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

Parut Suksai¹, Siriporn Tangsudjai¹, Ladawan Sariya¹, Tatiyanuch Chamsai¹, Poonyapat Sedwisai¹, Suppakorn Patumrattanathan², Luxsana Prasittichai², Passanan Cutter³, Parntep Ratanakorn¹ and Nareerat Sangkachai¹,*

¹The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotics Animals, Faculty of Veterinary Science, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand
²Department of National Parks, Wildlife and Plant Conservation, Chatuchak, Bangkok 10900, Thailand
³Conservation Biology Program, University of Minnesota, 187 McNeal Hall, 1985 Buford Avenue, St. Paul, MN 55108, USA

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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. The organisms attach to the cell surface of erythrocytes. Feline hemoplasmas were reclassified within genus *Mycoplasma* based on nucleotide analysis of 16S ribosomal (r) RNA gene sequences. The sequencing of the 16S
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 \(-20^\circ\text{C}\) 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
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\(^{27,28}\) (\textit{M. haemofelis} forward primer: 5'-TCG

Table 1. Specific primer sequences from multiplex PCR for the detection of three feline hemoplasma species\(^a\)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mhf-F</td>
<td>GAGGGATAATTATGATAGTACTTCGTG</td>
<td>\textit{M. haemofelis}</td>
</tr>
<tr>
<td>Mhf-R</td>
<td>CAATCTAGACATGTAGTATTCGGTG</td>
<td></td>
</tr>
<tr>
<td>CMhm-F</td>
<td>TCTGATTGAAAAGTTTTATTTTAG</td>
<td>\textit{Ca. M. haemominutum}</td>
</tr>
<tr>
<td>CMhm-R</td>
<td>TAATTCTAGACAAACGATCTATC</td>
<td></td>
</tr>
<tr>
<td>CMtc-F</td>
<td>GAAAAATTTGATGCTCCCTC</td>
<td>\textit{Ca. M. turicensis}</td>
</tr>
<tr>
<td>CMtc-R</td>
<td>GCCGAAACACAAATCCCGAC</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)reference number 20

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

AACGGAYYTGGTTTGGTTTCG-3', reverse primer: 5'-C
AAATGAATGTATTTTTAAATGCCCAC-3', Ca. M. haemominutum forward primer: 5'-AAGTCGAAC
GAAGGGTTTACT-3' and reverse primer: 5'-TTW
AATACGGTTTCAACTAGTACTTTCTCC-3', Ca. M. turicensis forward primer: 5'-GAACGTGCTCC
AAAAGGCAGTTAGC-3' and reverse primer: 5'-A
GAAGTTTCATTCTTGACACAATTGAA-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. 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

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. Co-infection
with both these hemoplasmas is commonly found
in wild felids. 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
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.

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

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number of fishing cats</th>
<th>M. haemofelis</th>
<th>Ca. M. haemominutum</th>
<th>Ca. M. turicensis</th>
<th>Co-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>8</td>
<td>0 (0.00)</td>
<td>1 (4.55)</td>
<td>0 (0.00)</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>0 (0.00)</td>
<td>1 (4.55)</td>
<td>0 (0.00)</td>
<td>1 (4.55)</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>0 (0.00)</td>
<td>2 (9.1)</td>
<td>0 (0.00)</td>
<td>3 (13.6)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive Mean ± SD</th>
<th>Mina</th>
<th>Maxb</th>
<th>Negative Mean ± SD</th>
<th>Mina</th>
<th>Maxb</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT (%)</td>
<td>40.18 ± 3.96</td>
<td>38</td>
<td>47</td>
<td>42.74 ± 15.22</td>
<td>33.2</td>
<td>52.8</td>
<td>0.379</td>
</tr>
<tr>
<td>WBC count (10³/μl)</td>
<td>13.36 ± 5.54</td>
<td>7</td>
<td>15.2</td>
<td>13.78 ± 6.42</td>
<td>7.4</td>
<td>23.7</td>
<td>0.872</td>
</tr>
<tr>
<td>RBC count (10⁶/μl)</td>
<td>6.62 ± 0.29</td>
<td>6.3</td>
<td>7</td>
<td>6.94 ± 2.51</td>
<td>5.5</td>
<td>9.28</td>
<td>0.301</td>
</tr>
<tr>
<td>HB (g/dl)</td>
<td>12.68 ± 0.86</td>
<td>12</td>
<td>14.1</td>
<td>13.73 ± 5.11</td>
<td>11.2</td>
<td>18.9</td>
<td>0.367</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>61.6 ± 3.51</td>
<td>58</td>
<td>67</td>
<td>62.07 ± 20.86</td>
<td>53</td>
<td>68</td>
<td>0.795</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.04 ± 1.05</td>
<td>18</td>
<td>20.2</td>
<td>19.71 ± 5.11</td>
<td>16.3</td>
<td>27.1</td>
<td>0.544</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>31.64 ± 1.16</td>
<td>30</td>
<td>32.2</td>
<td>31.74 ± 10.86</td>
<td>29.8</td>
<td>41.1</td>
<td>0.94</td>
</tr>
<tr>
<td>PLT count (10³/μl)</td>
<td>219.2 ± 109.26</td>
<td>116</td>
<td>404</td>
<td>246.33 ± 108.08</td>
<td>162</td>
<td>391</td>
<td>0.54</td>
</tr>
<tr>
<td>Total serum protein (mg%)</td>
<td>8.48 ± 0.99</td>
<td>7.5</td>
<td>9.9</td>
<td>9.5 ± 2.02</td>
<td>6.1</td>
<td>13.9</td>
<td>0.295</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>86.4 ± 37.11</td>
<td>55</td>
<td>141</td>
<td>105.91 ± 69.24</td>
<td>35</td>
<td>287</td>
<td>0.556</td>
</tr>
<tr>
<td>BUN (mg%)</td>
<td>25.74 ± 7.01</td>
<td>14</td>
<td>31.6</td>
<td>32.88 ± 10.75</td>
<td>16</td>
<td>57.3</td>
<td>0.181</td>
</tr>
<tr>
<td>CK (IU/L)</td>
<td>630.2 ± 628.45</td>
<td>297</td>
<td>1,740</td>
<td>508.24 ± 433.02</td>
<td>110</td>
<td>1,480</td>
<td>0.622</td>
</tr>
<tr>
<td>Creatinine (mg%)</td>
<td>1.45 ± 0.36</td>
<td>0.9</td>
<td>1.9</td>
<td>1.29 ± 0.42</td>
<td>0.5</td>
<td>2.09</td>
<td>0.454</td>
</tr>
</tbody>
</table>

a) Minimum value, b) Maximum value

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Feline hemoplasmas detected in fishing cats

*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 (99.6% 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. They may cause chronic to severe anemia or asymptomatic infection. In particular, *M. haemofelis* is a zoonotic pathogen that was detected in a human immunodeficiency virus (HIV)-infected case in Brazil. Wildlife is a major reservoir of zoonotic pathogens and source of public health problems in humans and animals. Wild felids have been suggested as important reservoir hosts of feline hemoplasmas, but free-roaming domestic animals such as feral dogs and cats can also transmit infectious diseases to wild animals. Feline hemoplasma infections have been previously identified in wild felids, but there was 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. Co-infection with both *Ca. M. haemominutum* and *feline retroviruses*, especially FeLV, can result in significant anemia. 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. The source of infection in fishing cats is still unknown.

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. Host-specific feline and canine hemoplasmas were considered because of unsuccessful experimental transmission of feline hemoplasmas in dogs. On the other hand, *M. haemofelis* infection was found in Darwin’s fox (*Lycalopex fulvipes*) in Chile. To confirm, differentiation between *M. haemofelis* and *M. haemocanis* clusters using the RNA subunit of the RNase P gene had been suggested.
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.

**Acknowledgements**

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