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Original Article

Subclinical Infections of *Anaplasma phagocytophilum* and *Anaplasma bovis* in Dogs from Ibaraki, Japan

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SUMMARY: We evaluated the prevalence of *Anaplasma* infection in 332 dogs from Ibaraki, Japan, using serological and molecular methods. An immunofluorescence antibody assay against *Anaplasma phagocytophilum* indicated that 7 of the 328 serum samples tested (2.1%) were positive for A. *phagocytophilum*. Screening by polymerase chain reaction (PCR) analysis demonstrated that 8 of the 331 peripheral blood samples tested (2.4%) were positive for Anaplasmataceae. Phylogenetic analysis of the partial 16S rRNA sequence of the PCR amplicons revealed that 6 sequences were most similar to the 16S rRNA sequence of a *Wolbachia* sp., and the remaining 2 to *A. bovis*. Further analysis by *A. phagocytophilum*-specific nested PCR demonstrated that 1 dog infected with *A. bovis* was also positive for *A. phagocytophilum*. This is the first study to report the dual infection of a dog in Japan with *A. bovis* and *A. phagocytophilum.*

INTRODUCTION

Members of the family Anaplasmataceae are obligate intracellular gram-negative bacteria. The family consists of 7 genera, *Anaplasma*, *Ehrlichia, Neorickettsia, Aegyptianella, Wolbachia*, "*Candidatus* Neoehrlichia," and "*Candidatus* Xanohaliotis" (1). Anaplasmosis is a tick-borne disease caused by *Anaplasma*, and is a globally emerging disease in both humans and animals (1). The major species of *Anaplasma* include *A. phagocytophilum*, *A. platys*, *A. centrale*, *A. marginale*, *A. bovis*, *A. ovis*, and *A. capra* (2). In particular, *A. phagocytophilum* is an important pathogen that causes zoonotic life-threatening diseases, including human granulocytic anaplasmosis (HGA), tick-borne fever in ruminants, equine granulocytic anaplasmosis, and canine granulocytic anaplasmosis (CGA) (3–5).

CGA has been reported in North America, Europe, and Asia since 1982 (5,6). We recently reported the first CGA case in Japan (7). This case showed typical symptoms of CGA, including fever and thrombocytopenia, although most dogs infected with *A. phagocytophilum* are asymptomatic (5). In previous serological and molecular studies on canine *Anaplasma* infection in Japan, some dogs were shown to have antibodies against *A. phagocytophilum* (8), although DNA fragments of *A. phagocytophilum* were not detected (9,10). The epidemiology of canine *A. phagocytophilum* infection in Japan remains unclear.

Against this backdrop, the present study tested peripheral blood from clinically normal dogs in Ibaraki, where the first CGA case in Japan was discovered, using serological and molecular methods, for canine *Anaplasma* infection.

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MATERIALS AND METHODS

Samples: We collected 331 ethylenediaminetetraacetic acid (EDTA)-treated peripheral blood samples and 328 serum samples from 332 dogs that had been presented at 6 private veterinary clinics located in Ibaraki Prefecture—i.e., 2 clinics in Tsukuba, and 1 each in Tsuchiura, Moriya, Shimotsuma, and Koga—between March 2016 and June 2017. Age, gender, breed, tick infestation, and clinical history of the dogs were recorded at each clinic. In each blood sample, the presence of heartworm (*Dirofilaria immitis*) antigen was assessed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Snap HW; IDEXX Laboratories Inc., Tokyo, Japan), and the platelet count was obtained with an automated hematology system (Celltac α; Nihon Kohden, Tokyo, Japan). A platelet count below $150 \times 10^3/\mu L$ was defined as thrombocytopenia. Blood and serum samples were stored at –20°C until use.

Immunofluorescence antibody assay (IFA): An IFA was conducted using an *A. phagocytophilum* IFA Substrate Slide (Veterinary Medical Research & Development, Pullman, WA, USA) according to procedures described in the literature (11). A 1:200 dilution of fluorescein isothiocyanate-labelled anti-canine IgG antibody (Rockland, Gilbertsville, PA, USA) was used as the secondary antibody. Sera were screened using a 1:20 dilution, and then titrated using serial 2-fold dilutions to determine end titers. *A. phagocytophilum*-positive serum from a CGA dog that had been confirmed by molecular and serological methods (7) was used as the positive control.

Polymerase chain reaction (PCR) and sequencing: DNA from EDTA-treated blood samples was extracted using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). Screening PCR, which amplified the partial 16S rRNA gene of Anaplasmataceae, was performed using EHR16SD and EHR16SR primers (Table 1) (12). In Anaplasmataceae-positive samples, semi-nested PCR with primer pairs fD1/EHR16SR and fD1/GA1UR was performed for the first and second amplifications, respectively (Table 1) (12). These primers cover the divergent region of the 16S rRNA gene near the 5′ end. *A. phagocytophilum*-specific nested PCR, which amplified a portion of the citrate synthase gene (*gltA*), was also performed using primer pairs APgltA-1F/APgltA-1084R (7) and APgl151F2/APgl756R2 (13) in Anaplasmataceae-positive samples (Table 1). PCR amplification, electrophoresis, purification, and direct sequencing were performed as previously described (7,12).

Data analysis: Homology searches based on partial gene sequences of the PCR products were performed using BLAST (National Center for Biotechnology Information). Phylogenetic trees were constructed based on alignments of 16S rRNA and *gltA* sequences using MEGA7 sequence analysis software (14). The neighbor-joining method was used to construct a phylogenetic tree. The stability of the tree was estimated by bootstrap analysis of 1,000 replications using the same program.

RESULTS

IFA testing revealed that 7 of the 328 serum samples (2.1%) had antibodies against *A. phagocytophilum* with titers of ≥ 20 (Table 2). Eight of the 331 peripheral blood samples examined by PCR screening (2.4%) were positive for Anaplasmataceae (Table 2). Of these, 1 dog was also positive for antibodies against *A. phagocytophilum*, with a titer of 1:20.

All 8 of the Anaplasmataceae-positive peripheral blood samples were also positive according to semi-nested PCR, and the nucleotide sequences of the PCR products (approximately 450 bp) were successfully determined. BLAST analysis revealed that 6 samples were 98.9% – 99.4% identical to a *Wolbachia* sp. from *Dirofilaria immitis* (AF088187), whereas 2 samples were 98.0% – 98.7% identical to *A. bovis* from a dog

Table 1. Primers used in the study

Target gene	Primer (5′ to 3′)	Reference
16S rRNA	EHR16SD: GGTACCYACAGAAGAAGTCC	(11)
	EHR16SR: TAGCACTCATCGTTTACAGC	(11)
	fD1: AGAGTTTGATCCTGGCTCAG	(11)
	GA1UR: GAGTTTGCCGGGACTTCTTCT	(11)
gltA	APgltA-1F: ATGGTAGAAAAAGCTGTTTTGAGTG	(7)
	APgltA-1084R: TCTTAGCACTATACCTGAGTAAAAG	(7)
	APgl151F2: GCTTGCAGATCAGAGATAACTTTCATTGAT	(12)
	APgl756R2: AGTGGCCACTCCYGCGCACAAACA	(12)

Table 2. Clinical characteristics of 14 dogs IFA-positive for *Anaplasma phagocytophilum* and/or PCR-positive for the family Anaplasmataceae screening PCR

No.	Area	Breed	Age	Sex	Tick bite	Heartworm antigen	Thrombo-cytopenia ($< 150 \times 10^3/\mu L$)	IFA titer (≥ 20)	PCR and 16S rRNA sequence
232	Tsukuba	Mix breed	6	Male	+	-	-	80	-
236	Tsukuba	Mix breed	UNK	Male	UNK	+	-	-	*Wolbachia* sp.
253	Tsuchiura	Shih Tzu	3	Cast	UNK	-	-	80	-
283	Ishioka	Wire fox Terrier	9	Spay	+	-	-	80	-
285	Tsukuba	Mix breed	3	Male	+	-	-	40	-
294	Joso	Mix breed	UNK	Male	UNK	+	-	80	-
299	Tsuchiura	Mix breed	2M	Male	+	-	-	40	-
306	Tsukuba	Mix breed	2	Female	UNK	+	-	-	*Wolbachia* sp.
309	Koga	Mix breed	7	Male	+	+	-	-	*Wolbachia* sp.
310	Koga	Pekingese	1	Male	+	+	-	-	*Wolbachia* sp.
311	Koga	Mix breed	17	Female	+	+	-	-	*Wolbachia* sp.
312	Koga	Mix breed	6	Male	+	+	-	-	*Wolbachia* sp.
335	Moriya	Mix breed	13	Cast	UNK	-	+($44 \times 10^3/\mu L$)	20	*Anaplasma bovis*
337	Moriya	Shiba Inu	5	Male	UNK	-	+($133 \times 10^3/\mu L$)	-	*Anaplasma bovis*

UNK, unknown.

in Hiroshima, Japan (HM131217) (9) (Fig.1). The sequences of 16S rRNA determined in this study have been deposited in GenBank with accession numbers LC431231 to LC431238.

A positive band from *A. phagocytophilum*-specific nested PCR was obtained from 1 dog in which *A. bovis* 16S rRNA was also detected. The sequence of this partial *gltA* gene (552 bp) was 97.1% identical to the *gltA* gene of *A. phagocytophilum* from the CGA dog in Ibaraki, Japan (LC334015) (7) (Fig. 2). The partial *gltA* sequence of this dog has been deposited in GenBank with accession number LC431239.

Heartworm antigen was detected in all 6 dogs that were positive for the 16S rRNA gene of a *Wolbachia* sp., and only 2 dogs that were positive for *A. bovis* had thrombocytopenia (Table 2).

DISCUSSION

Serological tests for canine *A. phagocytophilum* infection typically include IFAs and enzyme-linked immunoassay (EIAs) (3–5). The rate of seropositive results reflects the clinical suspicion that *A. phagocytophilum* is present (5). The rate of seropositive results in this study (2.1%) was higher than that reported in a previous nationwide serological study in Japan (0.2%) (8). However, much higher rates (50%–55%) have been reported in endemic areas around the world, especially in Germany, Portugal, and the United States (5). Antibodies used in IFAs and EIAs for *A. phagocytophilum* are known to cross-react with other *Anaplasma* and *Ehrlichia* spp. (5). In the present study, *A. bovis* was detected in 1 of 7 IFA-positive dogs, and it is possible that there was cross-reactivity of antibodies between *A. phagocytophilum* and *A. bovis*. Our findings also suggest the possibility that some dogs in Ibaraki may have experienced past infections with *Anaplasma* or *Ehrlichia* spp., including *A. phagocytophilum* and *A. bovis*.

In screening PCR for Anaplasmataceae, we detected a *Wolbachia* sp. in 6 dogs with heartworm antigen. *Wolbachia* spp. are known to be symbionts of heartworms (9), and thus this result may suggest a relationship between *Wolbachia* spp. and heartworm infection.

A. bovis was first reported in cattle, and infects circulating monocytes and tissue macrophages (14). DNA fragments of *A. bovis* have been detected in several animals including dogs and vector ticks in Japan (9,10,15). *A. bovis* infection is rare and may be subclinical. However, anemia, leukopenia, and thrombocytopenia may be observed among

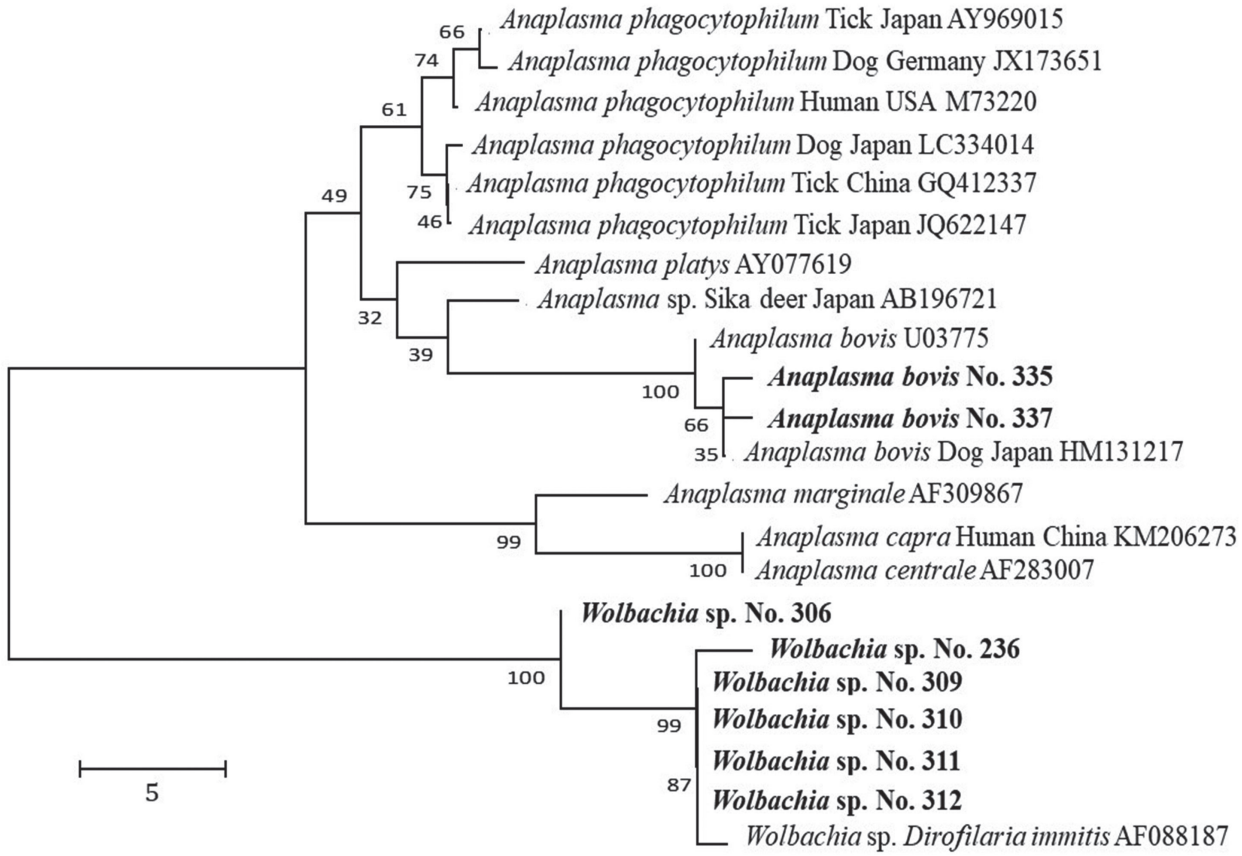

Fig. 1. Phylogenetic relationships of *Anaplasma bovis* and *Wolbachia* sp. from this study within the family Anaplasmataceae based on 16S rRNA. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications.

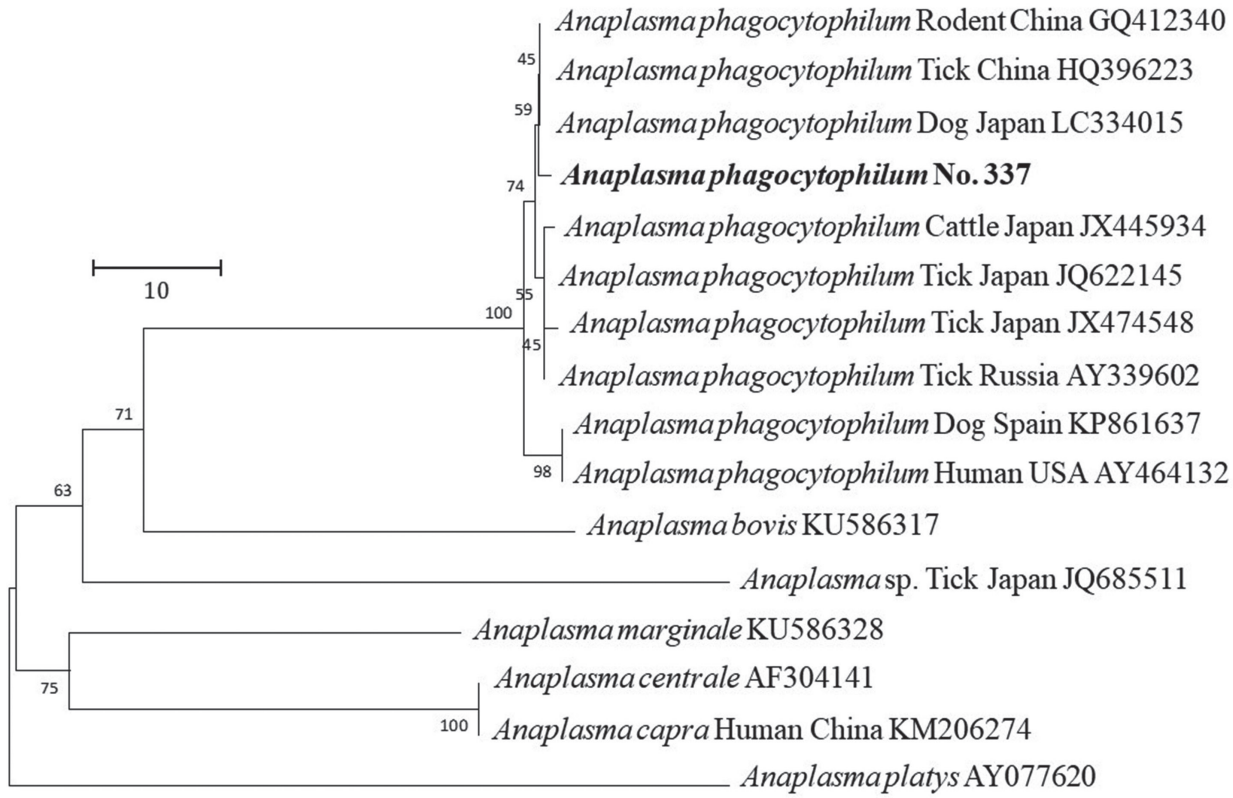

Fig. 2. Phylogenetic relationships of *Anaplasma phagocytophilum* from this study within the genus *Anaplasma* based on *gltA* gene. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1,000 bootstrap replications.

clinicopathological findings in cattle (15). Two dogs that were PCR-positive for *A. bovis* had thrombocytopenia, which may have been caused by subclinical infection with *A. bovis*.

The dual detection of *A. phagocytophilum* and *A. bovis* has been reported in ticks (16) and cattle (17) in Japan. In China, a recent study reported the triple detection of *A. phagocytophilum*, *A. bovis*, and *A. ovis* in dogs (18). In the present study, the 16S rRNA gene of *A. bovis* and the *gltA* gene of *A. phagocytophilum* were detected in 1 dog. Thus, the dog is expected to have been infected with both pathogens. To the best of our knowledge, this is the first report of subclinical and dual infection by *A. phagocytophilum* and *A. bovis* in a dog in Japan. In the phylogenetic analysis of both pathogens, the highest identities were found in dogs from Japan, indicating that these pathogens may be rather common in the country. Further studies on the reservoirs and vectors of these pathogens are warranted.

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Conflict of interest None to declare.

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