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Short Communication

First Molecular Detection and Phylogenetic Analysis of *Anaplasma phagocytophilum* from a Clinical Case of Canine Granulocytic Anaplasmosis in Japan

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SUMMARY: *Anaplasma phagocytophilum* DNA was detected from a dog with canine granulocytic anaplasmosis (CGA) in Japan. Phylogenetic analysis of the DNA using 16S rRNA, *gltA*, and *groEL* sequences revealed that the strain was nearly identical to *A. phagocytophilum* detected from *Apodemus agrarius* (black-striped field mouse) in China and Korea. To our knowledge, this is the first report of molecular detection and phylogenetic analysis of *A. phagocytophilum* from a clinical case of CGA in Japan.

Anaplasma phagocytophilum is a tick-borne pathogen that causes human granulocytic anaplasmosis (HGA), equine granulocytic anaplasmosis, and pasture fever or tick-borne fever in ruminants (1). The pathogen is also known to infect dogs and cause canine granulocytic anaplasmosis (CGA) (2). Infected dogs present mainly with fever and thrombocytopenia (2). In Japan, clinical cases of HGA have recently been reported (3). *A. phagocytophilum* DNA has been detected and phylogenetically analyzed in various animals, excluding dogs, in Japan (4,5). Definitive diagnosis of CGA has never been achieved in Japan. In the present study, we aimed to conduct phylogenetic analysis of an *A. phagocytophilum* strain isolated from a newly diagnosed case of CGA in Japan.

An 8-year-old male American Cocker Spaniel with a 3-day history of lethargy was brought to a private veterinary clinic in Moriya, Ibaraki Prefecture, Japan (Fig. 1), in November 2016. The dog had been reared indoors and had not traveled abroad. The routine vaccinations and heartworm and flea/tick chemoprophylaxis were up-to-date. Physical examination revealed a rectal temperature of 39.9°C (normal range: 37.7–39.2°C). There was no evidence of tick bites. Hematological tests revealed thrombocytopenia (94,000/μl; normal range: 200,000–500,000/μl). Leukocytes were not evaluated, as microscopic observation of blood smears was not performed. A peripheral blood sample was submitted to IDEXX Laboratories Inc. (Tokyo, Japan) for vector-borne pathogen-screening polymerase chain reactions (PCRs), including those for *Anaplasma, Babesia, Bartonella, Ehrlichia, Hepatozoon, Leishmania, Neorickettsia, Rickettsia,* and hemoplasmas on Day 1, and was found to be positive for *A. phagocytophilum*. The dog was treated with subcutaneous injections of 1.5 mg/kg prednisolone and 5 mg/kg enrofloxacin on Day 1; oral doxycycline (10 mg/kg q24h, for 28 days) was administered from Day 2. The dog rapidly recovered and the thrombocyte count increased to 294,000/μl by the first follow-up on Day 5. Ethylenediaminetetraacetic acid (EDTA) blood and serum samples obtained on Days 1 and 18 were preserved at −20ºC until further analysis. By the end of treatment, the dog had completely recovered. Serological examination performed using an *A. phagocytophilum* IFA Substrate Slide (Veterinary Medical Research & Development, Pullman, WA,USA) revealed that the titer had increased from < 1:20 on Day 1 to 1:40 on Day 18.

Genomic DNA was extracted from EDTA blood on Days 1 and 18 by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Full-length 16S rRNA was amplified with the fD1 and Rp2 primers (4) (Table 1). Partial sequences of the citrate synthase gene (*gltA*) and heat shock protein gene (*groEL*) were amplified with the APgltA-1F/APgltA-1084R primer pairs, modified from the primers reported by Ybañez et al. (6) and from EEgro1F/EEgro2R (7), respectively (Table 1). PCR amplification, electrophoresis, and amplicon purification were performed as previously described (4,6). The purified PCR products were submitted for direct sequencing performed using amplification and internal primers, including 519F, 519R, 907F, and 907R for 16S rRNA (newly designed), and EEgro3F, EEgro4R, EEgro5F, and EEgro6R for *groEL* (7) (Table 1). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The 16S rRNA, *gltA*, and *groEL* sequences determined in this study have been deposited in GenBank with the accession numbers LC334014, LC334015, and LC334016, respectively.

The DNA sequences were aligned and compared to those in the GenBank database by using BLAST (National Center for Biotechnology Information) to verify

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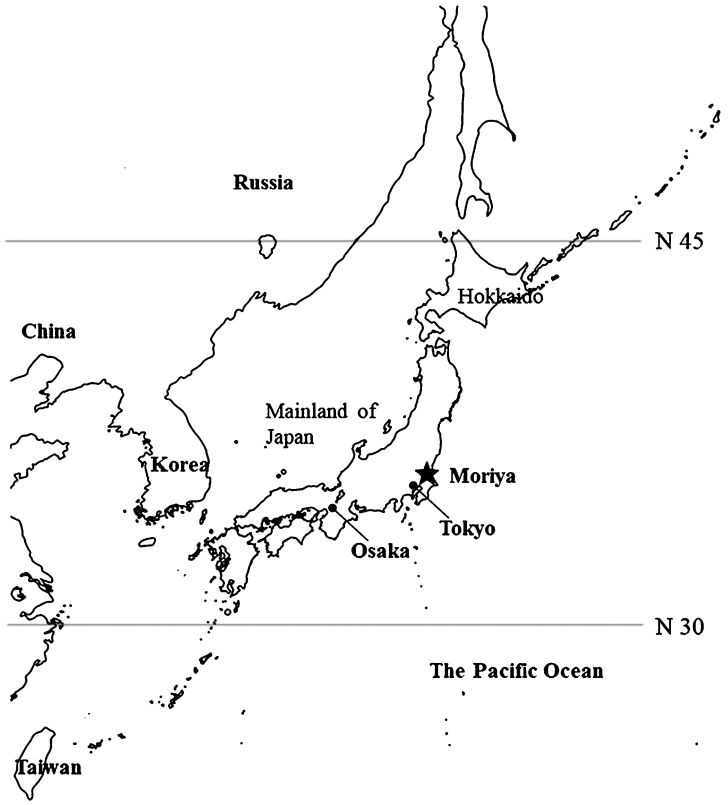

Fig. 1. Map of Moriya City (indicated by the asterisk), where the CGA dog was reared, located in the eastern part of mainland of Japan.

Table 1. Primers for detection of 16S rRNA, *gltA*, and *groEL* genes of *Anaplasma phagocytophilum*

Target gene	Primer (5′ to 3′)	Reference
16S rRNA	fD1: AGAGTTTGATCCTGGCTCAG	(4)
	Rp2: ACGGCTACCTTGTTACGACTT	(4)
	Sequencing internal primers	
	519F: CAGCMGCCGCGGTAAT′	In this study
	519R: ATTACCGCGGCKGCTG	In this study
	907F: AAACTYAAAKGAATTGACGG	In this study
	907R: CCGTCAATTCMTTTRAGTTT	In this study
gltA	APgltA-1F: ATGGTAGAAAAAGCTGTTTTGAGTG	Modified of (6)
	APgltA-1084R: TCTTAGCACTATACCTGAGTAAAAG	Modified of (6)
groEL	EEgro1F: GAGTTCGACGGTAAGAAGTTCA	(7)
	EEgro2R: CAGCGTCGTTCTTACTAGGAAC	(7)
	Sequencing internal primers	(7)
	EEgro3F: GCGAATGGAGACAAGAACATA	(7)
	EEgro4R: AGTTGCGTCTTTTGTGATTCG	(7)
	EEgro5F: AGCGAAGTTGAGGTGAAGGA	(7)
	EEgro6R: CGCTTCCTTAGCCTTGAGAA	(7)

that *A. phagocytophilum* DNA had been amplified (8). Phylogenetic trees of *A. phagocytophilum* were constructed on the basis of alignments of 16S rRNA, *gltA,* and *groEL* gene sequences with the sequence analysis software MEGA7 (9). The neighbor-joining method was used to construct the phylogenetic trees; their stability was estimated by bootstrap analysis of 1,000 replications with the same program.

During PCR analysis of the 16S rRNA, *gltA*, and *groEL* sequences, positive products were observed on Day 1 but not on Day 18. The 1464-bp 16S rRNA gene sequence was found to be 99.6–99.7% identical to sequences from a patient with HGA in the United States (M73220) and a dog with CGA in Germany (JX173651). The sequence of this amplicon showed higher identity (99.9%) to *A. phagocytophilum* detected from *Apodemus agrarius* (black-striped field mouse) in China (GQ412337) and Korea (KR611718) than to *A. phagocytophilum* from Japan (JQ622147: 99.5%) or to *Anaplasma* sp. Japan (AB196721: 89.7%; Fig. 2A). The sequence of the *gltA* PCR product (975 bp) was 100% identical to that of *A. phagocytophilum* detected from

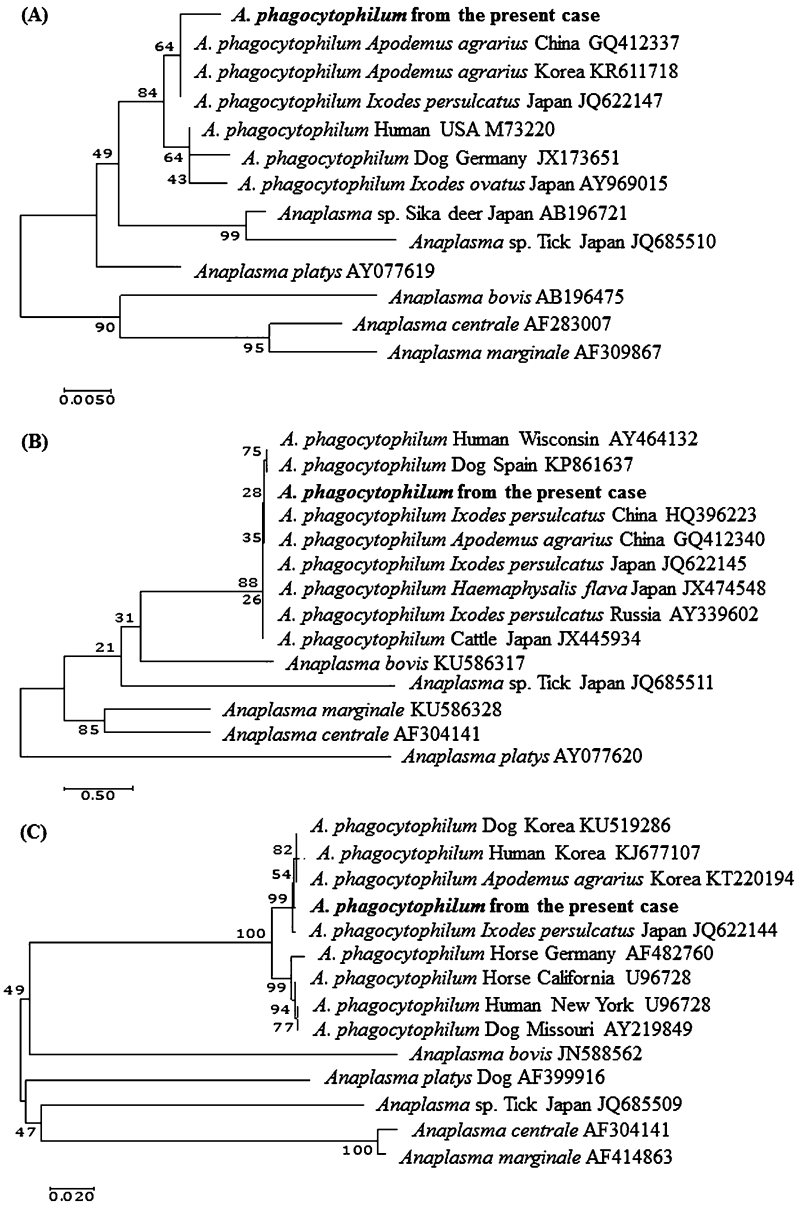

Fig. 2. Phylogenetic relationships of *A. phagocytophilum* from the present case within the genus *Anaplasma* based on (A) 16S rRNA, (B) *gltA*, and (C) *groEL*. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications.

A. agrarius in China (GQ412340) and 99.1–99.4% and 56.6% identical to those of *A. phagocytophilum* from Japan (JQ622145 and JX474548) and *Anaplasma* sp. Japan (JQ685511), respectively (Fig. 2B). The *groEL* sequence (1671 bp) was 99.8% identical to those of *A. phagocytophilum* detected from *A. agrarius* (KT220194) and a dog (KU519286) in Korea and 99.7% and 81.2% identical to those of *A. phagocytophilum* from Japan (JQ622144) and *Anaplasma* sp. Japan (JQ685509), respectively (Fig. 2C).

Multiple strains of *A. phagocytophilum* exist globally and have different host tropisms and pathogenicities (1). Pathogenic differences between 2 strains isolated in the United States are well known (1). The major strain, called Ap-ha, is infectious to humans, dogs, and horses; the infected individuals subsequently develop clinical signs. The reservoir hosts for this strain are rodents. The other strain, called Ap-V1, is infectious to wild and domestic ruminants but not to humans or rodents; the infected animals are asymptomatic. In Japan, 2 strains of *A. phagocytophilum* have been also reported on the basis of phylogenetic studies (4,6). Human-type *A. phagocytophilum* has been detected in human patients and ticks (10). To date, no information was available for *A. phagocytophilum* strains from dogs in Japan. In this study, we identified 16S rRNA, *gltA*, and *groEL* sequences of an *A. phagocytophilum* strain from a dog with CGA in Japan. Since the sequences were highly identical to those of *A. phagocytophilum* from patients with HGA in the United States, this strain could be classified as human-type *A. phagocytophilum* in Japan and its host tropism might be similar to that of Ap-ha in the United States.

Interestingly, the DNA sequenced in this study showed higher identity to *A. phagocytophilum* from *A. agrarius* in China and Korea than to *A. phagocytophilum* in Japan for all the 3 genes assessed (i.e., 16S rRNA, *gltA*, and

groEL). *A. agrarius* is considered to be one of the main reservoir hosts for *A. phagocytophilum* in Korea (11–13). Because *A. agrarius* is not found in mainland Japan (14), reservoir hosts for human-type *A. phagocytophilum* in Japan remain unknown. In Korea, *Haemaphysalis flava, Haemaphysalis longicornis, Ixodes nipponensis*, and *I. persulcatus* are the main vectors of *A. phagocytophilum* (15). These tick species are commonly found in Japan; it is supposed that the dogs are bitten by ticks when they are taken for walks and that the owners are unaware of the tick bites, thereby leading to lack of detection of the infection initially. Epidemiological surveys will be required to determine the reservoirs and vectors of *A. phagocytophilum* that infects dogs in Japan.

To our knowledge, this is the first report of molecular detection and phylogenetic analysis of *A. phagocytophilum* from dogs in Japan. Canine *A. phagocytophilum* infection in Japan is considered to be low risk, as in Korea (12,13), given the high phylogenetic identity revealed in this study and its lower prevalence relative to that in the United States and Europe (2). However, CGA might be underdiagnosed because most *A. phagocytophilum*–infected dogs are asymptomatic and the infection is self-limited (2). Therefore, in order to clarify the prevalence of this strain, further studies will need to be performed in the region in which this case of CGA was detected.

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Conflict of interest None to declare.

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