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Molecular detection of *Anaplasma phagocytophilum* from larvae of *Haemophysalis longicornis* in Ibaraki, Japan

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SUMMARY: DNA from 1084 ticks collected by flagging in vegetation in Tsukuba and Moriya (Ibaraki, Japan), where several cases of canine granulocytic anaplasmosis were found, was molecularly examined for infection with the family Anaplasmataceae. Twenty-six positive samples of Anaplasmataceae-specific PCR of partial 16S rRNA gene were subjected to semi-nested PCR covering the divergent regions the gene and sequence analysis. *Anaplasma phagocytophilum* was detected in three pools of *Haemophysalis longicornis* larvae, and *Anaplasma bovis* from a *Haemophysalis flava* male. Sequences of both amplicons had highest homologies to those from dogs in our previous studies in Ibaraki, respectively. These results suggest that genus *Haemophysalis* ticks are the candidate vectors of *Anaplasma phagocytophilum* and *Anaplasma bovis* in Ibaraki, Japan. [115 words]
Anaplasma phagocytophilum is a tick-borne intragranulocytic bacterium, causing human granulocytic anaplasmosis (HGA), equine granulocytic anaplasmosis (EGA), tick-borne fever (TBF) and canine granulocytic anaplasmosis (CGA) for humans, horses, ruminants and dogs, respectively (1). This pathogen has been emerging zoonotic threat in the world (2). A. phagocytophilum is mainly transmitted by hard ticks of Ixodes spp., including I. ricinus in Europe, I. scapularis, I. pacificus and I. spinipalpis in USA, and I. persulcatus and I. ovatus in East Asia and Russia (1-3).

In Japan, HGA was first reported in Kochi in 2013 (4) and CGA was first recognized in Tsukuba, Ibaraki in 2016 (5). Dogs infected by A. phagocytophilum were successively detected in Moriya, Ibaraki (6). In our previous epidemiological study, serological and molecular evidence of infection with Anaplasma phagocytophilum and/or Anaplasma bovis was found in subclinical dogs around Tsukuba and Moriya (7). Although DNA fragments of A. phagocytophilum and A. bovis have been detected from many tick species including Ixodes persulcatus, I. ovatus, I. nipponensis, Haemophysalis megaspinosa, H. formosensis, H. douglasi, H. longicornis and Amblyomma testudinarium in Japan (8-10), candidate vector ticks of both bacteria for dogs in Japan are still unknown.

Therefore, the objective of this study was to determine which tick species may transmit these bacteria to dogs using molecular techniques.

In April and May 2016 and April, June, July, September, October and November 2017, ticks were collected by flagging at vegetation around the areas, which A. phagocytophilum infected dogs were reared, in Tsukuba and Moriya, Ibaraki, Japan (Fig. 1). Ticks were morphologically identified to the species level and classified into various developmental stages (11). One adult tick, one to seven (median: 5) nymphs, or one to thirteen (median: 10) larvae of the same species and stage were fixed and pooled into one tube with 70% ethanol, respectively. Total DNA was extracted from each tube using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). DNA samples were stored at -20°C in 200μl of
TE buffer until use.

To detect A. phagocytophilum and A. bovis, we performed 16S rRNA gene-based PCR, electrophoresis, purification, and direct sequencing as described previously (7). Briefly, Screening PCR that amplified the partial gene sequence of Anaplasmataceae was performed with primers pairs EHR16SD/EHR16SR, and PCR-positive samples were subsequently subjected to semi-nested PCR covering the divergent regions of partial16S rRNA gene near the 5’ end with primer pairs fD1/EHR16SR and fD1/GA1UR for the first and second amplifications, respectively. Homology searches based on partial 16S rRNA 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 sequence using the sequence analysis software MEGA7. 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.

A total of 1084 ticks were collected, including Haemophysalis flava (42 females, 58 males, 266 nymphs and 181 larvae), H. longicornis (6 females, 2 males, 192 nymphs and 273 larvae), Ixodes nipponensis (3 females, 6 males, 52 nymphs and 2 larvae) and I. turdus (1 female). In sum, a total of 118 adults, 107 pools of nymphs and 47 pools of larvae were analyzed to detect family Anaplasmataceae.

Twenty-six samples were positive for screening PCR, including H. flava (10 females, 7 males, 1 pool of nymphs and 2 pools of larvae) and H. longicornis (1 male, 1 pool of nymphs and 4 pools of larvae) that were collected in Moriya. Twenty-three samples were positive for the semi-nested PCR and the partial 16S rRNA gene-sequences of these positive-PCR products were determined. BLAST analysis revealed that 19 samples were not belonging to the family Anaplasmataceae, whereas three pools from larval H. longicornis were 96.6-100% identical to A. phagocytophilum from a CGA dog in Moriya (LC334014) and one sample from a male H. flava was 99.7% identical to A. bovis from a
dog in Moriya (LC431238) (Fig. 2). These sequences of 16S rRNA determined in this study have been deposited in GenBank with accession numbers from LC457961 to LC457964, respectively.

To the best of our knowledge, this is the first report for molecular detection of *A. phagocytophilum* from larval *H. longicornis*. The larvae of genus *Ixodes* are not thought to transmit *A. phagocytophilum* to mammals since there is no evidence of transovarial (from adult ticks to eggs) transmission (1-3). In a previous study, *A. phagocytophilum* was also detected from larvae of *H. megalospinosa* [8]. The transovarial transmission of *Anaplasma* spp. was confirmed in *Dermacentor* ticks [12,13]. Although experimental confirmation for transovarial transmission of *Anaplasma* has not been demonstrated, these findings may suggest that *A. phagocytophilum* can be transovarially transmitted by *Hemaphysalis* ticks [8].

In our previous reports, no evidences of tick-bite were found in dogs infected with *A. phagocytophilum* and/or *A. bovis* [6,7], and infectious routes of both pathogens were unknown. In sight of the highest homologies between the sequences from ticks in this study and those from dogs in our previous studies [6,7], both pathogens might infect dogs by ticks around their habitation. Moreover, *H. flava* and *H. longicornis* might be the dominant tick species and the candidate vectors of *A. phagocytophilum* and *A. bovis* in Tsukuba and Moriya, Ibaraki, Japan. This situation resembles to those in Korea [14], as the strain of *A. phagocytophilum* in Ibaraki is genetically close to that in Korea [6]. Thus, this strain in Ibaraki might infect humans and be developed HGA since HGA patients have been recently reported in Korea [15].

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Fig. 1. Map of Tsukuba and Moriya in Ibaraki where ticks were collected in 2016 and 2017. Closed circles indicate collection sites.
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Fig. 2. Phylogenetic relationships of Anaplasma phagocytophilum and Anaplasma bovis from this study within the genus Anaplasma based on 16S rRNA gene. The tree was analyzed using nucleotide sequences by the neighbor-joining method and was supported by 1000 bootstrap replications.