 females of *H. campanulata*, 9 *Haemaphysalis* sp. (4 females and 5 nymphs), 2 females of *I. ovatus*, 1 female of *I. persulcatus*, 5 females of *Ixodes* sp. and 2 *Rhipicephalus sanguineus* (1 female and 1 nymph). Only one *I. persulcatus* tick was recovered from a cat and the rest of them were recovered from dogs. DNAs in Group 3 were detected from 14 females of *I. nipponensis* (7 from dogs and 7 from cats), 4 females of *I. ovatus* (1 from a dog and 3 from cats), 4 *H. flava* (3 females and 1 nymph,

3 from dogs and 1 from a cat), 3 females of *H. longicornis* (2 from dogs and 1 from a cat), 2 *Ixodes* sp. (1 female and 1 larva from 2 dogs) and 2 *Haemaphysalis* sp. (1 female from a dog and another from a cat). In Group 4, *R. canada*-like rickettsial DNA was detected from a female *Haemaphysalis* sp. recovered from a dog, and 'C. R. tarasevichiae'-like DNA was from a female *Ixodes* sp. recovered from a dog. Most of the unidentified ticks were partly- or fully-engorged larvae or nymphs.

DISCUSSION

In the present study, ticks recovered from dogs and cats were used for the epidemiological study of *Rickettsia* in Japan. One tick was selected from each dog and cat for screening purposes. All the selected ticks were fully- or semi-engorged adults or nymphs, meaning they contained peripheral blood of the canine or feline host. Thus, a positive PCR result would lead to two possibilities of infection, either the tick or the animal host. From the nested PCR results, 109 samples were positive and these ticks were

widely distributed throughout Japan. Successfully determined 102 rickettsial *gltA* sequences were divided into 4 groups based on their sequence analyses.

In Group 1, rickettsial DNAs closely related to *R. japonica* or *Rickettsia* spp. strains DT1 FLA1, and Hf151, were detected from 38 ticks, mainly in western Japan. DT1 and FLA1, isolated from *Dermacentor taiwanensis* and *H. flava*, respectively, were found to be closely related to *R. japonica* [6]. *Rickettsia* sp. Hf151 detected from *H. flava* is thought to be a new species of the SFG rickettsiae and is particular to Japan [5]. It is not clear whether the *Rickettsia* spp. strains DT1, FLA1 and Hf151 are pathogenic to humans and/or animals. As *R. japonica* is a well known pathogen of Japanese spotted fever in Japan [7], it is important to distinguish *R. japonica* from other related strains. Unfortunately the 322bp of the rickettsial *gltA* analyzed in this study was too short to determine the strains accurately. More sequence analyses using other genes should be carried out for this purpose. However, in the present study, rickettsial DNAs of *R. japonica* or closely related strains were detected from several prefectures, including Tokyo, Ishikawa, Kyoto, Nara, Okayama and Tottori, where the pathogens or JSF patients have never been detected before [5, 6, 8]. *D. taiwanensis* and *H. flava* are confirmed vectors of *R. japonica* [6] and *H. longicornis* is thought to be a potential vector of the pathogen [15]. In this study, 57.9% of Group 1 rickettsial DNA was detected from *H. longicornis*, the predominant tick species of dogs and cats. These results suggest that *H. longicornis* may be a vector of *R. japonica* and closely related strains.

In Group 2, *R. helvetica* and closely related strains IM1, IP2 and IO1, are included [2, 6]. In the present study, 33 detected DNA were found to be *R. helvetica* or closely related strains. The DNA was detected from Hokkaido, northern Japan, to Okinawa, southern most islands. Previously *R. helvetica* was known to exist only in European countries [1]. Recently, several *Rickettsia* spp. closely related to *R. helvetica* were detected from *Ixodes* ticks in Hokkaido, Akita, Fukushima, Kumamoto and Kagoshima prefectures [2, 6]. *R. helvetica* and its close strains were found to be more widespread in Japan than previously reported. It is possible that this group of *Rickettsia* might be more widely distributed than expected. Furthermore, most of the *R. helvetica* and related DNA were detected from *Haemaphysalis* ticks in this study, although suspected vectors of *R. helvetica* and related species are *Ixodes* spp [2]. Whether or not *Haemaphysalis* is a vector of *R. helvetica* or closely related strains in Japan remains to be seen, because it is not clear whether the rickettsial DNA came from the tick or the host. DNA fragments of *R. helvetica*-like strains were also detected from 2 *Rh. sanguineus* ticks recovered from 2 dogs in Okinawa, in this study. This is the first record where DNA of *R. helvetica* or a close strain was detected from *Rh. sanguineus* and also the first such finding in the sub-tropics. *Rh. sanguineus* is a tick that prefers canine hosts and is known to be a vector of *R. conorii*, an etiological agent of Mediterranean spotted fever [9]. How-

ever, *R. conorii* was not detected in this study.

R. akari is a pathogen of rickettsial pox and has been detected in the U.S.A., Ukraine, Korea and Slovenia [10]. Although *R. akari* has never been detected in Japan, *Rickettsia* spp. strains AT1 and In56 that are closely related to *R. akari* have recently been detected in Japan [5, 6]. The pathogenicity of these strains, however, remains to be elucidated. In the present study, 1 rickettsial DNA which showed 100% nucleotide sequence identity to strain AT1 and 28 rickettsial DNA which showed high nucleotide sequence identity to strain In56, was identified and categorized into Group 3. Rickettsial DNA in Group 3 was widely distributed from northern to southern Japan. More than half of the *R. akari* and related DNA were detected from *Ixodes nipponensis* and *Ixodes ovatus* ticks, in this study. These ticks may be possible vectors of this agent. Furthermore, cats were more related to this *Rickettsia* than other *Rickettsia* species, although direct evidence is required to demonstrate the relationship between cats, *Ixodes* spp. and this group of *Rickettsia*.

R. canada is widespread in northern America and its vector is *H. leptorispalustris*. The pathogenesis of *R. canada* is not clear, but there is a report that *R. canada* causes a Rocky Mountain spotted fever-like disease and acute cerebrovasculitis in humans [3]. *R. canada* or a related species has never been detected or isolated in Japan, thus this is the first record of this *Rickettsia* species in Japan. In this study, a DNA fragment similar to *R. canada* was detected from a tick recovered from a dog in Fukuoka prefecture, but the origin and history of overseas travel of the dog were both unknown. Another *R. canada*-like DNA was detected from an *Ixodes* sp. tick recovered from a dog in Hokkaido. The *gltA* sequence showed 99.8% similarity with 'C. *Rickettsia tarasevichiae*' which was recently detected from *I. persulcatus* in Russia [14]. In Russia, 9.27 to 20.5% of *I. persulcatus* have 'C. *R. tarasevichiae*', but its pathogenicity is still unclear [16]. 'C. *Rickettsia tarasevichiae*' has never been detected or isolated outside of Russia, including Japan. The dog from which the tick was recovered showed no symptoms except for heavy tick infestation. Although the origin and history of overseas travel of this dog were unknown, it is possible that 'C. *Rickettsia tarasevichiae*' is present in northern Japan where the tick vector *I. persulcatus* can be found [12].

Four groups of Rickettsiae, including possible new pathogens, were detected in this study. Although more epidemiological studies are required to clarify the distribution, vectors, and carriers of the Rickettsia, dogs and cats may be possible carriers of the agents. It is also important for both medical and veterinary workers to be aware of the variety of rickettsial pathogens present in Japan.

ACKNOWLEDGMENTS. The authors would like to acknowledge the technical expertise of the DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a Grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. They would also like

to thank Nippon Zenyaku Kogyo Co., Ltd. for collecting and sending the tick samples. This work was supported in part by Meril Japan Ltd., National Institute of Animal Health, and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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