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NOTE

Molecular study of feline hemoplasmas in free-ranging fishing cats (*Prionailurus viverrinus*) in Thailand

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Abstract

Feline hemoplasmas, consisting of *Mycoplasma haemofelis* (*M. haemofelis*), *Candidatus* Mycoplasma haemominutum (*Ca.* M. haemominutum), and *Candidatus* Mycoplasma turicensis (*Ca.* M. turicensis), cause feline infectious anemia and zoonoses. Using multiplex PCR and phylogenetic analysis based on 16S rRNA, 22 blood samples from fishing cats (*Prionailurus viverrinus*) living in Khao Sam Roi Yot National Park, Thailand were determined positive for *M. haemofelis* (13.6%)and *Ca.* M. haemominutum (22.7%). *M. haemofelis* and *Ca.* M. haemominutum infection can result severe anemia and asymptomatic, respectively. However, not all positive cases exhibit anemia symptoms. Future study of hemoplasma infection in wild felids is necessary for conservation and the preservation of public health in Thailand.

Key Words: feline hemoplasmas, fishing cat, *Prionailurus viverrinus*

Introduction

Feline hemoplasmas (previously known as *Haemobartonella felis*) are small (0.3–0.8 μm) and unculturable and lack cell-wall bacteria[29)]. The organisms attach to the cell surface of erythrocytes[29)]. Feline hemoplasmas were reclassified within genus *Mycoplasma* based on nucleotide analysis of 16S ribosomal (r) RNA gene sequences[17)]. The sequencing of the 16S

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rRNA gene is associated with the differentiation of feline hemoplasma isolates[26,31]. Feline hemoplasmas consist of *Mycoplasma haemofelis* (*M. haemofelis*)[17], *Candidatus* Mycoplasma haemominutum (*Ca.* M. haemominutum)[8], and *Candidatus* Mycoplasma turicensis (*Ca.* M. turicensis)[27,28]. Feline hemoplasmas are mainly transmitted by ectoparasites such as fleas and ticks[29]. General clinical signs of infection are lethargy, anorexia, dehydration, weight loss, mucosal pallor, anemia, tachypnoea, tachycardia, lymphadenopathy splenomegaly, and icterus[22,29]. Severe symptoms are found with *M. haemofelis* infection[29]. Feline hemoplasma infections have been reported in domestic cats from several countries[1,22].

Fishing cats (*Prionailurus viverrinus*) are one of nine native wild felid species in Thailand[19] and are listed as endangered on the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species[16]. While their distribution is discontinuous in Southeast Asia, northern India, and Sri Lanka[12], they are concentrated primarily in wetland habitats[12]. The expansion of human communities that overlap with the habitats of fishing cats is a significant threat[16] and has allowed interspecies transmission of feline hemoplasmas to occur between fishing cats and domestic cats. There is little information about feline hemoplasma infection in fishing cats, especially the nucleotide sequences used as the basis for analysis to determine molecular epidemiology. Therefore, the present study is focused on phylogenetic analysis to determine the genetic sequences of feline hemoplasma species identified in free-living fishing cats in southern Thailand.

Materials and methods

Ethylenediamine tetra-acetic acid (EDTA) and clotted blood samples were collected from 22 free-ranging fishing cats in Khao Sam Roi Yot National Park, Prachuap Khiri Khan Province, Thailand (Fig. 1) from February 2009 to January 2012. EDTA blood and serum samples were harvested and stored at −20°C until use. Profiles of the fishing cats, including gender, age, weight, date, location of capture, and condition of the animal, were recorded. The protocol of this study was approved by the Faculty of the Veterinary Science-Animal Care and Use Committee (FVS-ACUC) (Protocol No. MUVS-2009-05). Complete blood count (CBC) for each blood sample of the fishing cats was analyzed using the CELL-DYN 3700 (Abbott, Germany), and serum biochemistry was tested using the BS-300 Chemistry Analyzer (Mindray, China). Total DNA and total RNA were extracted from the EDTA samples using the DNeasy blood and tissue kit (QIAGEN, Germany) and the QIAamp viral RNA mini kit, respectively. Each sample was eluted with 50 µl of nuclease-free water. Specific primers for feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), feline herpesvirus (FHV), feline infectious peritonitis virus (FIPV), feline calicivirus (FCV), and *Toxoplasma gondii* (*T. gondii*) screening were designed according to previously reported directions[3,5,13,18,23].

DNA amplification and differentiation of the feline hemoplasma species (i.e., *M. haemofelis*, *Ca.* M. haemominutum, and *Ca.* M. turicensis) were performed by multiplex polymerase chain reaction (PCR) as described in a previous report[20]. Table 1 provides the specific primers for detection of the three feline hemoplasma species with the amplified 16S rRNA gene. The PCR product sizes were 190, 241, and 359 bp for *M. haemofelis*, *Ca.* M. haemominutum, and *Ca.* M. turicensis, respectively. The PCR reaction was carried out using the QIAGEN Multiplex PCR Kit (QIAGEN, Germany), 1X QIAGEN Multiplex PCR Master Mix, 0.4 µM of each primer, and 2 µl of template DNA. Nuclease-free water was added to make a final volume of 25 µl. The multiplex PCR cycling conditions were as follows: 95°C for 15 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The reactions were performed



Fig. 1. Map of sampling location for fishing cats in Thailand.

Table 1. Specific primer sequences from multiplex PCR for the detection of three feline hemoplasma species[a)]

Primer name	Primer sequence (5' to 3')	Target species
Mhf-F	GAGGGATAATTATGATAGTACTTCGTG	*M. haemofelis*
Mhf-R	CAATCTAGACATGTAGTATTCGGTG	
CMhm-F	TCTGATTGTAAAGTTCTTTTATTTAG	*Ca.* M. haemominutum
CMhm-R	TAATTCTAGACAAACGATATCTATC	
CMtc-F	GAAAAATTTGATGGTACCCTC	*Ca.* M. turicensis
CMtc-R	GCCGAAACACAAATCCCGAC	

[a)]reference number 20

using a DNA amplifier machine. The PCR products were resolved on 2% agarose gels and visualized under ultraviolet light.

Near-complete sequences of 16S rRNA gene of the feline hemoplasma species were sequenced from the positive samples. Species-specific primers for the pathogens followed previously reported designs[27,28)] (*M. haemofelis* forward primer: 5'-TCG

AACGGAYYTTGGTTTCG-3', reverse primer: 5'-C AAATGAATGTATTTTTAAATGCCCAC-3', *Ca.* M. haemominutum forward primer: 5'-AAGTCGAAC GAAGAGGGTTTACTC-3', reverse primer: 5'-TTW AATACGGTTTCAACTAGTACTTTCTCC-3', *Ca.* M. turicensis forward primer: 5'-GAACTGTCC AAAAGGCAGTTAGC-3' and reverse primer: 5'-A GAAGTTTCATTCTT GACACAATTGAA-3'). The primers amplified 1,309 bp, 1,354 bp, and 1,317 bp gene fragments of *M. haemofelis*, *Ca.* M. haemominutum, and *Ca.* M. turicensis, respectively. The PCR mixture contained 1X Dream Taq buffer, 0.2 mM dNTPs mix, 0.5 μM of each primer, 0.625 U Dream Taq DNA polymerase (Thermo Scientific, Lithuania), and 2 μl of template DNA. Nuclease-free water was added to make a total volume of 25 μl. The amplification conditions for *M. haemofelis* consisted of an initial denaturing step at 94°C for 5 min followed by 35 cycles at 94°C for 45 sec, 58°C for 45 sec, 72°C for 1 min, and a final extension step of 72°C for 10 min. The condition reactions for *Ca.* M. haemominutum and *Ca.* M. turicensis were similar to those for *M. haemofelis*, with the exception of the annealing step, which was optimized at 64°C for 45 sec and 60°C for 45 sec, respectively.

The DNA fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany). Each DNA fragment of *M. haemofelis*, *Ca.* M. haemominutum, and *Ca.* M. turicensis was ligated into the pGEM-T easy vector (Promega, USA). The calcium chloride method was used to transform the plasmid into competent *Escherichia coli* strain Top10F' (Invitrogen, USA). Transformants were selected in LB agar with X-Gal/IPGT and 100 μg/ml of ampicillin. Next, they were propagated in LB broth containing 100 μg/ml of ampicillin. Purified plasmids were prepared using the MiniPrep DNA preparation kit (QIAGEN, Germany) and sent to a capillary DNA sequencing laboratory (AITbiotech, Singapore) for nucleotide sequencing.

The near-complete nucleotide sequences were aligned using Clustal W, and the phylogenetic relationship of various hemoplasmas was constructed based on 16S rRNA gene sequences using the neighbor-joining method in the MEGA6 software[24]. The distances were evaluated by the maximum composite likelihood substitution model. The data sets used consisted of 1,000 bootstrap resamplings. All reference nucleotide sequences of hemoplasma species used for comparison were obtained from GenBank NCBI (http://www.ncbi.nlm.nih.gov/genbank).

Statistical analysis was performed using SPSS 18 for Windows (SPSS Inc., United States). Pearson's chi square was used to test the association of gender and feline hemoplasma infection. Results of CBC and biochemistry were expressed as mean ± standard deviation. Comparison of CBC and biochemistry data was examined by independent t test. Statistical significance was considered at $p < 0.05$.

Results

The 16S rRNA gene of feline hemoplasmas DNA was detected in five of the 22 (22.7%) samples. The results showed three *M. haemofelis* infections (13.6%) and five *Ca.* M. haemominutum infections (22.7%). All of the samples positive for *M. haemofelis* were co-infected with *Ca.* M. haemominutum. No samples were positive for *Ca.* M. turicensis. *M. haemofelis* and *Ca.* M. haemominutum infection can cause severe anemia and asymptomatic, respectively[29]. Co-infection with both these hemoplasmas is commonly found in wild felids[29]. Table 2 shows the overall results for feline hemoplasma determination by gender. No statistically significant difference in infection status ($p = 0.211$) was found for gender.

The samples were also determined negative for other organisms (FeLV, FIV, FCV, FHV, FIP, and *T. gondii*) by nucleic acid amplification and negative for blood parasites by microscopic examination. Table 3 presents the hematology and biochemistry values for the positive and negative groups. When comparing the positive and negative

Table 2. Positive results of feline hemoplasma in 22 free-ranging fishing cats using multiplex PCR

		Number of fishing cats	Number of positive fishing cats (%)			
			M. haemofelis[a]	*Ca.* M. haemominutum[b]	*Ca.* M. turicensis[c]	Co-infection[d]
Gender	Female	8	0 (0.00)	1 (4.55)	0 (0.00)	2 (9.1)
	Male	14	0 (0.00)	1 (4.55)	0 (0.00)	1 (4.55)
Total		22	0 (0.00)	2 (9.1)	0 (0.00)	3 (13.6)

[a]Positive results of *M. haemofelis* alone
[b]Positive results of *Ca.* M. haemominutum alone
[c]Positive results of *Ca.* M. turicensis alone
[d]Positive results of both *M. haemofelis* and *Ca.* M. haemominutum

Table 3. CBC values and serum biochemistry analysis in feline hemoplasma positive and negative groups

Variable	Positive			Negative			*p*-value
	Mean ± SD	Min[a]	Max[b]	Mean ± SD	Min[a]	Max[b]	
HCT (%)	40.18 ± 3.96	38	47	42.74 ± 15.22	33.2	52.8	0.379
WBC count (10^3/μl)	13.36 ± 5.54	7	15.2	13.78 ± 6.42	7.4	23.7	0.872
RBC count (10^6/μl)	6.62 ± 0.29	6.3	7	6.94 ± 2.51	5.5	9.28	0.301
HB (g/dl)	12.68 ± 0.86	12	14.1	13.73 ± 5.11	11.2	18.9	0.367
MCV (fl)	61.6 ± 3.51	58	67	62.07 ± 20.86	53	68	0.795
MCH (pg)	19.04 ± 1.05	18	20.2	19.71 ± 5.11	16.3	27.1	0.544
MCHC (g/dl)	31.64 ± 1.16	30	32.2	31.74 ± 10.86	29.8	41.1	0.94
PLT count (10^3/μl)	219.2 ± 109.26	116	404	246.33 ± 108.08	162	391	0.54
Total serum protein (mg%)	8.48 ± 0.99	7.5	9.9	9.5 ± 2.02	6.1	13.9	0.295
ALT (IU/L)	86.4 ± 37.11	55	141	105.91 ± 69.24	35	267	0.556
BUN (mg%)	25.74 ± 7.01	14	31.6	32.88 ± 10.75	16	57.3	0.181
CK (IU/L)	630.2 ± 628.45	297	1,740	508.24 ± 433.02	110	1,480	0.622
Creatinine (mg%)	1.45 ± 0.36	0.9	1.9	1.29 ± 0.42	0.5	2.09	0.454

[a]Minimum value, [b]Maximum value

groups, no significant differences were found in values for hematocrit (HCT), white blood cell (WBC) count, red blood cell (RBC) count, platelet (PLT) count, hemoglobin (HB), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total protein, alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine kinase (CK), and creatinine (Table 3).

Based on the 16S rRNA gene, the sequence identity matrix of this study was compared within isolates and between isolates from positive samples and reference samples from the GenBank database. Most feline hemoplasma sequences (n = 5) in the present study (FC 9, FC 10, FC 12, FC 18, and FC 21; accession numbers KU645931, KU645932, KU645933, KU645934, and KU645935, respectively) presented 97.1% to 99.1% similarity with isolates of *Ca.* M. haemominutum in domestic dogs, domestic cats, and wild felids, which includes isolates of domestic cats in Thailand (accession number EU285281). Three partial sequences of positive samples (FC 10, FC 12, and FC 21; accession numbers KU645928, KU645929, and KU645930, respectively) were closely related with the isolates of *M. haemofelis* (98.9% to 99.5%) in domestic cats in Thailand and showed 99.0% to 99.7% identity with those of domestic cats and wild felids in other geographic areas. The sequence similarity observed in positive *M. haemofelis* sequences in the present study ranged from 98.8% to 99.2%. Moreover, *M.*

haemofelis isolates in the positive fishing-cat samples had 99.1% to 99.5% identity with *M. haemocanis* isolates from dogs in the United States (accession number NR_074289), Italy (accession number GQ129119), Switzerland (accession number EF416568), and Japan (accession number AY529641) and a raccoon dog in Japan (accession number AB848714). *M. haemofelis* also showed high similarity (99.o% to 99.5%) with the human 16S rRNA gene sequence of *Mycoplasma* sp. in Brazil (accession number EU888930). The genetic relationship of feline hemoplasmas and other species of hemoplasmas was determined by phylogenetic tree analysis. Two distinct clusters of the near-complete 16S rRNA nucleotide sequences of positive samples were identified (Fig. 2). Five positive sequences (FC 9, FC 10, FC 12, FC 18 and FC 21) were contained in the *Ca.* M. haemominutum cluster. Three sequences (FC 10, FC 12 and FC 21) were grouped into the other cluster, which included both *M. haemofelis* and *M. haemocanis* isolates.

Discussion

Feline hemoplasmas have been isolated globally in domestic animals and wildlife and are an important cause of infectious anemia in felidae species[22]. They may cause chronic to severe anemia or asymptomatic infection[2,29]. In particular, *M. haemofelis* is a zoonotic pathogen that was detected in a human immunodeficiency virus (HIV)-infected case in Brazil[7]. Wildlife is a major reservoir of zoonotic pathogens and source of public health problems in humans and animals[15]. Wild felids have been suggested as important reservoir hosts of feline hemoplasmas[30], but free-roaming domestic animals such as feral dogs and cats can also transmit infectious diseases to wild animals[21]. Feline hemoplasma infections have been previously identified in wild felids[9,10,30]. In this study, the infection rate in fishing cats was lower than in domestic cats in Thailand (43 to 45%)[1,11]. This may be result of various host species, sample size, and location. All positive samples were infected with *Ca.* M. haemominutum. These results correlate with a previous suggestion that the prevalence of *Ca.* M. haemominutum is higher than that of other feline hemoplasmas[29]. Three positive fishing cat samples presented concurrent infection of *Ca.* M. haemominutum and *M. haemofelis*. Co-infection with feline hemoplasma species is generally found in free-ranging wild felids and domestic cats[1,11,29]. Conversely, the 16S rRNA gene of *Ca.* M. turicensis was not detected in the samples but has been reported in domestic cats in Thailand[20]. However, the fishing cats infected with feline hemoplasmas exhibited no clinical signs of anemia, such as pale mucus membranes, and had normal CBC values and serum biochemistry. It is possible that there is no association between feline hemoplasma infection and anemia, which is in agreement with previous observation[26]. Co-infection with both *Ca.* M. haemominutum and feline retroviruses, especially FeLV, can result in significant anemia[6]. Moreover, our study also indicated that the gender was not the risk factor. This contrasts with the point in previous reports that the gender was the factor for feline hemoplasma infection[22,25,29]. The difference may be affected by sample size.

The phylogenetic tree and sequence identity matrix based on 16S rRNA revealed two clusters in the present study. The majority cluster of feline hemoplasma infections in free-living fishing cats in Thailand was *Ca.* M. haemominutum. In the other cluster, *M. haemofelis* and *M. haemocanis* could not be distinguished. This finding agrees with other literatures[14,26]. Host-specific feline and canine hemoplasmas were considered because of unsuccessful experimental transmission of feline hemoplasmas in dogs[26]. On the other hand, *M. haemofelis* infection was found in Darwin's fox (*Lycalopex fulvipes*) in Chile[4]. To confirm, differentiation between *M. haemofelis* and *M. haemocanis* clusters using the RNA subunit of the RNase P gene had been suggested[26].

The source of infection in fishing cats is still

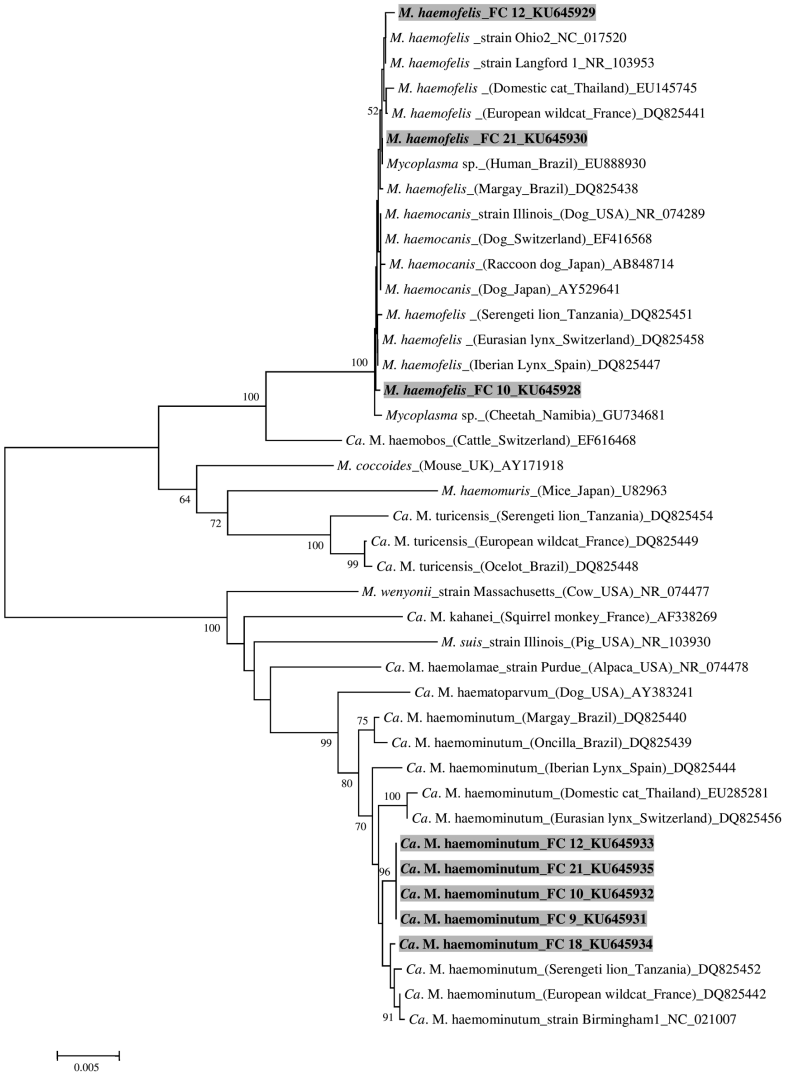


Fig. 2. Phylogenetic tree of the partial 16S rRNA sequences of feline hemoplasma species in free-ranging fishing cats and hemoplasma species from GenBank. The bold font represents isolates from the present study. Bootstrap values were calculated from 1,000 resamplings, and values over 50% are displayed. Percentages of bootstrap values were obtained on the nodes of the tree.

unknown but may be the result of cross transmission from domestic animals or infection from prey or vectors. Evidence from the surveillance of hemoplasma infection in other animals and vectors in the habitat of fishing cats may significantly support this hypothesis. Surveillance is necessary for the conservation of endangered species and the preservation of public health.

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