Molecular Survey of *Mycoplasma haemofelis* and ‘Candidatus Mycoplasma Haemominutum’ Infection in Cats in Yamaguchi and Surrounding Areas

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**ABSTRACT.** A molecular survey of hemoplasma (*Mycoplasma haemofelis* and ‘Candidatus Mycoplasma haemominutum’) in Yamaguchi Prefecture and surrounding areas was performed by using molecular methods. PCR-RFLP with *HindIII* revealed that 2 cats were infected with *M. haemofelis*, and 16 with ‘C. Mycoplasma haemominutum’ among 102 randomly selected cats. Partial 16S rRNA gene sequences of *M. haemofelis* and ‘C. Mycoplasma haemominutum’ determined in this study showed percent similarities of 98.3–99.8% and 96.4–100%, respectively, with those from other countries. Hemoplasma infections were more frequently detected in free-roaming cats than inside cats. Also, the status of FeLV infection was another significant risk factor for hemoplasma infection.

**KEY WORDS:** ‘Candidatus Mycoplasma haemominutum’, feline, *Mycoplasma haemofelis*.

The feline hemoplasmas, *Mycoplasma haemofelis* and ‘*Candidatus Mycoplasma haemominutum*’, were previously ascribed to *Haemobartonella felis* strains Ohio and California, respectively [13, 14], which cause hemolytic anemia, thrombocytopenia, fever and jaundice [3, 10]. Hemoplasma infection is usually diagnosed through the detection of the parasite on the surface of erythrocytes in blood smears stained with Giemsa and viewed under a microscope [1]. However, it is often difficult to confirm the infection, as the organisms resemble Howell-Jolly bodies or background debris [2]. It is even more difficult to distinguish *M. haemofelis* and ‘C. Mycoplasma haemominutum’ morphologically. A number of molecular techniques, including polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis, have recently been developed for feline hemoplasmas infection, and widely used for both diagnosis and epidemiological studies in the U.K. [16, 17], Spain [5], U.S.A. [7, 12], Israel [9], and Australia [4]. However, limited numbers of studies on feline hemoplasma infection in Japan have been published. Thus, the aim of the present study was to analyze the infection of hemoplasma in cats in Yamaguchi and surrounding area by using molecular methods.

A total of 102 feline patients were randomly selected from cats that presented at the Veterinary Teaching Hospital, Yamaguchi University, from March 2002 to March 2003. Twelve of 102 cats were presented for physical complaints, Yamaguchi University, from March 2002 to March 2003. Twelve of 102 cats were presented for physical complaints, and did not show any clinical problems. Thus these cats were categorized as a healthy group. The rest of the 90 cats showed one or more diseases. Blood treated with the anticoagulant EDTA was collected for PCR to detect hemoplasma. Clinical symptoms were recorded at the time of blood collection. Infection of feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) was occasionally checked using a SNAP FeLV/FIV Combo kit (IDEXX, U.S.A.). The significance of individual factors (age, sex, roaming, inside/outside, health condition, FIV and FeLV infection) as determinants for hemoplasma infection was investigated by maximum likelihood estimates of the chi square test derived from contingency table analysis. The critical probability was set at p<0.05.

Total DNA was extracted from the packed red blood cell fraction of each feline sample by a method described previously [11]. For a screening purpose, PCR amplification was performed in a 25-µl reaction mixture containing 5 µl of each DNA template with the primer set of *H. felis* F and *H. felis* R described by Jensen et al. [12], which can amplify both *M. haemofelis* and ‘C. Mycoplasma haemominutum’. These primers produce a 170-bp fragment in *M. haemofelis* and a 193-bp fragment in ‘C. Mycoplasma haemominutum’. Positive DNA controls for *M. haemofelis* and ‘C. Mycoplasma haemominutum’ were kindly supplied by Dr. S. Tasker (University of Bristol, UK). To confirm the nature of the amplification products of both *M. haemofelis* and ‘C. Mycoplasma haemominutum,’ the PCR products were digested with *HindIII* (New England Biolabs, U.S.A.) according to the recommendations of the manufacturer. The PCR products and digestion products were separated by using 3 % agarose gel electrophoresis. Partial 1 sequence of 16S RNA gene, including the divergent region near the 5’-end, were then determined for the screening-positive samples. A primer set, fD1 [19] and Hf513R (5’-ACG CCC AAT AAA TCC GAA TAA-3’), were used for the amplification, and nucleotide sequences of the PCR products were determined and analyzed by the method previously reported [11]. The GenBank accession numbers of 16S rRNA gene sequences used to analyze the percent identities and to construct a phylogenetic tree were as follows: *M. haemofelis* strains Australia 1: AY150976, Australia 2: AY150977.
H. INOKUMA ET AL.

UK5: AY150984, UK6: AY150985, France: AY150972, Illinois U.S.A.: U95297, North Carolina U.S.A.: AY069948, Oklahoma U.S.A.: AF178677, Ohio-Florida U.S.A.: U88563, South Africa: AF548631, ‘Candidatus Mycoplasma haemominutum’ strains UK 1: AY150980, UK 2: AY150981, Israel: AY150974, South Africa: AY150979, Birmingham U.S.A.: AF271154, California U.S.A.: U88564, Mycoplasma haemocanis strain Illinois: AF197337, Mycoplasma haemomuris: U82963, Mycoplasma suis: U88565, Mycoplasma ovis: AF338268 and Mycoplasma pneumoiae: M29061. The nucleotide sequences of the 16S rRNA gene of M. haemofelis detected from cats in this study have been deposited in the GenBank database under the following accession numbers as follows: Yamaguchi 4806; AY529629 and Yamaguchi 6287; AY529632. The accession numbers of the partial sequences of the 16S rRNA gene of ‘C. Mycoplasma haemominutum’ determined in this study were as follows: Yamaguchi 6167; AY529628, Yamaguchi 5091; AY529630, Yamaguchi 6424; AY529633, Yamaguchi 6532; AY529635, Yamaguchi 6571; AY529637, Yamaguchi 6584; AY529638, Yamaguchi 6683; AY529679, Yamaguchi 6763; AY529640, Hiroshima 6439; AY529634, Fukuoka 5884; AY529631 and Fukuoka 6566; AY529636.

Hemoplasma was detected in 18 (17.7%) out of 102 randomly selected cats by PCR screening. The healthy population (n=12) failed to amplify the target gene, while the positive ratio in currently ill cats was 20.0% (18 among 90 cats). Subsequent RFLP with HindIII yielded fragments of 76 and 117 bp for ‘C. Mycoplasma haemominutum,’ and no cut in M. haemofelis (Fig. 1). Digestion of the PCR products with HindIII revealed that 2 out of 18 positive cats were infected with M. haemofelis, and the remaining 16 with ‘C. Mycoplasma haemominutum’. One (Yamaguchi 6287) cat infected with M. haemofelis, showed typical clinical symptoms of feline hemobartonellosis, including hemolytic anemia, anorexia, jaundice and haematuria. But others did not show any signs of hemobartonellosis. Watanabe et al. reported that among 18 cats infected with hemoplasma in Japan, 12 cats (67%) were infected with M. haemofelis, 4 (22%) with ‘C. Mycoplasma haemominutum’, and 2 with both pathogens [18]. They used cats that showed clinical symptoms of hemoplasma infection. This may have caused the difference of the positive ratio in the two studies. As the pathogenesis of M. haemofelis is generally more severe than that of ‘C. Mycoplasma haemominutum’ [6, 20], the percentage of cats positive for M. haemofelis would be higher in symptomatic cats than in healthy or non-symptomatic cats.

Partial 16S rRNA gene sequences, approximately 500 bp in length were successfully determined for 13 of 18 hemoplasma-positive cats. The sequences of 2 isolates of M. haemofelis obtained from cats in Yamaguchi Prefecture were 100% identical with each other and showed percent identities of 98.3 to 99.8% with those of isolates from U.S.A., Europe, Australia, Israel and South Africa. The phylogenetic analysis showed that the 2 sequences of M. haemofelis determined in this study belong to the same

![Fig. 1. Results of PCR and RFLP from 5 hemoplasma-positive cats. (A) Screening by PCR produced a 193-bp fragment for ‘C. Mycoplasma haemominutum.’ (Lanes 1, 3 and 4) and a 170-bp fragment for M. haemofelis (Lanes 2 and 5). (B) Subsequent RFLP analysis with HindIII yielded fragments of 76 and 117 bp for ‘C. Mycoplasma haemominutum.’ (Lanes 1, 3 and 4) and no cut in M. haemofelis (Lanes 2 and 5).](image-url)
Fig. 2. Phylogenetic relationship of various hemoplasmas based on partial sequences of the 16S rRNA gene. The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 10% divergence. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown.
The transmission of feline hemoplasma was also found in a previous study on risk factors for hemoplasma infection in cats in Japan. 

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