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**A Running Head**: Dual infection of A. bovis and A. phagocytophilum
Names and addresses of domestic authors written in Japanese

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SUMMARY: The prevalence of Anaplasma infection in 332 dogs from Ibaraki, Japan, was evaluated by serological and molecular surveys. Immunofluorescence antibody assay (IFA) against Anaplasma phagocytophilum indicated that 7 (2.1%) of 328 dogs were positive for A. phagocytophilum. Screening PCR demonstrated that 8 (2.4%) of 331 dogs were positive for Anaplasmataceae. Phylogenetic analysis of the partial 16S rRNA sequence of PCR amplicons revealed that 6 sequences were most similar to the 16S rRNA sequence of Wolbachia sp. and the remaining 2 to Anaplasma 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. [125 words]
Introduction

Bacteria 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 for 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 for 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 on 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 had 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, for canine Anaplasma infection using serological and molecular methods.

Materials and methods

Samples: 331 EDTA-treated peripheral blood and 328 serum samples were collected from
332 dogs that presented to 6 private veterinary clinics, including 2 clinics in Tsukuba, and 1 each in Tsuchiura, Moriya, Shimotsuma, and Koga, located in Ibaraki Prefecture from March 2016 to June 2017. Age, gender, breed, tick infestation, and clinical history were recorded at each clinic. In each blood sample, the presence of heartworm (*Dirofilaria immitis*) antigen was assessed using a commercial ELISA kit (Snap HW, IDEXX laboratories Inc, Tokyo, Japan) and platelet count was obtained with an automated hematology system (Celltac α, Nihon Kohden, Tokyo, Japan). A platelet count below 150x10³/µL was defined as thrombocytopenia. Blood and serum samples were stored at -20°C until use.

**IFA:** IFA was conducted using an *A. phagocytophilum* IFA Substrate Slide (Veterinary Medical Research & Development, Pullman, WA, USA) and similar procedures previously performed (11). A 1:200 dilution of fluorescein isothiocyanate-labelled anti-canine IgG antibody (Rockland, 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 the CGA dog confirmed by molecular and serological methods (7) was used as the positive control.

**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 APglt151F2/APglt756R2 (13) in
Anaplasmaceae-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 the sequence analysis software MEGA7 (14). The neighbor-joining method was used to construct a phylogenetic tree. The stability of the tree was estimated by bootstrap analysis of 1000 replications using the same program.

**Results**

IFA testing revealed that 7 (2.1%) of 328 dogs had antibodies against *A. phagocytophilum* with titers of ≥20 (Table 2). Eight (2.4%) of 331 dogs examined by screening PCR 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 samples were also positive by 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 *Wolbachia* sp. from *Dirofilaria immitis* (AF088187), whereas 2 samples were 98.0-98.7% identical to *A. bovis* from a dog in Hiroshima, Japan (HM131217) (9) (Figure 1). These sequences of 16S rRNA determined in this study have been deposited in GenBank with accession numbers from LC431231 to LC431238, respectively.

The 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 a CGA dog in Ibaraki, Japan (LC334015) (7) (Figure 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 positive for the 16S rRNA gene of *Wolbachia* sp., and only 2 dogs positive for *A. bovis* had thrombocytopenia (Table 2).

**Discussion**

Serological tests for canine *A. phagocytophilum* infection typically include IFA and enzyme-linked immunoassay (EIA) (3-5). The rate of seropositive results reflects on the clinical suspicion existed for *A. phagocytophilum* (5). The rate of this study (2.1%) was higher than that reported in a previous nationwide serological study (0.2%) in Japan (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 IFA and EIA for *A. phagocytophilum* are known to cross-react with other *Anaplasma* and *Ehrlichia* species (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* species including *A. phagocytophilum* and *A. bovis*.

In screening PCR for *Anaplasmataceae*, we detected *Wolbachia* sp in 6 dogs with heartworm antigen. *Wolbachia* sp. is known to be a symbiont of heartworms (9), and thus this result may suggest a relationship of *Wolbachia* sp. with 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 the clinicopathological findings in cattle (15). Two dogs which 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 from 1 dog. Thus, the dog is expected to have been infected with both pathogens. To 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 with dogs in Japan, indicating that these pathogens may be rather common in the country. Further studies on reservoirs and vectors of these pathogens are warranted.

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Conflict of interest. This research was supported in part by a grant from Boehringer Ingelheim Animal Health Japan Ltd.

REFERENCES


11. Tabuchi M, Jilintai, Sakata Y, et al. Serological survey of *Rickettsia japonica* infection in


Figure Captions

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 1000 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 1000 bootstrap replications.
Table 1. Primers used in the study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>EHR16SD: GGTACCYACAGAAGAAGTCC</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>EHR16SR: TAGCACTCATCGTTTACAGC</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>fD1: AGAGTTTGATCCTGGGCTCAG</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>GA1UR: GAGTTTGCCGGACTTCTTCT</td>
<td>(11)</td>
</tr>
<tr>
<td>gltA</td>
<td>APgltA-1F: ATGGTAGAAAAAGCTGGTTTGAGTG</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>APgltA-1084R: TCTTAGCAGTACCTGAGTAAAG</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>APgl151F2: GCTTGAGATCAGAGATAACTTCATTGAT</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td>APgl756R2: AGTGGCCACTCCYGCGACAAACA</td>
<td>(12)</td>
</tr>
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Table 2. Clinical characteristics of 14 dogs IFA-positive for *Anaplasma phagocytophilum* and/or PCR-positive for the family *Anaplasmataceae* screening PCR

<table>
<thead>
<tr>
<th>No.</th>
<th>Area</th>
<th>Breed</th>
<th>Age</th>
<th>Sex</th>
<th>Tick bite</th>
<th>Heartworm antigen</th>
<th>Thrombocytopenia (&lt;150x10^3/uL)</th>
<th>IFA titer (&gt;=20)</th>
<th>PCR and 16S rRNA sequence</th>
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</thead>
<tbody>
<tr>
<td>232</td>
<td>Tsukuba</td>
<td>Mix breed</td>
<td>6</td>
<td>Male</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>236</td>
<td>Tsukuba</td>
<td>Mix breed</td>
<td>UNK</td>
<td>Male</td>
<td>UNK</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>253</td>
<td>Tsuchiura</td>
<td>Shih Tzu</td>
<td>3</td>
<td>Cast</td>
<td>UNK</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>283</td>
<td>Ishioka</td>
<td>Wire fox Terrier</td>
<td>9</td>
<td>Spay</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>285</td>
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<td>3</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>294</td>
<td>Joso</td>
<td>Mix breed</td>
<td>UNK</td>
<td>Male</td>
<td>UNK</td>
<td>+</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>299</td>
<td>Tsuchiura</td>
<td>Mix breed</td>
<td>2M</td>
<td>Male</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>306</td>
<td>Tsukuba</td>
<td>Mix breed</td>
<td>2</td>
<td>Female</td>
<td>UNK</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>309</td>
<td>Koga</td>
<td>Mix breed</td>
<td>7</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>310</td>
<td>Koga</td>
<td>Pekingese</td>
<td>1</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>311</td>
<td>Koga</td>
<td>Mix breed</td>
<td>17</td>
<td>Female</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>312</td>
<td>Koga</td>
<td>Mix breed</td>
<td>6</td>
<td>Male</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Wolbachia sp.</td>
</tr>
<tr>
<td>335</td>
<td>Moriya</td>
<td>Mix breed</td>
<td>13</td>
<td>Cast</td>
<td>UNK</td>
<td>-</td>
<td>+(44x10^3/uL)</td>
<td>20</td>
<td>Anaplasma bovis</td>
</tr>
<tr>
<td>337</td>
<td>Moriya</td>
<td>Shiba Inu</td>
<td>5</td>
<td>Male</td>
<td>UNK</td>
<td>-</td>
<td>+(133x10^3/uL)</td>
<td>-</td>
<td>Anaplasma bovis</td>
</tr>
</tbody>
</table>

UNK: unknown