A Feline Hemoplasma, '*Candidatus Mycoplasma haemominutum*', Detected in Dog in Japan

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ABSTRACT. We examined for '*Candidatus Mycoplasma haemominutum*' infection in 167 blood samples collected from domestic dogs between 2008 and 2009 in the Tohoku area, Japan, and found 5 (3.0%) were positive by PCR assay. This is the first demonstration of '*Candidatus Mycoplasma haemominutum*', a feline haemotropic mycoplasma, in the dogs raised in Japan.

KEY WORDS: 16S rRNA, canine, feline hemoplasma, mycoplasma.

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Hemoplasmas, hematropic mycoplasmas, infect the erythrocytes and are causative of hemolytic anemia and thrombocytosis in animal [16]. Currently, three hemoplasma species, *Mycoplasma haemofelis*, '*Candidatus Mycoplasma haemominutum*', and '*Candidatus M. turicensis*', and tentatively "*Candidatus M. haematoparvum*-like" strain are recognized in cats [4, 5, 19, 23]. On the other hand, there are two hemoplasma species in dogs, *M. haemocanis* [15], and '*Candidatus M. haematoparvum*' [18]. In general, the *Mycoplasma* species has the strict host specificity, so there have been only a few reports of the infections in an animal species not perceived as primary host.

The inability to culture the hemoplasma *in vitro* has hampered the possibilities of investigating the epidemiology and pathogenesis of this particular microbial agent [7]. Definitive diagnosis of hemoplasma infection has mainly been depend on microscopic examination of a thin Wright-Giemsa-stained blood smear, but this method and has low sensitivity and specificity because the organisms resemble Howell-Jolly bodies or back ground debris [2], and it is more difficult to distinguish between hemoplasma species. Thus the PCR is widely used to detect hemoplasmas because of its sensitivity [1, 21].

Haemoplasmosis caused by *M. haemofelis* can lead to life-threatening hemolytic anemia in cats, whereas clinical signs in haemoplasmosis caused by '*Candidatus M. haemominutum*' and '*Candidatus M. turicensis*' have been reported to be minor or absent [5]. Dogs are latently infected with hemoplasmas, and the presence of underlying disorders such as immunosuppressive, co-infection, splenectomy, overt disease, trigger the anemia by hemoplasmas [8, 14, 22].

Although there are a few epidemiological reports on

'*Candidatus M. haemominutum*' infections in dogs in only China [25] and France [12], there have been no examination on this particular hemoplasma species in Japan. Therefore, we examined for the presence of the feline hemoplasma '*Candidatus M. haemominutum*' infection in the dogs in Japan.

Ethylene diamine-tetraacetic acid (EDTA)-anticoagulated blood samples were collected from 167 pet dogs (*Canis lupus familiaris*) in the Miyagi Prefecture, Japan between 2008 and 2009. The whole blood samples were taken for clinical diagnostic and health check purposes, and the remainder was subjected to detection of '*Candidatus M. haemominutum*'. These dogs have no travel history and not been splenectomized. Date on age and sex and clinical information obtained for the dogs was incomplete. The contact history to the cats is unknown. Blood samples were stored at –80°C prior to analysis.

DNA was extracted from 200 µl anticoagulated whole blood in EDTA collected using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's instructions. DNA was eluted in 200 µl elution buffer and stored at –20°C until examination in the PCR assay.

The 16S rRNA gene of '*Candidatus M. haemominutum*' was amplified by using the following primers. A 654-bp fragment of the 16 rRNA gene was amplified using primers F1 (5'-GATTAATGCTGGTGGTATGC-3') and R1 (5'-CATTGAATTCCAGTATCTCC-3') [6]. A 403-bp fragment of the 16 rRNA gene was amplified using primers F2 (5'-TACTCTCTTAGTGGCGAACG-3') and R2 (5'-AATCAAGGCTTAATCATTTTC-3') [6]. PCR was performed in two steps as followed; the first with pairs of the outer primers (F1 and R1) and the second with the inner primers (F2 and R2). The mixture for each reaction contained 5 µl of extracted DNA samples and 45 µl of the PCR master mix, which consisted of 5 µl of 10X PCR buffer, 8 µl of 2.5 mM deoxynucleoside triphosphates, 10 pmol specific outer primers, 2 units of *Tth* DNA polymerase (TOYOBO, Tokyo, Japan) and one drop of mineral oil. PCR was per-

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formed with an initial denaturation of 94°C for 30 sec, followed by 30 cycles of heating at 94°C for 30 sec, at 55°C for 2 min, and at 72°C for 1 min. Then 1 µl of the first PCR products were transferred into a second tube containing 49 µl of the reaction buffer as described in the first round reaction, for the second round of the same 30 cycles of amplification, but with the pair of inner primers. The PCR products were detected by electrophoresis on a 1.0% agarose gel in Tris-acetate-EDTA buffer and stained with ethidium bromide (0.4 µg/ml) and visualized under UV transilluminator, BioDoc-I Imaging System (UVP, Upland, CA, U.S.A.). Positive samples were directly subjected to DNA sequencing for identification of hemoplasma species. The nucleotide sequence of the partial 16S rRNA gene has been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession number AB550430.

A PCR product was amplified from the five dogs, with expected band size on a 1.0 % agarose gel (Fig. 1). Three are adult dogs suffering from multi-centric lymphoma, starvation, atopic dermatitis, respectively. The other two dogs are puppy (about three months old) and have no sign of disease. No PCR products were amplified from the other 162 dogs, which did not develop any clinical sign of haemolytic anemia due to '*Candidatus M. haemominutum*' infection. Comparison of the obtained sequence (a dog suffered from lymphoma) with those corresponding sequences available in the GenBank database revealed the 98% homology to the 16S rRNA gene of '*Candidatus M. haemominutum*' (Fig. 2). We can not gain sequence of the other 4 PCR products because of insufficient PCR product in the first step PCR.

In this study, we first demonstrated that feline hemoplasma '*Candidatus M. haemominutum*' infected dogs in the northern part of Japan. The relatively low prevalence in dogs in this study, as compared to the feline infection rate of '*Candidatus M. haemominutum*' in Japan [6], may be based on the present study included healthy dog population. The study has certain limitations because of the relatively small number of case analyzed.

These dogs infected with hemoplasma had been kept in the ectoparasite-free conditions and no clinical signs of

Fig. 1. A representative PCR result on detection for feline '*Candidatus M. haemominutum*' in the dogs. Lane 1 shows 200-bp DNA Ladder (TaKaRa-Bio, Shiga, Japan); Lanes 2–6 show 403-bp DNA fragment. Lanes 2, 3 and 4 dogs suffered from multi-centric lymphoma, starvation, atopic dermatitis, respectively. Lanes 5 and 6; healthy puppy; Lane 7; negative control (water).

hemolytic anemia by '*Candidatus M. haemominutum*'. It is known that '*Candidatus M. haemominutum*' infection rarely results in significant clinical signs and anemia is not usually apparent in cat [5]. Similarly, dogs infected with '*Candidatus M. haemominutum*' lacked any clinical signs of hemolytic anemia. The pathogenicity of '*Candidatus M. haemominutum*' may be not severe in the dogs.

The transmission route of '*Candidatus M. haemominutum*' from the cats to the dog is currently unknown. Blood-sucking arthropods (e.g., fleas, ticks) have been suspected as natural means of hemoplasma transmission [24]. However, there were not so much tick infestations due to the cold climate in the Tohoku area compared to the southern parts in Japan. It is also possible that the infection was occurred by horizontal transmission from infected cats. There is some

Fig. 2. Phylogenetic tree based on the 16S rRNA sequence comparison among feline and canine hemoplasmas. Genetic distances were computed with CLUSTAL W [14]. Hemoplasma sequences used in this analysis were as follows: '*Candidatus M. haematoparvum*' (EF416569), '*Candidatus M. haemominutum*' (AB550430, AF271154, AM691834, DQ157142, U88564, AY150974, AY297712), '*Candidatus M. turicensis*' (DQ825454), *M. haemocanis* (EF416568), and *M. haemofelis* (U88563). Out-group unit was *M. pneumoniae* (U00089). Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the evolutionary distance value of 0.1 (a single nucleotide substitution per 10 nucleotides). AB550430 represents a hemoplasma strain detected from a dog (suffered from multi-centric lymphoma) in the present study.

evidence for direct transmission of hemoplasma in cat [24]. In dog, direct transmission of hemoplasmas is one the likely routes. There is also the possibility of transplacental infection [13]. Since we detected ‘*Candidatus M. haemominutum*’ from the two puppies of three months old, there is another likely route of the vertical transmission of ‘*Candidatus M. haemominutum*’ in dogs. The mechanisms of transmission of hemoplasma between dogs and cats have yet to be defined and need further exploration. Zhuang *et al.* [25] suggested the possibility that a dog was one of the hosts for the feline hemoplasma ‘*Candidatus M. haemominutum*’. However, it is likely that the presence of some underlying conditions (e.g., immunosuppressive, immature) may destroy the strict host specificity in mycoplasma infections, as previously reported in the cases of hemoplasma infection in human [3, 10, 11, 17]. Also, our results may indicate that dogs represent one of the hosts for the feline ‘*Candidatus M. haemominutum*’.

In conclusion, we first showed feline hemoplasma ‘*Candidatus M. haemominutum*’ infected with dogs in Japan.

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