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NOTE

A molecular survey of *Mycoplasma haemocanis* in dogs and foxes in Aomori Prefecture, Japan

Sasaki, M.[1], Ohta, K.[1], Matsuu, A.[2], Hirata, H.[3], Ikadai, H.[1*] and Oyamada, T.[1]
[1] Department of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan, [2] Department of Veterinary Internal Medicine, Faculty of Agriculture, Tottori University, Koyama-Minami, Tottori 680-8553, Japan, [3] School of Veterinary Medicine, Rakuno-Gakuen University, Ebetsu 069-8501, Japan
*Correspondence to: Ikadai, H., Department of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University, Towada, Aomori, 034-8628, Japan.
Tel.: +81-176-23-4371; fax: +81-176-25-0165; *E-mail* : ikadai@vmas.kitasato-u.ac.jp

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Mycoplasma haemocanis is an epierythrocytic microbe that causes canine haemobartonellosis. *M. haemocanis* is transmitted by haematophagous arthropod vector and can be transmitted directly through blood (Pryor *et al*., 1975). The dogs are infected latently and usually become carriers without presenting symptoms. However, other factors such as splenectomy or immunosuppression may trigger immune-mediated hemolytic anemia (Bundza *et al*., 1976; Chalker, 2005; Kemming *et al*., 2004; Kenny *et al*., 2004; Lester *et al*., 1995; Pryor *et al*., 1975). *M. haemocanis* was previously classified in the order *Rickettsiales*, and named *Haemobartonella canis*. However, recent sequence analysis of 16S rRNA and the RNase P gene showed that this pathogen is a member of the genus *Mycoplasma* (Chalker, 2005; Kenny *et al*., 2004; Tasker *et al*., 2003). Consequently, *H. canis* was renamed *M. haemocanis*. This pathogen is distributed widely (Chalker, 2005), but detailed epidemiological evidence has not been reported.

M. haemocanis infection is diagnosed by detecting pathogens in a Giemsa-stained peripheral blood smear under the microscope. However, this method is difficult because the organism is small (0.2 - 0.6 μm in diameter) (Sykes *et al*., 2005) and a method for artificial culture of *M. haemocanis* has not been established (Chalker, 2005). A recently reported PCR method with high sensitivity and specificity for detecting pathogens is useful for diagnosing *M. haemocanis* (Chalker, 2005). The similarity between the 16S rRNA gene of *M. haemocanis* and *M. haemofelis* is 99.3 - 99.7% (Birkenheuer *et al*., 2002; Brinson *et al*., 2001; Tasker *et al*., 2003), indicating that *M. haemocanis* 16S rRNA can be detected using PCR and the same method as used for the detection of *M. haemofelis* (Brinson *et al*., 2001).

We used a PCR method with a primer set that amplifies the *M. haemofelis* 16S rRNA gene to demonstrate the distribution of *M. haemocanis* in an epidemiological survey of dogs, foxes, and raccoon dogs in Aomori Prefecture in Japan.

Between July 2002 and July 2003, peripheral blood samples were collected randomly from 913 dogs in Aomori Prefecture. These blood samples were taken into EDTA or heparin tubes and kept at 4°C. DNA was extracted from 100 μL of blood from each dog using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences, UK), and stored in 50 μL of TE buffer at -20°C. Whole blood samples or clots were obtained from the carcasses of 12 foxes and four raccoon dogs in Aomori Prefecture, and the samples were stored at -80°C. DNA was extracted using the GFX Genomic Blood DNA Purification Kit and stored in 50 μL of TE buffer at 4°C.

M. haemocanis was detected using primer sets identical to primers that amplify *M. haemofelis* 16S

rRNA. Forward primer H. felis-f1 (5´-GACTTTGGTTTCGGCCAAGG- 3´) and reverse primer H. felis-r3 (5´-CGAAGTACTATCATAATTATCCCTC- 3´) were used in the first PCR (Brinson *et al*., 2001). And primer set fo2 (5´-AGCTTTTAAAGCCTTGGGG- 3´) and ro2 (5´-TGCTTCCCGTAGGAATATG-3´) were structured for the nested PCR.

The DNA extracted from blood samples was added to a reaction mixture containing 10 pmol of each primer H. felis-f1 and H. felis-r3, 5 U/μL of AmpliTaq Gold DNA polymerase (Applied Biosystems, US), 2.0 mM dNTP (Applied Biosystems), 10 × PCR Gold Buffer (150 mM Tris-HCl [pH 8.0], 500 mM KCl) (Applied Biosystems), 25 mM $MgCl_2$ Solution (Applied Biosystems), and adjusted to 25 μL with ultra pure distilled water. The amplification conditions were as follows: 95°C for 10 min, 40 cycles of denaturation at 95°C for 45 sec, primer annealing at 52°C 45 sec, and amplification at 72°C for 1 min, followed by final extension at 72°C for 10 min. The nested PCR was performed using primers fo2 and ro2 under the same conditions as the first PCR. PCR products were separated by electrophoresis on a 1.5% TBE agarose gel and stained with ethidium bromide. *M. haemocanis* was expected to produce a fragment of 348 bp in nested PCR.The amplified DNA samples from two randomly selected dogs and a fox were used to determine their sequence. One microliter of the amplified DNA was cloned into a pCR ® 2.1-TOPO ® vector using a TOPO TA Cloning ® Kit (Invitrogen, US), and the sequences were determined with an ABI PRISM ® 310 genetic analyzer (Applied Biosystems). The obtained sequences were analyzed using Genetyx ® Version. 8 (Genetyx, Japan).

The nested PCR using the primer set produced the expected 348 bp fragments. The sequences of PCR products from two dogs and a fox in Aomori Prefecture showed 100% homology to the sequences of *H. canis* 16S rRNA gene (GenBank accession nos. AF197337 and AF407208) and *M. haemocanis* 16S rRNA gene (AY529641). The sequences of *M. haemocanis* 16S rRNA gene from the United States, Germany, United Kingdom, France, the Middle East are reported to have little diversity (Chalker, 2005; Tasker *et al*., 2003).

In Aomori Prefecture, Japan, 37 of 913 dogs (4.1%) and one of 12 foxes (8.3%) showed a band corresponding to the 348 bp fragment that was positive for *M. haemocanis* (Table 1). Of the 913 dogs examined, 73 were Tosa dogs, and 25 (34.2 %) of these were positive for *M. haemocanis*. The incidence of *M. haemocanis* was 1.4 % (12/840) in other dogs. All but one of the 25 Tosa dogs infected with *M. haemocanis* were male. *M. haemocanis* is transmitted directly through blood (Pryor *et al*., 1975), and the transmission of *Babesia gibsoni* infection in dogs in Aomori Prefecture might occur from the direct transmission of blood during dogfights (Ikadai *et al*., 2004). Therefore, *M. haemocanis* may also be transmitted by contamination of blood. We also found *M. haemocanis* infection in one fox in Aomori Prefecture, suggesting that this pathogen may be transmitted between dogs and foxes through an arthropod vector.

Table 1. The incidence of *M. haemocanis* in dogs, foxes and raccoon dogs in Aomori Prefecture, Japan.

		No. of examined	*M. haemocanis* positive
Dogs	Tosa dogs	73	25 (34.2%)
	Other breeds	840	12 (1.4%)
	total	913	37 (4.1%)
Foxes		12	1 (0.83%)
Raccoon dogs		4	0 (0%)

In conclusion, a PCR method was used to identify potential carrier dogs of *M. haemocanis* in Aomori Prefecture. Infection by *M. haemocanis* was demonstrated in a fox. In dogs, factors such as splenectomy or immunosuppression might cause overt disease (Bundza *et al*., 1976; Chalker, 2005; Kemming *et al*., 2004; Kenny *et al*., 2004; Pryor *et al*., 1975). Transfusion is also reported to trigger clinical signs such as hemolytic anemia (Lester *et al*., 1995; Pryor *et al*., 1975). The mixed infection with other pathogens such as *B. gibsoni* may cause transformation of erythrocytes and anemia (Sonoda *et al*., 1978). *B. gibsoni* infection in Tosa dogs has been reported in Aomori Prefecture (Ikadai *et al*., 2004). This suggests that *M. haemocanis* infection occurs in combination with, and plays a role in aggravating the symptoms of, other infections. Further research is needed to investigate the route of infection of *M. haemocanis* in dogs.

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