

## Short Communication

# Detection of *Anaplasma bovis* DNA in the Peripheral Blood of Domestic Dogs in Japan

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**SUMMARY:** The prevalence of *Ehrlichia* and *Anaplasma* in 1,427 dogs from 32 Japanese prefectures was evaluated by PCR and DNA nucleotide sequencing. PCR screening demonstrated that 18 dogs (1.3%) were positive for *Anaplasmatidae*. Sequence analysis revealed that 14 of the amplicons were most closely related to *Wolbachia* spp., symbionts of *Dirofilaria immitis*, whereas three were identified as *Anaplasma bovis*. The remaining amplicon could not be sequenced. Almost the entire sequence of 16S rRNA (1,452 bp) from one of the positive specimens was determined, and subsequent phylogenetic analysis confirmed that the detected sequence was that of *A. bovis*. This is the first detection of *A. bovis* DNA fragments in dogs. Species-specific nested PCR showed that 15 (1.1%) of the 1,427 dogs involved in this study were positive for *A. bovis*. The geographical distribution of these dogs ranged from Aomori Prefecture in northern Japan to Kagoshima Prefecture in the south. The relationship between *A. bovis* infection and clinical disease is not yet clearly understood.

Members of the family *Anaplasmatidae* belong to the order *Rickettsiales* and comprise the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Aegyptianella*, *Wolbachia*, and 'Candidatus Neoehrlichia', which are obligate intracellular Gram-negative bacteria (1,2). *Anaplasma* and *Ehrlichia* are important emerging tick-borne pathogens in both humans and animals (3). The organisms of greatest clinical importance in dogs include *Ehrlichia canis*, *Anaplasma platys*, and *Anaplasma phagocytophilum*. These bacteria are distributed worldwide, and molecular evidence of these pathogens has been detected in Japan. DNA fragments of *E. canis* were detected in a dog from Kagoshima Prefecture in southern Japan (4), although the only confirmed case of *E. canis* infection occurred in a dog that had been transferred from Indonesia (5). *A. platys* DNA has been detected in dogs in Okinawa and Yamaguchi Prefectures (6-8) and in ticks recovered from Fukushima, Miyazaki, and Kagoshima Prefectures (9). Recently, DNA fragments from *A. phagocytophilum* were detected in deer, cattle, and ticks in Japan (10-13), although not in dogs. There have been no case reports of domestic dogs in Japan infected with these pathogens.

Several new species of *Ehrlichia* and *Anaplasma* have been reported recently in Japan, including *Ehrlichia muris* (14), *Ehrlichia* sp. isolated from *Ixodes ovatus* (15), 'Candidatus *Ehrlichia shimanensis*' (11), and *Anaplasma bovis* (11,16). These species may cause infection in dogs, although the pathogenicity of these organisms in these animals is unknown. Positive PCR results for *Ehrlichia* sp. isolated from *I. ovatus* have

been reported in Yamaguchi Prefecture (7), although no epidemiological studies of *Anaplasma* and *Ehrlichia* infection in dogs have been conducted at a national level in Japan. For this reason, we evaluated the prevalence of *Ehrlichia* and *Anaplasma* in dogs from most regions in Japan using PCR and DNA nucleotide sequencing methods. We also undertook the molecular characterization of the *A. bovis* detected in dogs in this study.

Blood samples were collected from 1,427 domestic dogs between January 2006 and June 2008 and processed in animal hospitals from 32 prefectures (Hokkaido, Aomori, Miyagi, Fukushima, Tochigi, Ibaraki, Tokyo, Chiba, Kanagawa, Ishikawa, Yamanashi, Shizuoka, Aichi, Mie, Osaka, Kyoto, Shiga, Nara, Wakayama, Hyogo, Tottori, Shimane, Hiroshima, Yamaguchi, Tokushima, Kochi, Fukuoka, Nagasaki, Kumamoto, Oita, Miyazaki, and Kagoshima). All of the animals involved in this study were routinely active outdoors. The clinical status and epidemiological information, including breed, sex, age, tick infestation, and clinical history, were collected by the veterinarians treating these animals. Blood samples were collected from each animal in EDTA tubes for subsequent DNA extraction, and then stored at -20°C until transfer to Obihiro University of Agriculture and Veterinary Medicine. DNA was extracted from the EDTA blood samples using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA samples were stored at -20°C in 200 µl of TE buffer until further analysis.

Screening PCR was performed using the group-specific primer pair EHR16SD and EHR16SR, which amplifies the 16S rRNA gene of the family *Anaplasmatidae* (17). When a strongly positive band was detected after PCR, the products were purified using the QIAquick PCR purification kit (Qiagen). Direct sequencing of PCR products and analysis of the sequences obtained were performed as described previously (9).

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Homology searches based on the sequences of the PCR products were performed using BLAST (National Center for Biotechnology Information).

Additional PCR amplifications using the primer sets FD1 and EHR16SR, or EHR16SD and Rp2, were performed to determine nearly full-length sequences of the 16S rRNA genes of some positive samples (18). Distance matrix calculations and construction of phylogenetic trees were performed using the ClustalW program (version 1.8) in the DNA data bank of Japan (DDBJ; Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). Distance matrices for the aligned sequences, with all gaps ignored, were calculated using the Kimura two-parameter method, and the neighbor-joining method was used to construct a phylogenetic tree. The stability of this tree was estimated by bootstrap analysis for 100 replications using the same program. The tree figure was generated using the Tree View program (version 1.6.6). The GenBank accession numbers of the 16S rRNA gene sequences used to construct phylogenetic trees and to analyze percent identities were as follows: *A. bovis* strain South Africa, U03775; *A. bovis* detected from sika deer in Shimane, Japan, AB211163; *A. bovis* detected from *Haemaphysalis longicornis* in Nara, Japan, AB196475; *A. bovis* detected from cattle in Okinawa, Japan, EU368730-EU368732; *A. phagocytophilum* strain Webster, U02521; *A. phagocytophilum* strain CAHU-HGE2, AF093789; *A. ovis*, AF414870; *A. platys*, AY077619; *A. marginale*, AF309867; *A. centrale*, AF283007; and *E. canis*, M73221.

Eighteen (1.3%) of the 1,427 samples examined by screening PCR were clearly positive for *Anaplasma* spp. The partial 16S rRNA gene-sequences of these positive-PCR products were determined using 345 bp, excluding the primer region. BLAST analysis revealed that 14 samples from Shimane, Kochi, Fukuoka, Oita, Nagasaki, Kumamoto, and Miyazaki were most

closely related to *Wolbachia* spp., whereas three samples from Hiroshima and Fukuoka were closely related to *A. bovis*. We were unable to determine the sequence of the one remaining positive sample.

As *Wolbachia* spp. are known to be symbionts of *Dirofilaria immitis* (heartworm) (19), we speculated that the positive-PCR results might be related to *D. immitis* infection. A previous study also detected DNA fragments of *Wolbachia* spp. from dogs in Okinawa, Japan (4). Dogs that were positive for *Wolbachia* spp. lived in western Japan, which is an endemic area for filarial nematodes. The 14 dogs which tested positive for *Wolbachia* spp. in our study were also found to be positive for *D. immitis* infection using an enzyme immunoassay kit (IDEXX canine snap 4D test; IDEXX Laboratories, Westbrook, Maine, USA).

Determination of nearly full-length sequences of the 16S rRNA genes of *A. bovis* positive samples was attempted using additional PCR. For one sample (Hiroshima-Z27), we successfully sequenced 1,452 bp of the 16S rRNA gene, excluding the primer region. This sequence has been deposited in GenBank under the accession no. HM131217. The sequence demonstrated a nucleotide identity of 99.5% with the 16S rRNA gene of *A. bovis* detected in South Africa (U03775) and 99.4% with *A. bovis* from cattle in Okinawa, Japan (EU368731) and from deer in Japan (AB196475). These sequences clustered in the same clade as *A. bovis* in the 16S rRNA gene-based phylogenetic tree (Fig. 1). This is the first detection of *A. bovis* DNA fragments in dogs, although such DNA has recently been isolated from ticks, cattle, and deer in Japan (11–13).

A species-specific nested PCR was performed using the primer sets EC9 and EC12A for the first amplification and AB1f and AB1r for the second amplification to determine the percentage of dogs infected with *A. bovis*. This method has been described previously (11). This nested-PCR evaluation suggested that 15 dogs (1.1%)

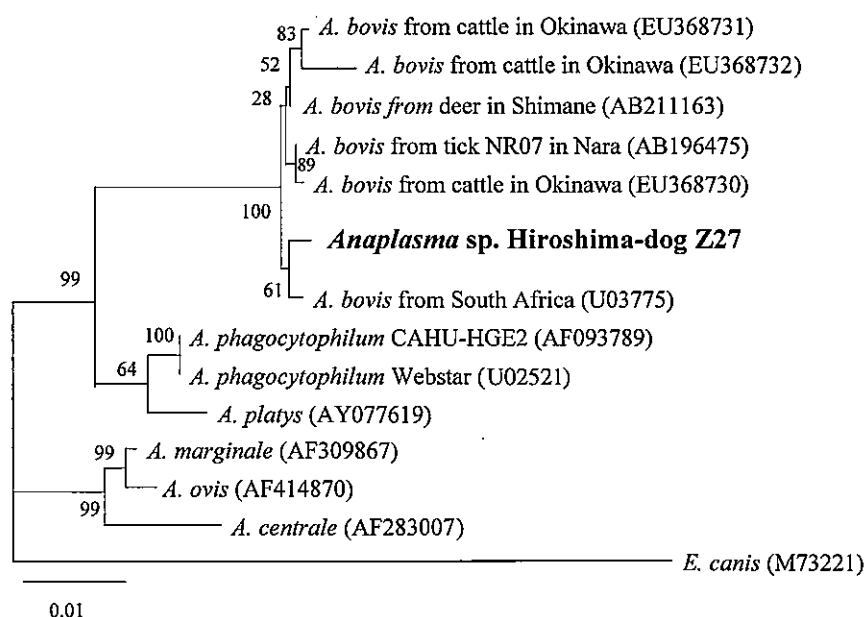


Fig. 1. Phylogenetic relationships of various *Anaplasma* spp. based on nucleotide sequences of the 16S rRNA gene. Scale bar indicates genetic distance (0.01 substitutions/site). The *Anaplasma* sp. detected in this study is shown in bold type.

Table 1. Clinical characteristics of 14 dogs positive for *Anaplasma bovis*

ID	Prefecture	Breed	Age (yr)	Sex	Tick infestation	History of diseases
J18	Aomori	Setter	7	M	NR	Filariasis
N4	Chiba	Mix	10	F	—	None
N34	Chiba	Border collie	3	M	NR	None
O10	Tokyo	NR	NR	NR	NR	NR
H28	Wakayama	Mix	11	M	NR	None
97	Tottori	Siberian Husky	NR	NR	NR	Heart failure
Z27	Hiroshima	Mix	8	F	+	None
Z37	Hiroshima	Plott hound	5	F	+	None
36	Yamaguchi	Kishu	2	M	NR	Pemphigus
136	Tokushima	Mix	7	M	+	None
138	Tokushima	L.Retriever	8	M	+	None
139	Tokushima	S.Sheep Dog	8	M	+	None
D27	Fukuoka	Shiba	3	M	NR	None
D37	Fukuoka	Mix	5	M	NR	None
8	Kagoshima	NR	NR	NR	NR	NR

NR, not recorded; F, female; M, male; L.Retriever, Labrador retriever; S.Sheep Dog, Shetland sheepdog.

were positive for *A. bovis*. Five of the resulting 15 amplicons were selected at random and their nucleotide sequences determined to confirm the nested PCR results. Sequences of all five samples were identical and showed 100% identity with *A. bovis*. The clinical characteristics of these 15 dogs are shown in Table 1. Their geographical distribution dogs ranged from Aomori Prefecture in the north to Kagoshima Prefecture in the south, although 10 of the 15 dogs lived in western Japan. Tick infestation was reported for five dogs. *Hyalomma* spp., *Rhipicephalus appendiculatus*, and *Amblyomma variegatum* are known vectors of *A. bovis* (20–22), although these particular ticks have not been detected in Japan. However, *A. bovis* DNA has been detected in *H. longicornis* ticks collected in Japan and Korea (11,23). This latter tick species has a geographical distribution ranging from northern to southern Japan and targets a wide variety of host animals (24). Unfortunately, we were unable to identify the tick species found on the dogs in the present study. As there is no evidence for transmission of the pathogen to dogs by ticks, further epidemiological studies are required to clarify the relationship between *A. bovis* infection in dogs and ticks.

A dog (J18) in Aomori Prefecture tested positive for both *A. bovis* and *Wolbachia* sp., although the effect of this dual infection on pathogenicity could not be determined. Furthermore, although one of the dogs which tested positive for *A. bovis* had a history of heart failure, the pathogenicity of *A. bovis* for dogs remains unknown. Veterinarians should be alert to the possible health risks posed by this agent in dogs.

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**Conflict of interest** None to declare.

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