

Molecular Detection of Spotted Fever Group Rickettsia in Feral Raccoons (*Procyon lotor*) in the Western Part of Japan

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ABSTRACT. Rickettsial infection in feral raccoons (*Procyon lotor*) in the western part of Japan (Shimane, Fukuoka, Saga and Nagasaki Prefectures) was surveyed by a nested polymerase chain reaction (PCR) assay detecting the rickettsial citrate synthase (*gltA*) gene. Four of one hundred and ninety-four feral raccoon spleens (2.1%) were positive for *Rickettsia* spp. One *gltA* gene sequence was identical to *R. helvetica*, whereas the other 3 sequences were identical and had the highest similarity (98.4%) to *R. amblyommii*. Simultaneously, we determined a partial sequence of the rickettsial 17-kilodalton (17K) genus-common antigen gene in the later 3 raccoon samples. Their sequences were identical and had the highest similarity (98.5%) to *Rickettsia* sp. HJ126. Based on the sequences of *gltA* and 17K antigen genes, these raccoons might be infected with spotted fever group (SFG) rickettsia most closely related to *R. amblyommii* and/or *Rickettsia* sp. HJ126. Feral raccoons may be a susceptible reservoir for SFG rickettsiae in Japan.

KEY WORDS: Japan, raccoon, spotted fever group rickettsia.

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Raccoons (*Procyon lotor*), which are native to North America, have been imported as pets into Japan since the 1970s. Subsequently, the release and escape of pet raccoons resulted in an establishment of feral populations throughout Japan. The growing population of naturalized raccoons causes significant damage to the agriculture and ecosystem. Furthermore, it is of increasing concern that raccoons may increase the risk of infection with life-threatening pathogens to humans and animals. Indeed, raccoons are widely known as a reservoir of rabies virus and *Baylisascaris procyonis* [5, 11]. In Japan, some pathogens have been detected in feral raccoons, including gastrointestinal helminths, *Babesia microti*-like parasite and spotted fever group (SFG) rickettsiae [9, 13, 14]. Under such economic and public health problems, official control programs of feral raccoons have recently initiated in multiple prefectures of Japan.

SFG rickettsiae are obligate intracellular, gram-negative bacteria, transmitted by tick infestation to humans and animals. In Japan, since the first patient infected with *R. japonica* was reported in Tokushima Prefecture in 1984 [10], rickettsiosis has been noticed as a significant emerging infectious disease. Thereafter, cases caused by *R. helvetica*, *R. tamurae*, and *R. heilongjiangensis* have been also reported in Japan [1, 7, 17]. In wildlife, infection with *R. japonica* or *R. heilongjiangensis*, *R. felis* and *R. helvetica* was reported in feral raccoons in Hokkaido, Japan [13]. However, rickettsial agents in feral raccoons have not been surveyed in other

areas of Japan. Because of frequent case records of human rickettsiosis in Kyushu Island and Shimane Prefecture [1, 16], in the present study, we conducted a survey of rickettsial infection in feral raccoons in these areas by molecular methods.

A total of 194 raccoons were captured under official control programs between August 2009 and October 2011 in Shimane (n=34), Fukuoka (n=4), Saga (n=135) and Nagasaki (n=21) Prefectures, Japan. Spleen specimens were collected from these raccoons, and genomic DNA was extracted with a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, MO, U.S.A.). DNA samples were stored at -20°C prior to use. Nested polymerase chain reaction (PCR) for detecting the rickettsial citrate synthase (*gltA*) gene was carried out with the primers RpCS.877p and RpCS.1273r for the primary amplification, and RpCS.896f and RpCS.1258n for the secondary amplification [6, 12]. The first round of PCR was performed in a 20 µl reaction mixture containing GoTaq® Green Master Mix (Promega, Madison, WI, U.S.A.), 0.2 µM each primer, and 4 µl of DNA template. The amplification condition was initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 1 min, with final extension at 72°C for 5 min. For the secondary amplification, 0.5 µl of the product from the primary amplification was used as a template. The amplification condition was the same as in the first round, except for annealing at 56°C. DNA extracted from *R. conorii* (kindly provided by Dr. H. Inokuma in Obihiro University of Agriculture and Veterinary Medicine) and distilled water were used as positive and negative controls, respectively. In samples positive for *gltA* gene, semi-nested PCR for detecting the rickettsial 17-kilodalton (17K) genus-common antigen gene was also performed with the primers Rr17k.1p and Rr17k.539n for

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the primary amplification, and Rr17k.90p and Rr17k.539n for secondary amplification [8]. The amplification condition was the same as described above. The resulting PCR product was electrophoresed on 1.5% agarose gel, and visualized by ethidium bromide under ultraviolet light. The amplicon was purified from the gel using the RECOCHIP (TaKaRa, Kyoto, Japan), and then subjected to direct DNA sequencing with the BigDye[®] Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequences were compared with those from known *Rickettsia* species and strains in NCBI GenBank nucleotide database by a BLAST search.

Four of one hundred and ninety-four raccoons (2.1%) were positive for rickettsiae. In positive samples, 3 raccoons (Saga 006, Saga 007, and Saga 089) were collected in Saga Prefecture (2.2%), and one (Shimane 042) in Shimane Prefecture (2.9%) (Table 1). The 322 bp of the *gltA* gene, excluding the primer regions, was successfully sequenced. The sequence of *gltA* gene in Saga 089 (DDBJ/EMBL/GenBank accession number AB700588) was identical to *R. helvetica* (HM371185). The sequence was also highly similar (99.7%) to *R. asiatica* strain IO-1 (AB114797). The other 3 sequences (AB699870, AB699871 and AB699872) were identical and had the highest similarity (98.4%) to *R. amblyommii* (DQ517290) in a BLAST search (Table 2).

Simultaneously, the 17K genus-common antigen gene in 3 samples (Saga 006, Saga 007, and Shimane 042) was successfully amplified and then sequenced, whereas no amplification could be detected for Saga 089. The 410 bp of the 17K genus-common antigen gene, excluding the primer regions, in Saga 006 (AB699873), Saga 007 (AB699874), and Shimane 042 (AB699875) showed the same nucleotide sequence and the highest homology (98.5%) with *Rickettsia* sp. Hj126 (AB114810) in a BLAST search (Table 2). Based on the sequences of *gltA* and 17K genus-common antigen genes, these raccoons might be infected with SFG rickettsia most closely related to *R. amblyommii* and/or *Rickettsia* sp. Hj126.

Table 1. Prevalence of rickettsial *gltA* gene in feral raccoons

Prefecture	Number of raccoons	PCR positive
Saga	135	3 (2.2%)
Nagasaki	21	0 (0%)
Fukuoka	4	0 (0%)
Shimane	34	1 (2.9%)
Total	194	4 (2.1%)

Little information is available on the epidemiology of SFG rickettsiae in raccoons. To the best of our knowledge, this is the first report of SFG rickettsiae in feral raccoons in the western part of Japan. In this study, 4 of 194 raccoons (2.1%) were positive for rickettsiae, which was equivalent to 1.9% in a previous report in Hokkaido, Japan [13]. The number of cases with rickettsiosis in the western part of Japan, including Kyushu Island and Shimane Prefecture, is known to be a great deal more than that in the eastern part of Japan [1]. Nationwide epidemiological surveys are needed to elucidate the role of feral raccoons in the occurrence of rickettsiosis in Japan.

In the present study, *R. helvetica* was detected in 1 of 194 feral raccoons (0.5%). *R. helvetica* is known to be pathogenic to humans [4, 17], and widely exist in ticks collected from dogs and cats from Hokkaido to Kyushu Islands [6]. Interestingly, the pathogen was also detected in 10 of 699 feral raccoons (1.4%) in Hokkaido, Japan [13]. Feral raccoons may be one of susceptible host of *R. helvetica* in Japan.

In contrast, SFG rickettsia most closely related to *R. amblyommii* and/or *Rickettsia* sp. Hj126 was detected in 3 of 194 raccoons (1.5%). *Rickettsia* sp. Hj126 is an uncultured rickettsia, which has been detected in *Ixodes ovatus* and *Haemaphysalis flava* in Japan, but the animal hosts and the pathogenicity are unknown [8]. In contrast, *R. amblyommii* has been found in *Amblyomma* ticks, *Dermacentor nitens*,

Table 2. Nucleotide sequence similarities of *gltA* and 17K genus-common antigen genes from PCR-positive raccoons with those from known *Rickettsia* spp.

Gene	Sequence length	Sample ID	Nucleotide sequence similarity*
<i>gltA</i>	322 bp	Saga 089	<i>R. helvetica</i> ; 100% <i>R. asiatica</i> strain IO-1; 99.7%
		Saga 006, Saga 007, Shimane 042	<i>R. amblyommii</i> ; 98.4% <i>R. slovacica</i> , <i>R. aeschlimannii</i> , <i>R. raoultii</i> ; 98.1% <i>R. africae</i> , <i>R. heilongjiangensis</i> , <i>R. japonica</i> , <i>R. montanensis</i> , <i>R. parkeri</i> , <i>Rickettsia</i> sp. ARANHA; 97.8%
17K	410 bp	Saga 006, Saga 007, Shimane 042	<i>Rickettsia</i> sp. Hj126; 98.5% <i>R. rhipicephali</i> , <i>Rickettsia</i> sp. ARANHA, <i>Rickettsia</i> sp. R300; 98.3% <i>R. massiliae</i> , <i>R. rickettsii</i> , <i>R. amblyommii</i> , <i>R. africae</i> , <i>R. japonica</i> , <i>R. parkeri</i> ; 98.0% <i>R. conorii</i> , <i>R. slovacica</i> , <i>R. peacockii</i> , <i>R. honei</i> ; 97.8%

*DDBJ/EMBL/GenBank accession numbers are as follows; *gltA*: *R. helvetica* (HM371185), *R. asiatica* strain IO-1 (AB114797), *R. amblyommii* (DQ517290), *R. slovacica* (CP002428), *R. aeschlimannii* (HM050276), *R. raoultii* (JQ792127), *R. africae* (CP001612), *R. heilongjiangensis* (CP002912), *R. montanensis* (CP003340), *R. japonica* (AP011533), *R. parkeri* (JQ906783), *Rickettsia* sp. ARANHA (AY360216), 17K genus-common antigen; *Rickettsia* sp. Hj126 (AB114810), *R. rhipicephali* (DQ865207), *Rickettsia* sp. ARANHA (AY360215), *Rickettsia* sp. R300 (AY472039), *R. massiliae* (CP000683), *R. rickettsii* (CP000766), *R. amblyommii* (AY375162), *R. africae* (CP001612), *R. japonica* (AP011533), *R. parkeri* (CP003341), *R. conorii* (AE006914), *R. slovacica* (CP002428), *R. peacockii* (CP001227), *R. honei* (AF027124).

D. variabilis and *Rhipicephalus sanguineus* in the United States and Central and South America [3, 15]. Although the animal hosts of *R. amblyommii* have not been identified, dogs and horses have been suggested as the susceptible animals [3]. Moreover, *R. amblyommii* has been implicated as a potential human pathogen [2]. Taken together, it is highly conceivable that *R. amblyommii* and the related *Rickettsia* spp., such as the SFG rickettsia detected from 3 raccoons, can pose a threat to public health. Therefore, the epidemiology in terrestrial mammalian hosts and vectors, and the pathogenicity to humans and animals should be investigated.

In conclusion, we first demonstrated the prevalence of rickettsial infection in feral raccoons in the western part of Japan. The population growth and the expansion of living area of feral raccoons can result in an increase in the risk of infection of tick-borne pathogens, such as SFG rickettsiae, in humans and animals. Further investigation and surveillance of rickettsial infection in feral raccoons are needed to clarify their epidemiologic importance.

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