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Phylogenetic Analysis of Spotted Fever Group Rickettsia Gene from Ticks and Human Patients in Tottori Prefecture, Japan

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ABSTRACT

Background Japanese spotted fever (JSF) is a tick-borne bacterial febrile disease caused by *Rickettsia japonica* characterized by fever, rash, and occasional death. The number of patients in Japan and the Tottori Prefecture has been increasing over the past 20 years. Most cases were found in Eastern Tottori; however, the distribution of patients has expanded to the Central and Western regions. Ticks carried by wild animals may be the cause, but the prevalence of *R. japonica* in ticks has not yet been analyzed.

Methods Ticks were collected by flagging-dragging from 16 sites in Tottori, Japan. The ticks were morphologically classified and DNA was extracted. The 17-kDa antigen gene was amplified using nested PCR. PCR amplicons from ticks and JSF patients were sequenced and phylogenetically compared.

Results In total, 177 ticks were collected and identified as *Haemahysalis, Ixodes, Amblyomma, and Dermcentor*. The Spotted Fever Group Rickettsia (SFGR) was detected in *Haemahysalis* and *Amblyomma* spp. using PCR, with positivity rates of 36.8% and 33.3%, respectively. DNA sequencing and phylogenetic analysis revealed that positive ticks harbored *R. japonica*, *P. raoultii*, and other Rickettsiae species; however, the patient's samples were restricted to *R. japonica*. Similar to the incidence of JSF, the rate of *R. japonica*-positive ticks was higher in the Eastern region; however, *R. japonica*-positive ticks were also detected in the Western region.

Conclusion *R. japonica* sequences had been found in ticks collected in Tottori Prefecture. Ticks harboring *R. japonica* were found in the Eastern and Western parts of Tottori Prefecture and the sequences were identical to the human cases. Only the *R. japonica* sequence has been detected in patients with spotted fever symptoms, even though ticks were harboring various SFGRs.

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Abbreviations: JSF, Japanese spotted fever; SFGR, Spotted Fever Group Rickettsia

Key words Japanese spotted fever; tick; Tottori Prefecture; *Rickettsia japonica*; Spotted Fever Group Rickettsia

Rickettsia is a Gram-negative, rod-shaped bacterium known to be an obligate intracellular organism that can parasitize both vertebrate and arthropod hosts.[1] Rickettsiae are classified into four groups: the transitional group (TRG), spotted fever group (SFG), typhus group (TG), and ancestral group (AG).[2] SFGR is known to cause several human diseases such as Rocky Mountain spotted fever, Mediterranean spotted fever, and mild-to-moderate spotted fever, and so on.[1, 3]

Japanese spotted fever (JSF) is a tick-borne disease caused by *Rickettsia japonica*: the disease was first described in Tokushima Prefecture in Southwestern Japan and named by Mahara in 1985.[4] The causative pathogen was isolated and named *R. japonica* for the new species in 1992.[5] JSF distributes mainly in Japan, but there are also several reports of patients in South Korea, Thailand, and China.[6–8] The main symptoms of JSF are high fever and rash after 2-8 days of the tick bite and if treatment is delayed, it sometimes causes death, especially in the elderly.[9] More than 300 patients and occasional death cases are reported annually, and the number of cases is increasing year by year.[10] In recent times, the geographic distribution of JSF patients has been expanding.[11] The reason for this expansion is not clearly understood. It can be attributed to devastated farmlands and forests that force wild animals toward human habitats, often bringing ticks with them.[12] These wild animals are recognized as the reservoir of *R. japonica*, which would be infected by the Rickettsia harboring tick-bites and transmit the Rickettsia to other uninfected ticks and make them infectious. According to a report, wild Japanese deer (*Cervus nippon*) in the Shimane Peninsula showed 92.7% positivity for antibodies against the *R. japonica* antigen.[13] During activities in field sites, such as farming, forestry, or hiking, people have the chance to be bitten by ticks. Further reduction of the rural area population is expected in the future, meaning that more people would be exposed to the danger of tick bites and JSF infection.

For 15 years since 2007, 55 cases of JSF have been

reported in Tottori Prefecture, and the case number has been increasing since 2015.[14] Iwami town, located in prefectural east-end seaside, which used to be a hotspot of JSF in Tottori Prefecture, accounts for 69.1% of all cases. But after the first two cases were reported in Yonago city in 2017, sporadic cases were found in the Central and Western parts such as Kurayoshi city, Yonago city, and Sakaiminato city. The reason for expansion is not fully understood, but the distribution area of ticks carrying *R. japonica* might be expanding as wild animals move, as already described above. So far, *R. japonica* in ticks has not been identified in the Tottori Prefecture, even though the local Prefectural Institute attempted some surveys. There is one report that ticks collected from dogs and cats from all over Japan were examined, and some SFGR sequences close to *R. japonica* were reported in Tottori origin samples but there was no detailed description about identity of the sequence.[15] On the other hand, JSF patients were only diagnosed using PCR band positivity. Since no further analysis was carried out to identify Rickettsia species, there was no detailed information about the pathogens.

R. japonica belongs to the Spotted Fever Group Rickettsia (SFGR), and some of the species cause spotted fever in humans worldwide.[16] *R. japonica* is thought to be a symbiotic organism of ticks and habitats mainly in the digestive tract, such as the salivary gland, midgut, and ovary.[17] But not all SFGR are pathogenic,[1] and *R. japonica* functions as a symbiotic bacterium, which may benefit the host tick.[18] Because ticks suck blood three times in their life, there would be the chance to transmit *R. japonica* from one animal to another, but basically, it is maintained within the body of the tick and transmitted to the next generation via the egg.[17] To understand the reason for the expansion of JSF in Tottori Prefecture, first of all, the survey of ticks and human patients is important for clarifying the actual state of the SFGR population.

In this study, we examined SFGR prevalence in ticks collected from a wide area of the Tottori Prefecture and analyzed SFGR-specific molecular sequences. Clinical samples from patients diagnosed with JSF were also analyzed and compared with tick samples to determine the relationship between ticks harboring SFGR and human pathogenic Rickettsia in Tottori Prefecture.

MATERIALS AND METHODS

Sample collection

Ticks were collected at 16 points in four towns or cities in the Tottori Prefecture: Iwami Town, Tottori City (Fukube and Ketaka), Yurihama Town, and Yonago City from 2017 to 2019. These points were selected throughout the Tottori Prefecture for characteristic terrains between the mountains and coasts, where JSF patients have been reported frequently. Tick collection sites were precincts of shrines, road sides, and around farmlands. The precise locations where the ticks were collected were recorded using a GPS receiver O-GPS1 linked to a digital camera (PENTAX, Tokyo, Japan). Ticks were collected by the dragging-flagging method.[19] A 60 cm long and 45 cm wide white felt flag was spread out on the ground and dragged slowly over the grasses or bushes to capture ticks. The ticks attached to the felt were transferred to 70% ethanol in a collection tube using tweezers, and stored at room temperature until use. The date and location of collection were recorded for each tick.

Tick species identification

The collected ticks were examined using a stereomicroscope (OLYMPUS SZX7, Olympus Corp., Tokyo, Japan) and classified based on their morphological characteristics.[20, 21]

Genomic DNA extraction

DNA extraction and purification were performed individually for adult ticks. For ticks in the nymph stage, DNA was extracted individually or from a pool of two to four individuals. Ticks in the larval stages were treated individually or from a pool of two to eight individuals. The ticks were washed with deionized distilled water and cut using a surgical knife for extraction. DNA was purified using the QIAamp DNA Mini Kit or Qiagen DNA Easy Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified genomic DNA was solubilized in Tris-EDTA buffer and preserved at –20 °C until use.

Genomic DNA from patients

Nineteen patients with symptoms of spotted fever, such as high fever and rash, were hospitalized for diagnosis and treatment in the Tottori Prefecture from 2015 to 2018. The patients were quickly treated with tetracycline after whole blood and/or eschar samples were collected for diagnosis at the Tottori Prefectural Institute of Public Health and Environmental Science. DNA was purified from the blood and eschar separately and nested PCR was performed using specific primer sets to diagnose JSF and Tsutsugamushi disease (Scrub typhus). The remaining DNA samples were used to analyze and determine sequences, and each sample was provided without attaching any information about the patients. DNA samples were used to amplify Rickettsia sequences. Patients were able to opt out of this study by their

Table 1. Polymerase chain reaction primers and conditions to detect reckettsia in ticks and patient samples

Table 1a. Primers

Primer name		Primer sequences (5'-3')	Amplicon size (bp)	References
1st	R1	TCAATTCACATTGCCATT	533–539	Anderson BE, et al.[23]
	R2	TTTACAAAATTCTAAAAACC		
2nd	Rj5	CGCCATTCTACGTTACTACC	375	Furuya Y, et al.[22]
	Rj10	ATTCTAAAAACCATATACTG		

Table 1b. Reaction mixture

Components	Volume
10xPCR Buffer	5 μL
2.5 mM dNTPs	4 μL
Primer 1 (50 mM)	1 μL
Primer 2 (50 mM)	1 μL
DDW	33.75 μL
Ex Taq (TAKARA)	0.25 μL
Template	5 μL
Total	50 μL

Table 1c. PCR conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	94 °C	45 sec	
Annealing	52 °C	30 sec	35
Extension	72 °C	45 sec	
Final extension	72 °C	7 min	1

own will. This study was approved by the Institutional Ethical Review Board of the Tottori University Faculty of Medicine (No. 21A023).

Rickettsia detection and sequencing

To detect SFGR, nested PCR was performed to amplify the 17-kDa antigen gene.[22, 23] The nested-PCR conditions and primer sets are shown in Table 1. Positive PCR products were cloned into the pGEM-T Easy TA-cloning vector plasmid (Promega, Madison, WI), and one or two positive colonies were propagated in 3 ml of liquid culture medium. Plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN), sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA), and samples were sequenced by FASMAC (Kanagawa, Japan). The sequence data obtained were analyzed using MacVector 18.5 software (MacVector Inc., Apex, NC) prior to phylogenetic analysis.

Phylogenetic analysis

The standard *R. japonica* lineage YH-M and other SFGR sequences were obtained from the DDBJ/EMBL/GenBank databases. Sequences of Rickettsia from ticks and patients and human pathogenic Rickettsia sequences from the database were aligned using the MEGA X software.[24] The neighbor-joining method using the Kimura 2-parameter model was adopted to construct the phylogram. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.[25] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and expressed in units of the number of base substitutions per site.[26] This analysis involved 130 nucleotide sequences. All positions with gaps or missing data were eliminated (complete deletion). The final dataset contained 252 positions.

Blastx analysis

The obtained sequences of the 17kDa antigen were analyzed using a blastx search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastx) to determine homology with known sequences in the genome database. The query nucleotide sequence was automatically translated into an amino acid sequence and compared with the data in the protein database.

RESULTS

Tick collection and identification

In total, 104 specimens (177 ticks) were collected from 16 sites in four towns or cities in the Tottori Prefecture, as shown on the map (Fig. 1). Morphological identification under a binocular stereomicroscope revealed that the ticks consisted of *Haemahysalis*, *Ixodes*, *Amblyomma,* and *Dermcentor*. *Haemaphysalis* ticks

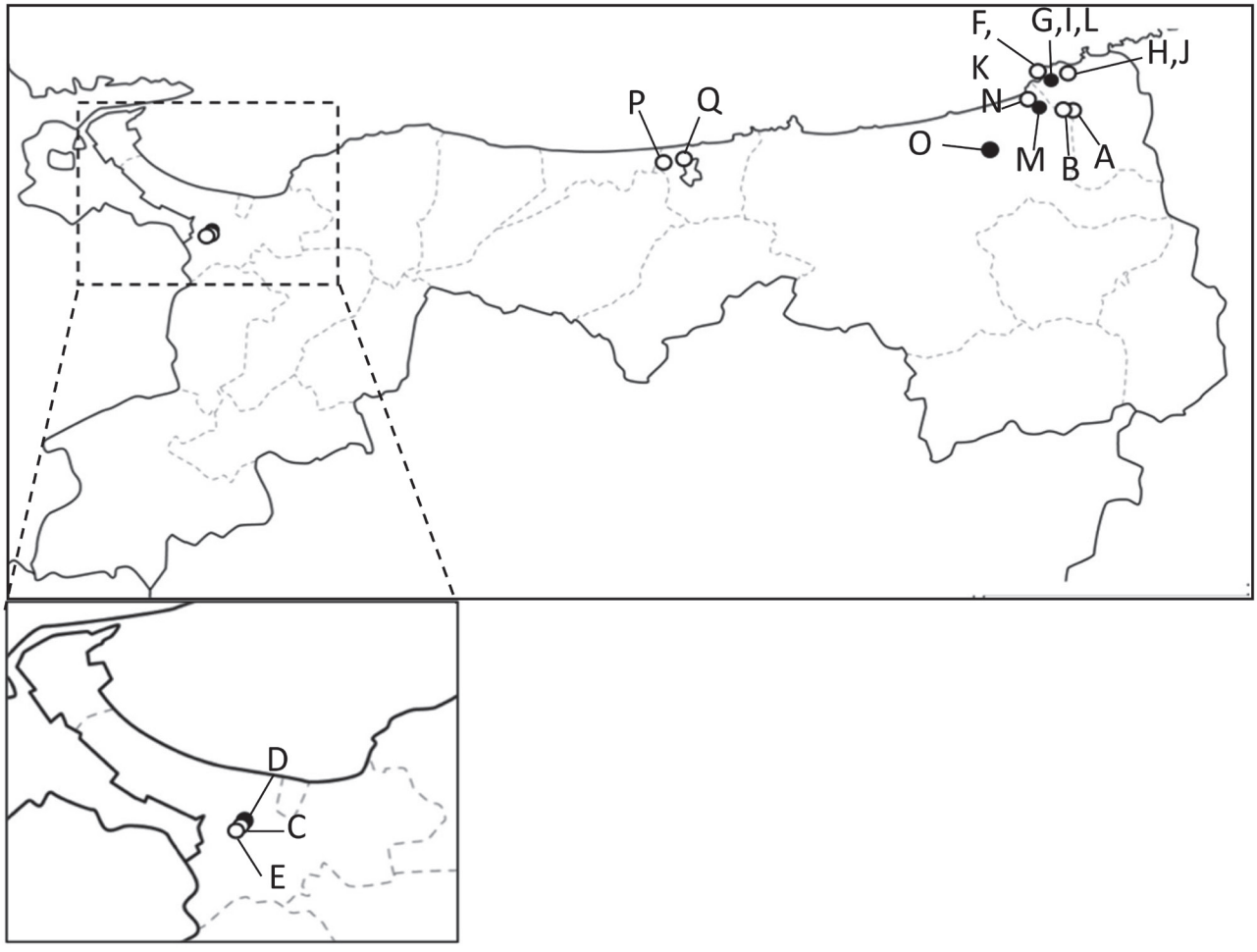

Fig. 1. Map of tick collection sites in Tottori Prefecture. Black and white circles represent the sites where *R. japonica* positive and negative ticks were collected, respectively. A letter for each site corresponds to tick name.

were dominant (94 of 104 samples, 91.3%). The stages and sex of the ticks are shown in Table 2, and representative photographs of the ticks collected at each life stage are shown in Fig. 2.

Detection and identification of SFGR from collected ticks and patients

PCR results as 17-kDa antigen band positive, that is, SFGR (including *R. japonica*)-positive samples, were detected in 36 of the 104 specimens (34.6%) (Table 2) and in all human samples by PCR. To identify the Rickettsia species, the amino acid sequences of the 17-kDa antigen derived from ticks and patients obtained by sequencing were aligned (Fig. 3). In this study, *R. japonica* was defined as having a 17-kDa antigen gene that showed 100% identity with *R. japonica* YH-M strain (GenBank accession number: AP011533), including synonymous mutations. We succeeded in detecting pathogenic *R. japonica* in 11 ticks (10.6% of the total, 30.6% of SFGR-positive ticks). In contrast, all sequences from the 19 patients perfectly matched the *R. japonica* YH-M sequence. In addition, eight samples from ticks showed predicted amino acid sequences identical to *R. raoultii* (Fig. 3), which is a causative bacterium of tick-borne lymphadenopathy (TIBOLA) in Europe and China.[3, 27]

The 17-kDa antigen gene fragment on the SFGR genome was detected in almost all *Haemahysalis* spp. ticks, with only one exception, and the *R. japonica* sequence was detected only in *Haemahysalis* spp. (Table 2). SFGR and *R. japonica* were detected from all stages of *Haemahysalis* spp. (Fig. 2). A regional analysis revealed that many ticks harboring Rickettsia were found in the Eastern part of Tottori Prefecture, with a considerably high positivity rate of 76.5% in Ketaka (Table 3). The *R. japonica* positivity rate was higher in the Eastern sites, at approximately 17% in Fukube and Ketaka. SFGR and *R. japonica* were also detected in the Western part of

Table 2. Rickettsia-carrying rate of collected ticks in Tottori Prefecture by species and stage

Species (spp.)	Stage (sex)	Number	PCR positve sample	
			(Number)	(%)
Haemaphysalis	Total	95	35	36.8%
	Larva	46	20	43.5%
	Nymph	28	10	35.7%
	Adult (male)	4	1	25.0%
	Adult (female)	17	4	23.5%
Ixodes	Total	5	0	0%
	Larva	0	0	0%
	Nymph	1	0	0%
	Adult (male)	1	0	0%
	Adult (female)	3	0	0%
Amblyomma	Total	3	1	33.3%
	Larva	2	1	50.0%
	Nymph	1	0	0%
	Adult (male)	0	0	0%
	Adult (female)	0	0	0%
Dermacentor	Total	1	0	0%
	Larva	0	0	0%
	Nymph	0	0	0%
	Adult (male)	0	0	0%
	Adult (female)	1	0	0%
Total		104	36	34.60%

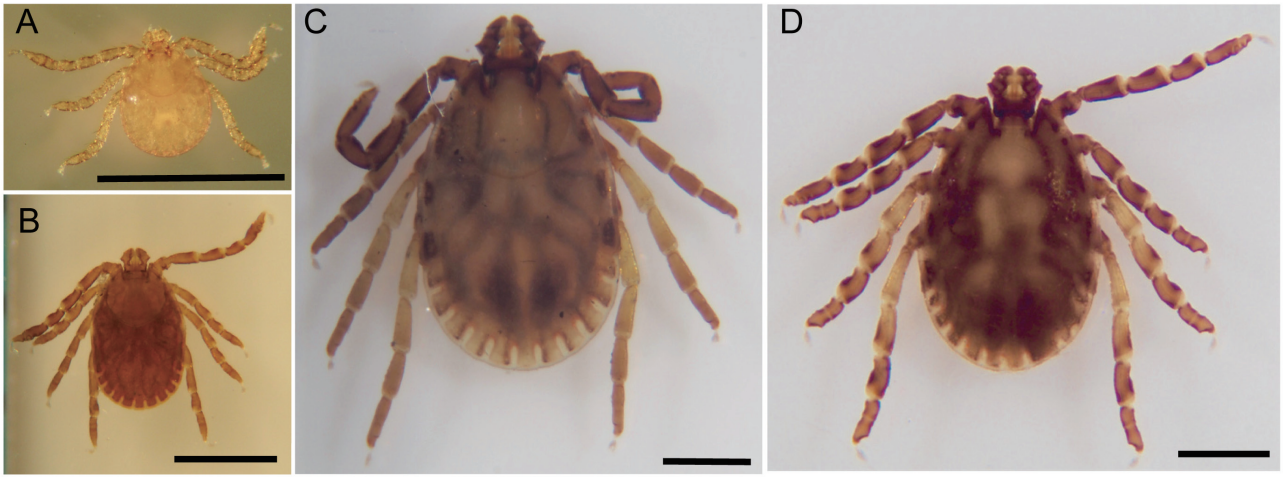

Fig. 2. All life stages of *Haemahysalis* spp ticks collected from field sites in this study. A: larva, B: nymph, C: adult /female, and D: adult /male. Scale bars = 1 mm.

the prefecture, although the prevalence rate was lower than that in the Eastern part. From the collected ticks, *R. japonica* was not detected in the Central part of Tottori Prefecture.

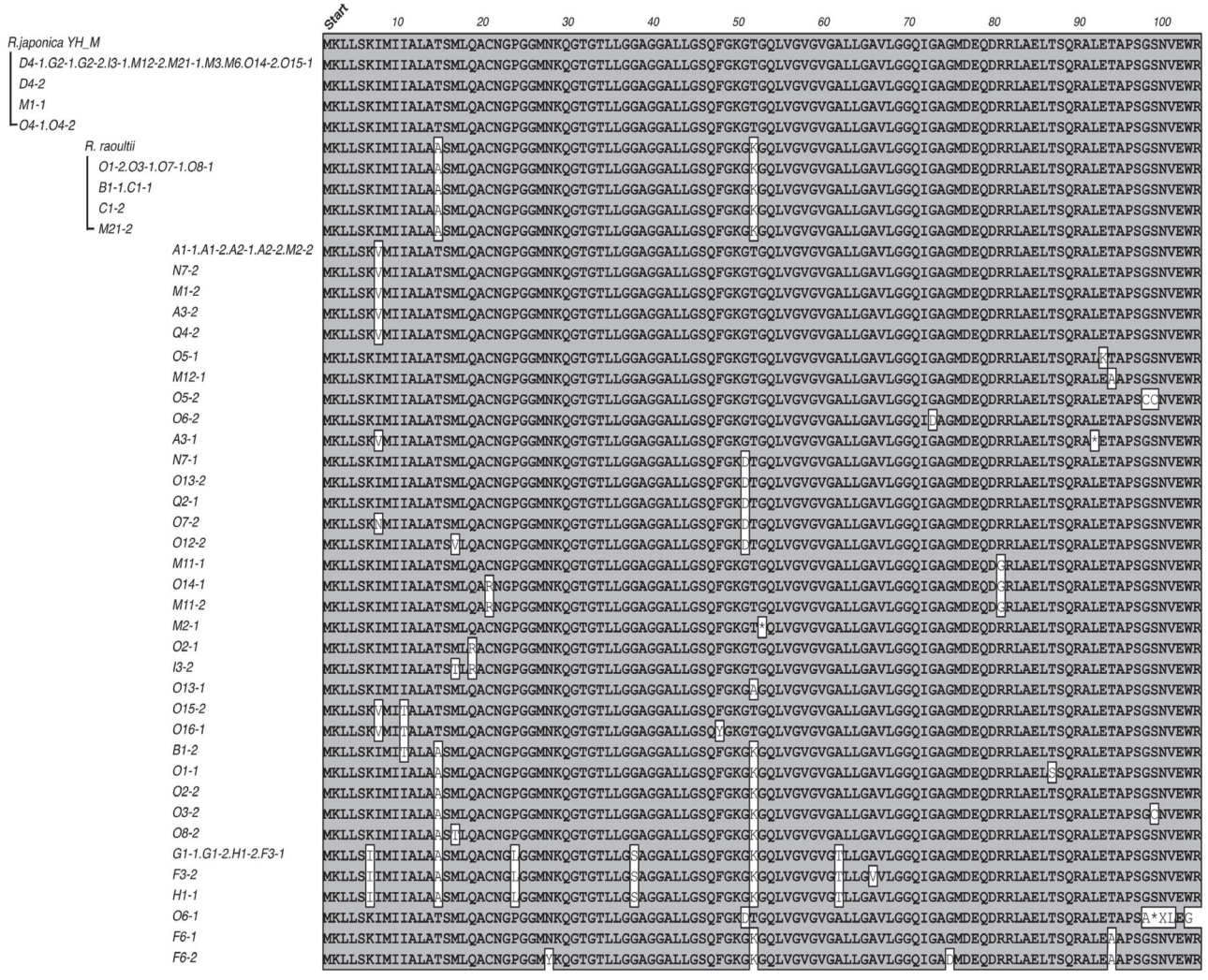

Fig. 3. Alignment of the amino acid sequence of 17-kDa antigens among SFGR from collected ticks, *R. japonica* and *R. raoultii* obtained from the database as standard sequences are also shown. The blocked area shows an identical sequence to the *R. japonica* YH-M sequence.

Table 3. Rickettsia-carrying rate of collected ticks in 4 cities and towns of Tottori prefecture

Area	Number	PCR positve samples		*R. japonica* positve samples	
		(Number)	(%)	(Number)	(%)
Iwami	31	10	32.3%	2	6.4%
Fukube*	29	9	31.0%	5	17.2%
Ketaka*	17	13	76.5%	3	17.6%
Yurihama	9	2	22.2%	0	0%
Yonago	18	2	11.1%	1	5.6%
Total	104	36	34.60%	11	10.6%

* in Tottori city

Classification of Rickettsia derived from ticks

To classify the 36 Rickettsia species isolated from ticks, we performed a phylogenetic analysis of 17-kDa antigen gene sequences from ticks and patients with known

human pathogenic SFGR sequences (Fig. 4). Sequences from ticks showed that some groups of the largest clade were identical or closely related to *R. japonica*. In patients, most of the samples were identical to *R. japonica*, but some samples had non-synonymous substitutions, such that an amino acid sequence change of the protein occurred. However, in all the patients, at least one sample was identical to *R. japonica* and showed JSF symptoms. The remaining sequences from ticks that did not match *R. japonica* seemed to form four clades, and one clade contained *R. raoultii* which may cause lymphadenopathy, similar to the amino acid sequence alignment. Samples which were identical to *R. longicornii* / *R. jingxinensis* were also detected, but there has not been any reports of human virulence.[28, 29] We also identified two SFGR in different clades that were detected in one tick sample (M21, O2, and O7), respectively. The list of ticks and rickettsia identified by BLASTX analysis is shown in Table 4.

DISCUSSION

In this study, we used field tick and JSF patient samples to detect the SFGR sequence: 1) ticks were collected from the field and we amplified *R. japonica* sequences that were identical to the human pathogen from ticks for the first time in Tottori Prefecture. We guessed that *R. japonica* harboring ticks are already established in Tottori Prefecture; 2) *R. japonica* harboring ticks were found from Eastern to Western Tottori, the distribution area being much wider than expected; 3) we revealed all clinically and PCR diagnosed JSF patient samples contained in the *R. japonica* sequence, and this may suggest a correlation between distribution of ticks and human cases; and 4) we detected several different SFGR sequences from ticks, and some of the sequences were identical to possible human pathogen *R. raoultii*, but no similar sequences were found in human JSF diagnosed samples.

We focused on tick-borne SFGR, especially *R. japonica*, which causes Japanese spotted fever, with an increasing number of cases in Japan. In Tottori Prefecture, the number of JSF cases has been increasing since 2015, mainly in the Eastern part of the prefecture. Eventually, areas of the reported cases began to expand in the prefecture, but there were no reports of *R. japonica* detection in ticks. In addition, no information on patient pathogens has been reported. Therefore, a survey of tick-carrying SFGR and an analysis of the Rickettsia sequence from human cases needed to be performed.

A total of 177 ticks were captured in the field from 16 sites in Tottori Prefecture. By 17kDa antigen PCR amplification and sequencing, we detected the identical sequence to *R. japonica* from ticks. This clearly shows *R. japonica* harboring ticks have been established in Tottori Prefecture for a long time. Around these sites, sighting information and/or traces of wild boar were often found. Ticks that suck blood from wild animals, including wild boars, have recently expanded their habitats closer to households. We think this is mostly because encroachment in forests and mountains has been increasing driving wildlife into urban areas.

SFGR harboring ticks could be detected from a wide area in Tottori Prefecture. *R. japonica* harboring ticks were mainly found in the Eastern Tottori, where most of the patients were also reported. Even though a lesser number of SFGR-positive ticks were detected in Western Tottori, it has been estimated that they have always been present throughout the whole prefecture. Thus, a variety of regional residents are exposed to risk of infection. And it should be emphasized that these *R. japonica* harboring ticks were collected on the ground or in bushes at shrines or on the roadside where local people have daily access; sometimes we even found traces of wild animals - mainly wild boar - around these spots. Our results show a higher prevalence of *R. japonica* than that in other reports.[30] And this report is also the first to confirm *R. japonica* from JSF patient samples by sequencing. All patients harbored at least one sequence that was 100% identical to *R. japonica*. The *R. japonica* genome is reported to show little diversity[31] but sometimes shows some degree of substitution, and patients having symptoms typical of JSF were also hospitalized. Therefore, we decided to treat these samples as *R. japonica* as well.

We detected *R. japonica, R. raoultii* and other Rickettsia species that could be amplified from the *R. japonica*-specific primers from ticks. At least one known human pathogenic SFGR, *R. raoultii* has been detected in ticks,[16] although there have been no reports of tick-borne lymphadenopathy in Japan. *R. raoultii* has been detected in ticks in several different areas in Japan,[32] but no detection in patients has been reported, although spotted fevers other than JSF have been reported sporadically in Japan.[30, 33] And to date, there have been no reports of non-JSF spotted fever cases in Tottori Prefecture. However, JSF patients in Tottori were diagnosed with typical symptoms of spotted fever. Therefore, the pathogenesis for those unclassified SFGRs hasn't been delineated yet. We have to consider the possibility of non-JSF like symptoms in the cases that were not diagnosed as JSF but were caused by unidentified Rickettsiae. These cases may have unique symptoms without being diagnosed as typical spotted fever.

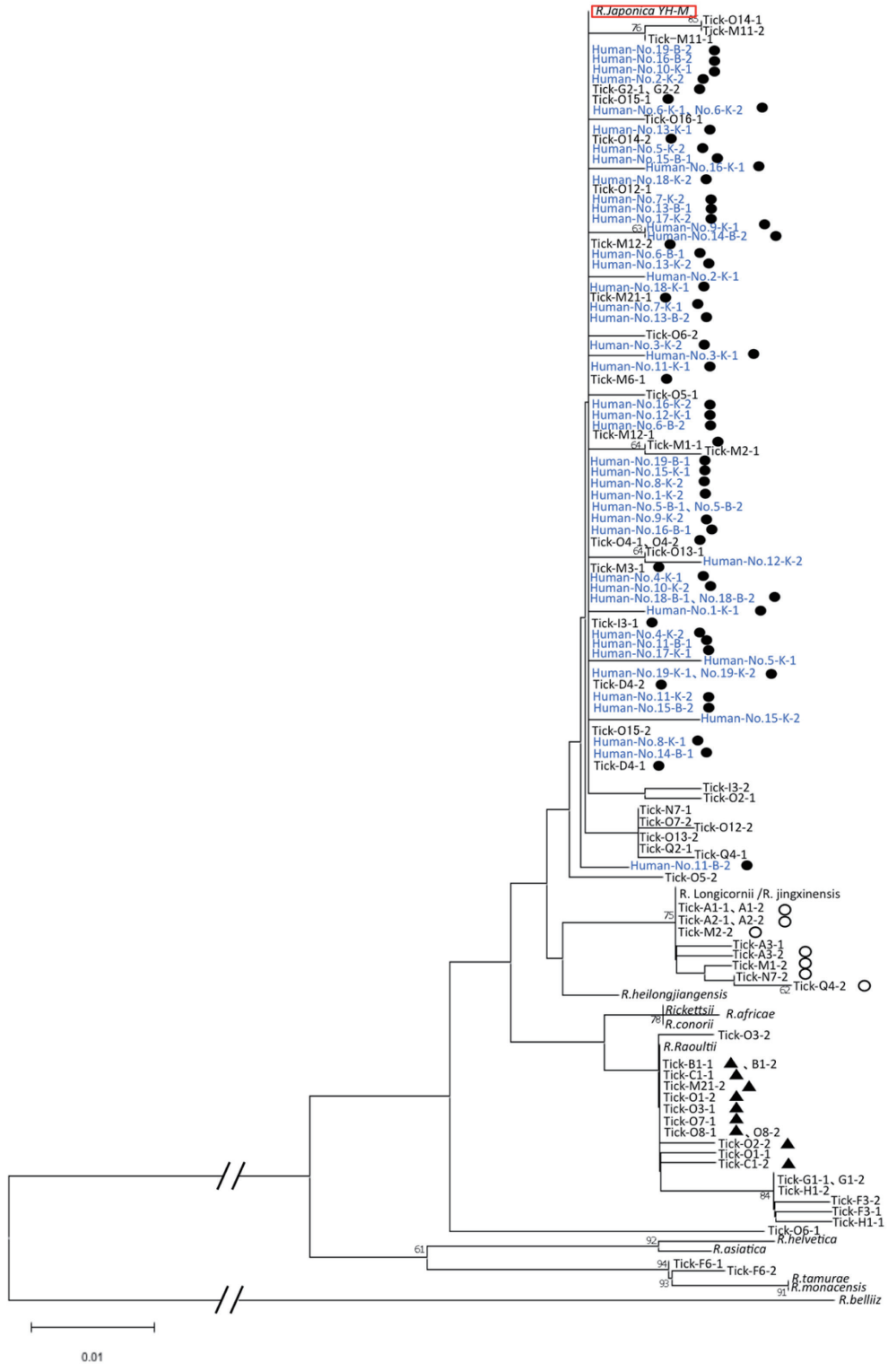

Fig. 4. Phylogenetic analysis of Rickettsiae based on 17-kDa antigen gene sequences of ticks and patient samples. Analysis was implemented by MEGA X software. Neighbor-joining phylogenetic tree construction and bootstrap analysis were performed according to the Kimura 2-parameter distances method. The human samples were named Human-No. X-B/K-clone number and shown in blue letters. B stands for blood samples, and K for eschar samples. Tick samples were named Tick- with a combination of the location (indicated by uppercase letter)-specimen number-clone number and shown in black letters. Black dots showed 100% identity with *R. japonica*. White dots showed 100% identity with *R. longicornii / R. jingxinensis.* Black triangle showed 100% identity with *R. raoultii*.

Table 4. List of collected ticks and Rickettsia analyzed using blastx

	Ticks			
Sample No.	Colection site	Genus	Stage/Sex	Species of Rickettsiae
G2-1	Iwami	*Haemaphysalis* spp.	Adult/F	*R. japonica*
G2-2	Iwami	*Haemaphysalis* spp.	Adult/F	*R. japonica*
I3-1	Iwami	*Haemaphysalis* spp.	Larva	*R. japonica*
M1-1	Fukube	*Haemaphysalis* spp.	Adult/F	*R. japonica*
M3-1	Fukube	*Haemaphysalis* spp.	Nymph	*R. japonica*
M6-1	Fukube	*Haemaphysalis* spp.	Adult/F	*R. japonica*
M12-2	Fukube	*Haemaphysalis* spp.	Larva	*R. japonica*
M21-1	Fukube	*Haemaphysalis* spp.	Nymph	*R. japonica*
O4-1	Ketaka	*Haemaphysalis* spp.	Larva	*R. japonica*
O4-2	Ketaka	*Haemaphysalis* spp.	Larva	*R. japonica*
O14-2	Ketaka	*Haemaphysalis* spp.	Larva	*R. japonica*
O15-1	Ketaka	*Haemaphysalis* spp.	Nymph	*R. japonica*
B1-1	Iwami	*Haemaphysalis* spp.	Larva	*R. raoultii*
M21-2	Fukube	*Haemaphysalis* spp.	Nymph	*R. raoultii*
O1-2	Ketaka	*Haemaphysalis* spp.	Larva	*R. raoultii*
O2-2	Ketaka	*Haemaphysalis* spp.	Larva	*R. raoultii*
O3-1	Ketaka	*Haemaphysalis* spp.	Larva	*R. raoultii*
O7-1	Ketaka	*Haemaphysalis* spp.	Larva	*R. raoultii*
O8-1	Ketaka	*Haemaphysalis* spp.	Larva	*R. raoultii*
A1-1	Iwami	*Haemaphysalis* spp.	Larva	*R. longicornii/R. jingxinensis*
A1-2	Iwami	*Haemaphysalis* spp.	Larva	*R. longicornii/R. jingxinensis*
A2-1	Iwami	*Haemaphysalis* spp.	Larva	*R. longicornii/R. jingxinensis*
A2-2	Iwami	*Haemaphysalis* spp.	Larva	*R. longicornii/R. jingxinensis*
A3-2	Iwami	*Haemaphysalis* spp.	Larva	*R. longicornii/R. jingxinensis*
M1-2	Fukube	*Haemaphysalis* spp.	Adult/F	*R. longicornii/R. jingxinensis*
M2-2	Fukube	*Haemaphysalis* spp.	Nymph	*R. longicornii/R. jingxinensis*
N7-2	Fukube	*Haemaphysalis* spp.	Nymph	*R. longicornii/R. jingxinensis*
F3-1	Iwami	*Haemaphysalis* spp.	Adult/F	*Rickettsiae* spp.
G1-1	Iwami	*Haemaphysalis* spp.	Adult/M	*Rickettsiae* spp.
G1-2	Iwami	*Haemaphysalis* spp.	Adult/M	*Rickettsiae* spp.
H1-1	Iwami	*Haemaphysalis* spp.	Nymph	*Rickettsiae* spp.
H1-2	Iwami	*Haemaphysalis* spp.	Nymph	*Rickettsiae* spp.
Q4-2	Yurihama	*Haemaphysalis* spp.	Nymph	*R. longicornii/R. jingxinensis*
D4-1	Yonago	*Haemaphysalis* spp.	Larva	*R. japonica*
D4-2	Yonago	*Haemaphysalis* spp.	Larva	*R. japonica*
C1-1	Yonago	*Haemaphysalis* spp.	Larva	*R. raoultii*
C1-2	Yonago	*Haemaphysalis* spp.	Larva	*R. raoultii*

To prevent the further increase of JSF patients, it is important to prevent residents from being bitten by ticks. To achieve this goal, wild animals must be kept away from human habitats by any means necessary. We also found ticks harboring a variety of Rickettsia, but patients with human spotted fever showed only *R.*

japonica sequences. This discrepancy may be due to differences in pathogenicity; however, this remains to be addressed in future studies. A survey of wild animals that may play a role as sources of diseased ticks is also important.

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The authors declare no conflict of interest.

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