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 *Hind*III 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*.

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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 examination or vaccination, 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- μ l reaction mixture containing 5 μ l 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 *Hind*III (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 rRNA 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,

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 pneumoniae*: 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 *Hind*III 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 *Hind*III 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 *Hind*III 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).

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.

Table 1. Risk factor analysis for hemoplasma infection in cats

Factors	Numbers of cats	Numbers of cats infected		
		<i>M. haemofelis</i>	' <i>C. M. hemominutum</i> '	Negative
Roaming				
In	36	0	1	35
Out	52	2	14	36
Unknown	14	0	1	13
<i>p</i> value (chi square)	0.0263			
FeLV infection				
Positive	4	1	2	1
Negative	63	1	8	54
Unknown	35	0	6	29
<i>p</i> value (chi square)	0.0263			

clade as *M. haemofelis* strains of other countries (Fig. 2). In contrast, 11 sequences of '*C. Mycoplasma haemominutum*' determined in this study showed more variable percent identities: 96.4 to 100% with those of isolates from U.S.A., European countries, Israel and South Africa; however, they all belong to the same clade of '*C. Mycoplasma haemominutum*' (Fig. 2).

Hemoplasma infections were more frequently detected in free-roaming cats than inside cats (Table 1, $P=0.0263$). This was also found in a previous study on risk factors for hemoplasma infection [8]. Transmission of feline hemoplasma by blood-sucking arthropods is considered by many to be the primary mode of transmission in cats [4], although such transmission has not been established experimentally except in the case of transmission of *M. haemocanis* by *Rhipicephalus sanguineus* [15]. It is also thought that transmission occur by cat-bite wounds [3]. The higher positive rate in free-roaming cats may reflect the higher possibility of exposure to blood-sucking arthropods or cat-bites. In addition, the status of FeLV infection was another significant risk factor for hemoplasma infection (Table 1, $p=0.0023$). There were no other significant differences in the frequency of hemoplasma infection according to age, sex, overall health condition, or FIV infection (data not shown). Although the reason why FeLV infection is related to hemoplasma infection in cats has not been determined yet, similar findings have been reported by others [7–9]. More recent reports have shown that both FeLV and FIV infections dramatically increase the possibility of hemoplasma infection [7, 9]. As only a small numbers of cats were used for the analysis in the present study, greater numbers of cats should be examined to clarify the relationship between virus infection and hemoplasma infection in cats in Japan.

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