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Prevalence of Feline Hemotropic Mycoplasmas Among Feral Cats in Korea by use of a PCR assay

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Abstract : We determined the prevalence of feline hemotropic mycoplasma species including '*Candidatus* Mycoplasma haemominutum', *Mycoplasma haemofelis*, and '*Candidatus* Mycoplasma turicensis' in naturally infected feral cats in Jeonju, Korea. Forty six feral cats were evaluated by PCR assay targeting the 16S rRNA gene sequence. Nine cats (19.6%) were positive for '*Candidatus* Mycoplasma haemominutum', 2 cats (4.3%) were positive for '*Mycoplasm a haemofelis*', and 1 cat (2.2%) was infected with both '*Candidatus* Mycoplasma haemominutum' and *Mycoplasma haemofelis*. '*Candidatus* Mycoplasma turicensis' was undetected. Partial 16S rRNA gene sequences of *Mycoplasma haemofelis* were closely (> 96%) related to those from other countries. The amplification of hemoplasma DNA in these samples confirmed the presence of '*Candidatus M. haemominutum*' and *M. haemofelis* in Korea.

Key words **:** Hemotropic *mycoplasma*, feral cat, Korea, PCR

Introduction

Haemotropic mycoplasmas, previously known as *Haemobartonella* and *Eperythrozoon*, are small size (0.3-0.8 μm), non-culturable bacteria that infect the cytoplasmic membrane of erythrocyte often inducing hemolytic anemia (18,30). Infected cats occasionally develop thrombocytopenia, pyrexia, and jaundice (23). The disease is known as feline infectious anemia due to the associated signs.

Two strains (Ohio and California) of *Haemobartonella felis* (*H. felis*) were considered causal agents of feline haemobartonellosis (9). However, a study showed that *H. felis* is genetically more closely related to mycoplasma based on the 16S rRNA gene sequence (9). The two strains have been reclassified as *Mycoplasma haemofelis* (*M. haemofelis*) and '*Candidatus* Mycoplasma haemominutum' ('*Candidatus M. haemominutum*'), respectively. Recently, '*Candidatus* Mycoplasma turicensis' ('*Candidatus* M turicensis') was detected in Switzerland (32) and, subsequently, in cats from Australia, the UK, and South Africa (33).

Cats infected with *M. haemofelis* often have hemolytic anemia during acute or chronic infection with concurrent disease (1,11,12). Infection with '*Candidatus M. haemominutum*' and '*Candidatus* M turicensis' may result in a decrease of red blood cell parameters, but typically anemia is not found in the absence of concurrent disease (10,11,32).

Diagnostic methods of haemotropic mycoplasma infection include microscopic examination of blood smear and polymerase chain reaction (PCR). Microscopic identification of microorganisms adhered on the cytoplasmic membrane of red blood cells on Wright-Giemsa-stained peripheral blood smears is diagnostic of haemotropic mycoplasma infection in cats (8,11,20). However, this method is very insensitive for detecting haemotropic mycoplasma (20,30). On the other hand, PCR analysis is more sensitive and specific for the detection of three mycoplasmas than direct microscopic examination of blood smear (11,30). A number of studies on PCR amplification of 16S RNA gene sequences are reported for *Mycoplasma* spp. PCR analyses can discriminate between '*Candidatus M. haemominutum*', *M. haemofelis* and '*Candidatus* turicensis'. Few studies have reported PCR analysis for the detection of hemotropic mycoplasma species in Korea. Besides, infection of '*Candidatus* M turicensis' has not been previously reported in domestic cats from Korea, to the best of our knowledge.

The aim of the present study was to assess the prevalence of the three hemotropic mycoplasma species in feral cats by PCR assay in Jeonju, Korea.

Materials and Methods

Cats

Between June and September 2014, 46 feral cats were selected from those admitted to the Trap-Neuter-Return (TNR) program in Jeonju, Korea. All selected cats were Korean Domestic Shorthair.

Blood samples and hematological evaluation

The blood samples were collected in EDTA-anticoagulant tubes for complete blood counts (CBC). CBC was performed with the ABC vet counter (ABX, Hematologie, Montpeillier, France). Packed cell volume was measured by

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manual micro-hematocrit tube centrifugation. Blood smears were prepared for microscopic examination. For the PCR test, the remaining sample of whole blood in the EDTA tube was stored at −20°C until DNA extraction.

PCR amplification of the 16S rRNA fragments

PCR and sequence analysis were performed as below: 1) screening mycoplasma infection using universal primer, 2) species-specific PCR was done to identify *M. haemofelis*, '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' from the positive samples of procedure 1), and 3) sequence analysis from the PCR amplicons.

DNA was extracted from 200 μL of whole blood using the G-spin™ Total DNA Extraction Kit (Intron biotechnology, Korea) according to the manufacturer's instructions. Purified DNAs were stored at −20°C and used as the template for PCR amplification.

The 16S rRNA gene was amplified using universal primer fHf1 (forward, 5'ACG CGT CGA CAG AGT TTG ATC CTG GCT3') and rHf2 (reverse, 5'CGC GGA TCC GCT ACC TTG TTA CGA CTT3') (20,32). The PCR was performed with 1 μL of template DNA in a 25 μL reaction mix containing DnaUs Taq DNA polymerase, magnesium, deoxynucleotide triphosphate (dNTPs), glycerol (LeGene Biosciences, San Diego, CA, USA) and 2 μL of 10 μM of each primer. The cycling conditions consisted of an initial denaturation of 2 min at 94°C, followed by 40 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 50°C, 2 min extension at 72°C, and 10 min final extension at 72°C.

Samples with approximately 1,400 to 1,500 base pair (bp) amplification product in the above PCRs were subjected to a PCR with species-specific primer for 16S rRNA amplification. The PCR was performed with 3 μL of template DNA in a 25 μL reaction mix and 1.5 μL of 10 μM of each primer. Primers were used targeting 16S rRNA gene: CA-B2 (5'CTG GGA AAC TAG AGC TTC GCG AGC3'), 00CR-rl (5'ATG GTA TTG CTC CAT CAG ACT TTC G3'), and OH-Ok (5'ATG CCC CTC TGT GGG GGA TAG CCG3') that produced a 273 base pair (bp) product from *M. haemofelis* and 202 bp amplicon from '*Candidatus M. haemominutum*', respectively (35). The thermal program comprised 94°C for 5 min; 35 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec. '*Candidatus* Mycoplasma haemominutum' DNA was used as a positive control and a reagent negative control (reagents with no DNA) was included in each PCR run to monitor contamination (35).

For '*Candidatus* M turicensis', the PCR was performed using Cmt1 (5'GAA CTG TCC AAA AGG CAG TTA GC3') and Cmt2 (5'AGA AGT TTC ATT CTT GAC ACA ATT GAA3') yield 1,317 bp product of the 16S RNA gene (32). The thermal program comprised 94°C for 2 min; 40 cycles of 94°C for 45 sec, 51°C for 45 sec, and 72°C for 2 min; and finally, 72°C for 10 min.

PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and photographed using the CoreBio i-MAX Gel Image Analysis System (CoreBio System, Seoul, Korea).

Nucleotide sequencing of amplified nucleotides

In order to confirm the PCR procedure and phylogenetic comparison, PCR sequence was analyzed from the PCR positive samples. PCR amplicons for subsequent sequencing were purified by the PureLink™ Quick Gel Extaction Kit (Invitrogen™, Germany) according to the manufacturer's instructions. Purified DNAs and primers mentioned above (fHf1, rHf2) were sent to Genotech (Daejeon, Korea) for sequencing.

Phylogenetic and similarity calculation analysis

Sequence homology searches were made using the National Center for Bio-technology Information (National Institute of Health) BLAST network service.

Results

Hematological variables

A total of 46 blood samples were analyzed of which eight cats were anemic and 38 cats were non-anemic. The CBC results in cats with positive PCR were shown in Table 1. The RBC count, hemoglobin concentration, PCV, MCV, MCHC of the 46 cats were described. Of the eight cats positive for '*Candidatus M. haemominutum*' alone, two cats were ane-

Table 1. Hematologic variables of 46 feral cats categorized by feline hemoplasma infection

		WBC ($\times 10^6$/μL) (17.0-22.0)	RBC ($\times 10^9$/μL) (5.80-10.70)	Hgb (g/dL) (9.3-15.6)	PCV (%) (25-47)	MCV (fl) (41-51)	MCHC (g/dl) (31.0-35.0)	RDW (%) (17.0-22.0)	Platelet ($\times 10^6$/μL) (17.0-22.0)
Negative (n = 36)	Mean	18.83	7.55	11.50	31.54	45.31	33.93	17.29	256.06
	SD	11.00	1.39	1.75	5.03	3.32	1.55	0.83	139.54
CMhm (n = 8)	Mean	22.9	7.17	10.91	30.50	45.25	34.23	17.84	299.5
	SD	10.51	1.90	2.31	7.02	3.03	2.92	1.57	155.34
Mhf (n = 1)		14.1	6.19	10.5	28	53	31.9	17.6	214
Mhf and CMhm (n = 1)		17.1	7.84	10.9	28	40	35.1	16.3	152

CMhm, '*Candidatus* Mycoplasma haemominutum'
Mhf, *Mycoplasma haemofelis* (*M. haemofelis*)

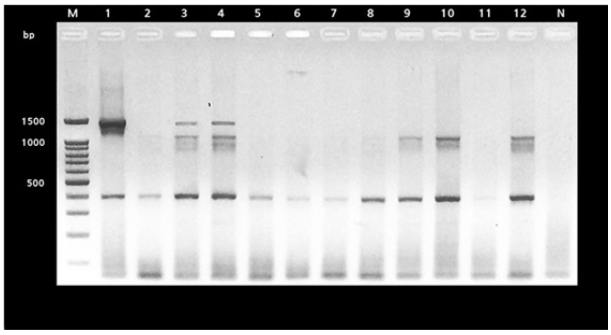

Fig 1. Amplification of bacterial 16S rRNA with primer set, fHf1 and rHf2. Lane M: molecular size marker; Lane N: reagent negative control, Lane 1, 3, and 4: approximately 1500-bp DNA fragments of 16S rRNA.

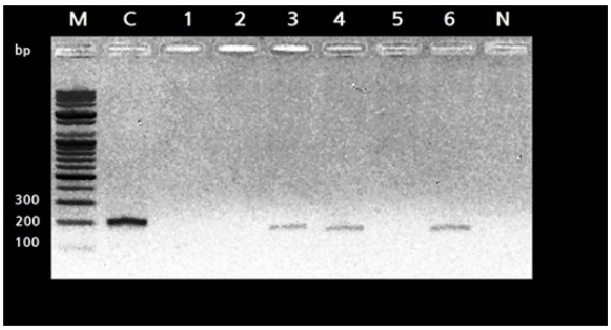

Fig 2. PCR amplicons of feline hemoplasma 16S rRMA gene. Lane M; molecular size marker. Lane C; '*Candidatus* Mycoplasma haemominutum' positive control: GenBank accession no. U88564, Lane N; reagent negative control, Lane 3, 4, and 6; 202-bp DNA fragment.

mic and six cats were non-anemic. A cat positive for *M. haemofelis* alone was non-anemic. A cat positive for both species (*M. haemofelis* and '*Candidatus M. haemominutum*') was non-anemic.

Pathogen identification by microscopic examination

All blood samples were examined on microscopy. No organisms were found on the peripheral blood smear with Diff-Quik stain.

PCR with universal primers and species-specific primers

Using the universal primers fHf1 and rHf2, we were able to amplify an approximately 1,500 bp fragment of the 16S rRNA indicating that the DNA preparations were suitable for amplification. Hemoplasma-like DNA was detected in 10/46 of blood samples (Fig 1). PCR using the species-specific primer set identified 9 samples positive for '*Candidatus M. haemominutum*' (Fig 2). PCR amplicons those were positive for the universal primers set but negative for species-specific primers set were purified and sent for DNA sequencing to Genotech (Daejeon, Korea).

Phylogenetic and similarity homology of amplified nucleotide sequences

Partial sequences of 16S rRNA gene of *M. haemofelis* in this study showed 96-98% identity with those from the published sequences of 7 different isolates from different geographical origins. (GenBank accession numbers: *M. haemofelis* strains UK: NR103953.1, Ohio: CP002808.1, Yamaguchi: AY-529632.1, UK6: AY150985.1, Australian: AY150976.1, Porto Alegre: EU930823.1, French: AY150972.1). The phylogenetic tree of *M. haemofelis* 16S rRNA gene was presented in Fig 3.

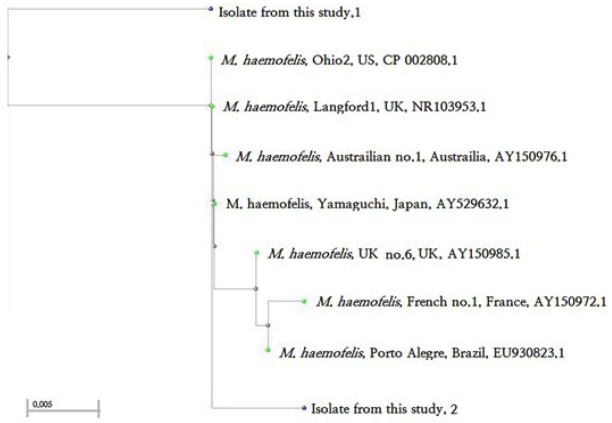

Fig 3. Phylogenetic relationship of *Mycoplasma haemofelis* based on partial sequences of the 16S rRNA gene. The neighbor-joining method was used to construct the phylogenetic tree. GenBank accession numbers were shown.

Discussion

We investigated the prevalence of hemoplasmas in 46 feral cats in Jeonju, located in southwestern area in Korean peninsula. The amplification of hemoplasma DNA in these samples confirmed the presence of natural infection of '*Candidatus M. haemominutum*' (19.6%) and *M. haemofelis* (4.3%) in this area. '*Candidatus* M. *turicensis*' was not detected in this study. A previous study from feral cats in Sung-Nam city, located in central area in Korea reported the prevalence of '*Candidatus M. haemominutum*' and *M. haemofelis* of 15.7% and 9.7%, respectively (35). The prevalence of '*Candidatus M. haemominutum*' was lower than that of the present study, whereas *M. haemofelis* was higher than previous study. Geographical variation, different sample number, retroviral infection status, gender ratio, different strain, different types of vector and age might have affected this difference. It is reported that male cats, FeLV /FIV infected cats were at higher risk for feline hemoplasma infection (19). Larger-scaled study considering these variables should be done for a further feline hemoplasma prevalence study.

Most of the prevalence studies worldwide reported that '*Candidatus M. haemominutum*' was the most common hemoplasma, with lower prevalence of '*Candidatus* M turicensis' and *M. haemofelis* (25). The prevalence of hemoplasmas infection in our samples was similar to that reported worldwide in feral cats. The prevalence worldwide ranged from 8.5% to 23.2% for '*Candidatus M. haemominutum*', from 2% to 26% for *M. haemofelis*, and from 2.7% to 6.7% for '*Candidatus* M turicensis' (7,15,16,18,19,22,25,26,30,31). We also detected co-infection of '*Candidatus M. haemominutum*' and *M. haemofelis* in one cat included in the present

study. The prevalence of co-infection reportedly ranges from 2.3% to 6.5% (16,19,28). The major factor with the most crucial impact on hemoplasmosis prevalence was not determined due to lack of information on the environmental circumstance of cats surveyed in previous studies. Differences in prevalence among countries can be explained by geographical variation, such as climate, vector distribution, and the cat population surveyed (22). In addition, direct comparisons of prevalence results are of limited value because of differences in subject number, health status, inclusion criteria, statistical methods, stage of infection, or the combinations, resulting in differences between studies (23).

A high prevalence of hemoplasmas in feral cats is typical as outdoor access is a recognized risk factor for infection (22). A study in Spain corroborated that outdoor access is a risk factor for infection (OR = 3.8) (21). In the US, the prevalence of hemoplasmas infection between feral cats (19) and client-owned cats (16, 24) was not significantly different. On the other hand, in Italy, the prevalence of hemoplasmas infection of feral cats in the TNR program (22) was higher than that of client-owned cats (17). Similarly, in a study from the US, the hemoplasma prevalence was 19.7% in cats with outdoor access but 3.6% in cats not allowed outdoors (13). One of the major limitations of this study was lack of data on prevalence among client-owned cats in Korea. However, interestingly, the 18.5% of feral cats had been adopted by TNR program in Korea in 2015, and the adoption rate is increasing now according to the animal protection management system in Korea (http://www.animal.go.kr/portal_rnl/index.jsp). It is hard to state feline hemoplasmas are absent in Korean cats based on this study. Clinicians must also be aware that feline hemotropic mycoplasmas are resident bacteria in Korea.

The partial sequences of 2 isolates of *M. haemofelis* obtained from cats in the study showed 96 to 98% identity with those of isolates from the published sequences of 7 different isolates from other countries. Although we did not perform full-length sequencing of isolates from this study, isolate (GenBank accession number; Korea: EF198142) of a prevalence study of feral cat in Korea (35) differed from our finding. This suggests that there could be several variants of *M. haemofelis* in Korea as well as in other countries (6,15,28).

Two samples had an approximately 1,300 to 1,500 bp amplification fragment with the universal primers indicating priming of most eubacteria with these universal primer sites (20). We used species-specific primers sets in order to differentiate species as well as identify co-infection, because it is difficult to diagnose co-infection of hemoplasmas using PCR with genus-specific primers. However, the species-specific primer set (00CR-rl, OH-Ok1) did not amplify the expected 273-bp fragment of the 16S rRNA gene. The amplification capacity of blood-derived nucleic acid samples can be dramatically reduced or blocked by PCR inhibitory substances including heme and lactoferrin (4). IgG present in human plasma was identified as a major inhibitor of diagnostic PCR in blood (3). Inhibition can arise from other endogenous sources, including eubacterial DNAs. Eubacterial DNAs may reduce the effective application of PCR (34). Eubacaterial DNAs including *Eperythorzoon suis, Mycoplasma genitalium,* and *Bartonella bacilliformis* are expressed on approximately 1500-bp using universal primers fHf1 and rHf2 (20). Thus, a possible cause of failure to amplify the 16S rRNA may be due to eubacaterial DNA.

By microscopic examination, organisms were not detected in any blood smear in this study. A study on 30 client-owned cats reported positive microscopic identification of only two of the nine PCR-positive samples (8). In a study on experimental inoculation of hemotropic mycoplasma, the organisms were not visible for 0-7 days, but after eight days of intravenously inoculation with whole blood (30). This discrepancy in microscopic observation of organisms can be explained by the lack of a readily identifiable bacteremia in latent and chronic infection, the rapid loss of bacteremia at the time of onset of clinical signs in acutely infected cats, and disappearance of infected RBCs from circulation in as little as 2 hours (14,29). Also, microscopy as a diagnostic procedure has many drawbacks since pathogens may be confused with artifacts or lost after EDTA tube collection of blood (5).

M. haemofelis infected cats may develop clinical signs of diseases, including fever, inappetence, lethargy, and anemia (2,9), and hemolysis is primarily extravascular and results in a severe regenerative anemia (2). However, in chronically infected cats, bacteremia is low or absent and the cat may be asymptomatic. In the present study, two cats testing positive for *M. haemofelis* were not anemic. Additionally, a prevalence study of 260 feral cats in Italy indicated that mean hematocrit value (SD) of *M. haemofelis* infected cats and hemoplasma-noninfected cats was 24.8 (3.1) and 25.5 (4.4), respectively (22). In a follow-up study, a cat infected with *M. haemofelis* remained healthy throughout despite FeLV coinfection (31). Thus, there is no association between PCR positive results and initiation of disease (2). These discrepancies are also due to a variety of pathogenicities, with some isolates consistently inducing hemolytic anemia whereas others with few noticeable clinical signs (27). In addition, the stage of infection when sampled cannot be determined. Such cats might be carriers for the microorganisms and could manifest clinical signs including hemolytic anemia and lethargy on first infection (35). Because generalized prediction of disease outcome following natural hemoplasma infection cannot be made, PCR results should be interpreted in conjunction with clinical signs and hematological findings (27,35).

Conclusively, clinicians must be aware that feline hemotropic mycoplasmas are resident bacteria in Korea. In addition, because generalized prediction of disease outcome following natural hemoplasma infection cannot be made, PCR results should be interpreted in conjunction with clinical signs and hematological findings.

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