AGRICULTURE AND NATURAL RESOURCES

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Research article

Molecular detection of *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis' of stray cats residing in Bangkok monasteries, Thailand

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Article Info

Article history:
Received 17 December 2020
Revised 23 April 2021
Accepted 28 April 2021
Available online 30 June 2021

Keywords:
Bangkok,
'*Candidatus* Mycoplasma haemominutum',
'*Candidatus* Mycoplasma turicensis',
Mycoplasma haemofelis,
Stray cats

Abstract

Feline hemoplasmosis, an important disease, causes anemia and fatality in felids worldwide. It is caused by the hemotropic bacterial parasites: *Mycoplasma haemofelis* (*Mhf*), '*Candidatus* Mycoplasma haemominutum' (*CMhm*) and '*Candidatus* Mycoplasma turicensis' (*CMt*). In Thailand, many stray cats are frequently seen in monasteries. The current study investigated the infection rate and risk factors associated with hemoplasma infections of stray cats in Bangkok monasteries, Thailand. Polymerase chain reaction was used for detection of *Mhf*, *CMhm* and *CMt* in 1,488 samples from stray cats randomly collected in 50 districts of Bangkok. Risk factors associated with feline hemoplasmosis were also collected and analyzed for correlation with infection. Out of the 1,488 stray cats, 16.1% (239/1,488), 24.5% (365/1,488) and 1.6% (23/1,488) were infected with *Mhf*, *CMhm* and *CMt*, respectively. Mixed infections with two or three hemoplasma species were found in 3.9% (58/1,488) of cats. The risk factor analysis indicated that sex, age, external parasite infestation, environmental conditions and number of cats per monastery were significantly ($p < 0.05$) associated with feline hemoplasma infections. Feline hemoplasmas were found in 29.4% (129/439) of the monasteries sampled from the 50 districts. In the 29.4% of hemoplasmas-positive monasteries, *CMhm* (100%), *Mhf* (96%) and *CMt* (34%) were identified. The results revealed that feline hemoplasmas were commonly found among stray cats in Bangkok, suggesting the possibility of these animal being a vector for the spread of diseases among outdoor cats.

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https://doi.org/10.34044/j.anres.2021.55.3.12

Introduction

Hemoplasmas are hemotropic bacterial parasites without a cell wall that can cause anemia in cats and other felids throughout the world (Krengel et al., 2013). Feline hemoplasma is evidently transmitted through biting wounds associated with flea infestations and via direct contact (Grindem et al., 1990; Willi et al., 2005; Tanahara et al., 2010). Three major hemoplasmas have been reported: *Mycoplasma haemofelis* (*Mhf*, formerly *Haemobartonella felis*), '*Candidatus* Mycoplasma haemominutum' (*CMhm*) (Foley and Pederson, 2001) and *Candidatus* Mycoplasma turicensis' (*CMt*) (Willi et al., 2005). To date, it has not been possible to culture these bacterial parasites *in vitro*. Polymerase chain reaction (PCR) techniques for the detection of feline hemoplasma infections have been developed (Jensen et al., 2001) and are now widely used for both diagnosis and phylogenetic studies. Phylogenetic differentiation of hemoplasma species based on 16S rRNA has been successfully developed and used in evolutionary studies (Tasker et al., 2003). However, there are limited numbers of studies on hemoplasma infections in Thailand.

Abandoned cats are a major problem in many countries worldwide including Thailand, as these cats have no owners and so do not receive general health care, vaccination, deworming or ectoparasite control. Importantly, they are a source of vector-borne diseases, including infectious bacterial and viral diseases, which are veterinary problems as well as a zoonotic problem to pet animals in communities (Do et al., 2020). In Thailand, colonies of stray or semi-domesticated cats can be seen in public areas and especially in monasteries where the animals have access to food and a safe place to stay. Bangkok, in particular, has high numbers of stray animals, especially in monasteries due to economic, cultural and religious factors (Food and Agriculture Organization of the United Nations (FAO), 2011). The representative population in the current study was stray cats in Bangkok monasteries that were living and roaming in public places such as monasteries, public parks, schools, fresh markets and along roadsides (Mitmoonpitak and Tepsumethanon, 1998; Do et al., 2020). Stray cats in monasteries are potential reservoirs for vector-borne pathogens including hemoplasmas due to the lack of health care. Therefore, this study aimed to investigate the prevalence of *Mhf*, *CMhm* and *CMt* infections of stray cats in Bangkok using PCR. In addition, the risk factors associated with hemoplasma infections of the stray cats were analyzed and evaluated.

Materials and Methods

Study areas

The study was undertaken in the metropolitan area of Bangkok. Of the 439 monasteries located in 50 districts of Bangkok, 140 monasteries were randomly selected based on the records of cats in each monastery. At least three monasteries in each district were randomly selected and 10 feline samples per monastery were randomly collected, where a district contained only 1 or 2 monasteries, 30 or 15 cats, respectively, were sampled from each monastery.'

Samples and data collection

In total, 1,488 jugular blood samples (each 3–5 mL) were randomly collected from the sampled stray cats living in the monasteries. An aliquot of each blood sample was preserved with sodium citrate and ethylene diamine tetraacetic acid (EDTA) or PCR, and a thin blood smear made. A questionnaire was designed to collect data on the sampled cats including age, gender, number of cats per monastery and external parasites. Habitat conditions of the cats were observed based on their feeding hygiene and resting and defecating areas and categorized as good, fair or poor. The record of cats per monastery was categorized as unclear (no obvious information), low (1–10 cats/monastery), medium (11–20 cats/monastery), and high (> 20 cats/monastery). Any external parasites found were collected and identified based on their morphological taxonomy**.** All work in the study met the International Guiding Principles for Biomedical Research Involving Animals (the Council for International Organizations of Medical Sciences (CIOMS)). This study was approved by the Ethics Committee of Kasetsart University, Bangkok, Thailand (Approval no. ACKU64-VET-025).

Microscopic examination

Blood smears were stained using Modified Giemsa and examined under a light microscope at 100× magnification. External parasites were rinsed in water and examined using a stereo microscope.

DNA extraction

A sample (200 µL) of the whole blood was used for genomic DNA extraction using a Genomic DNA Purification Kit (Gentra Puregene Blood Kit; Qiagen; USA) according to the manufacturer's instructions.

Identification of hemoplasmas using polymerase chain reaction

Polymerase chain reaction-restriction fragment length polymorphisms detection of M. haemofelis and 'Candidatus M. haemominutum'

Primers were used that targeted the 16S rRNA gene for *Mhf* and *CMhm* as designed by Jensen et al. (2001) and Tasker et al. (2003). The forward primer (Hf-F, 5'-ACG AAA GTC TGA TGG AGC AAT A-3') and reverse primer (Hf-R, 5'-ACG CCC AAT AAA TCC GRA TAA T-3') produced amplicons of 170 bp of *Mhf* and 193 bp of *CMhm*. Briefly, 25 µL of the PCR mixture contained 1X PCR buffer, 2.5 mM $MgCl_2$, 10 *p*Mol of each primer, 0.2 mM dNTP each, 2.5 U of *Taq* DNA polymerase (Invitrogen; USA) and approximately 1 µg of genomic DNA template. PCR amplification was performed using a Px2 thermal cycler (Thermo Scientific; USA) with 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 30 s, followed by 72°C for 3 min. PCR products were detected in 1.5% agarose gel electrophoresis. Restriction fragment length polymorphisms (RFLPs) were investigated to differentiate between *Mhf* and *CMhm*. The positive samples were incubated with endonuclease enzyme HindIII (Fermentas; USA) at 37°C for 12 hr, revealing a 170 bp of undigested PCR product of *Mhf* and two fragments of 76 and 117 bp for *CMhm*.

Polymerase chain reaction detection of hemoplasmas

PCR for the detection for hemoplasma 16S rRNA was used to provide longer PCR products (approximately 557–595 bp) than the primer pair for the amplification of *Mhf* and *CMhm* which generates PCR products that are too short to perform PCR-RFLPs. The PCR products were amplified as described by Criado-Fornelio et al. (2003). These PCR products were also used for nucleotide sequencing and phylogenetic study.

Polymerase chain reaction detection of 'Candidatus M. turicensis'

Specific primers targeting the 16S rRNA gene based on the reference *CMt* DNA sequence (accession number: DQ825450) were designed by Tasker et al. (2003). A forward primer (Mtuf, 5'-CAT CAG ACA GAA GGG GGA TTT AAA GGT G-3') and reverse primer (Mr, 5'- AGA AGT TTC ATT CTT GAC ACA ATT GAA -3') were used for amplification and yielded 1,230 bp of PCR product (Fig.1). Conventional PCR was performed as described by Tasker et al. (2003). The amplification conditions consisted of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min, followed by 72°C for 10 min.

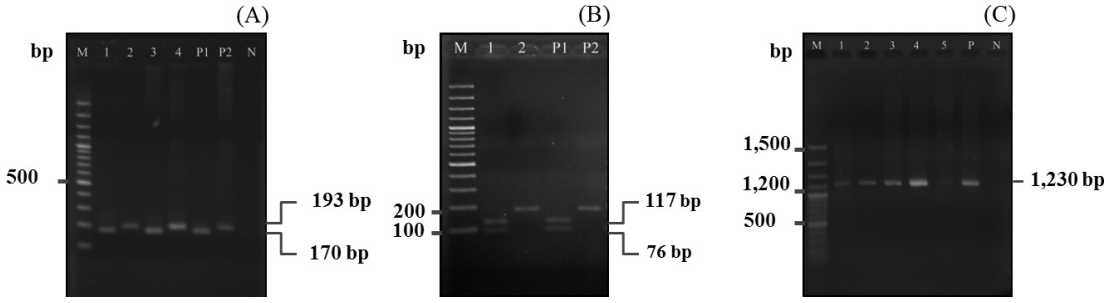

Fig. 1 Detection of feline hemoplasmas using molecular methods: (A) amplification of 16S rRNA gene for *Mhf* (170 bp) and *CMhm* (193 bp) based on conventional polymerase chain reaction (PCR), where lanes 1 and 3 = *Mhf* positive cats, lanes 2 and 4 = *CMhm* positive cats, lane P1 = *Mhf* positive control, lane P2 = *CMhm* positive control and lane N = negative control; (B) restriction fragment length polymorphisms-PCR using HindIII restriction enzyme showing results between undigested (*Mhf*) and digested to 76 bp and 117 bp (*CMhm*), where lane 1 = *CMhm* positive cats, lane 2 = *Mhf* positive cat, lane P1 = *CMhm* positive control and lane P2 = *Mhf* positive control; (C) amplification of 1,230 bp, partial sequence of 16S rRNA gene from *CMt* positive cases using current study-specific primers, where lanes 1–5 = *CMt* positive cats, lane P = *CMt* positive control and lane N = negative control

Cloning and sequencing analysis

All PCR products were cut from the agarose gel and purified using a PCR purification kit (Qiagen; USA) and then were inserted into multi cloning sites of the T&A cloning vector. Briefly, the expected purified PCR products were added into a mixture of 1 µL of ligation buffer A, 1 µL of ligation buffer B, 2 µL of T&A cloning vector (25 ng), 3 µL of PCR product and 1 µL of T4 DNA ligase (3 Weiss units/µL). The mixture was prepared by pipetting and the reaction was incubated at 16°C for 18 hr. The inserted vector was transformed into *E. coli* JM 109 strain (RBC Bioscience; Taiwan) using a heat shock technique at 42°C for 1 min in a water bath and then immediately incubated on ice for 20 min. Then, 900 µL of Luria-Bertani (LB) broth was added into the cell suspension and the cell was incubated at 37°C for 1 hr followed by spreading the cell precipitate on an LB agar plate containing 50 µg/mL of ampicillin plus 100 µL of 100 mM IPTG and 20 µL of 50 mg/mL X-gal and then incubating at 37°C for 16 hr. The white and single colony of the inserted vector was picked up and used for plasmid extraction with an AxyPrep Plasmid Miniprep Spin Kit (Axygen; USA). The purified inserted vector was used for the DNA sequencing template in an Applied Biosystem sequencer (First Base Inc.; Singapore). DNA sequences were blasted in the National Center for Bio Informatics (NCBI; USA).

Phylogenetic analysis

The 16S rRNA sequences from the current study consisting of *Mhf* (6 sequences), *CMhm* (7 sequences) and *CMt* (2 sequences) were submitted to the NCBI with the accession numbers EU145745, MW907597–MW907601, EU285281, MW907602–MW907607, EU789558 and EU789559, respectively. Nucleotide sequences were aligned using CLASTALX (Version 2, EMBL-EBI; UK). Phylogenetic trees were constructed using the MEGA 6 software (Tamura et al., 2013) with the neighbor-joining method. The dataset was reassembled 1,000 times to generate bootstrap percentage values. *Mycoplasma muris, M. coccoides*, *M. pneumoniae*, *M. suis*, *M. wenyonii* and *Candidatus* Mycoplasma haemolamae 16S rRNA sequences were used as the out-group in the phylogenetic analysis.

Statistical analysis of risk factors

Categorical variables of gender, external parasite infestations, number of cats per monastery and environmental conditions were used for testing the relation with hemoplasma detections. The null hypothesis is that each of categorical variables is independent of the hemoplasma infections. Statistical and Risk factor analysis was performed using EpiInfo Version 9.0 (EpiInfo; CDC; USA). Values of χ^2 and probability values were calculated, Mann-Whitney/Wilcoxon two-sample test was used for nonparametric statistics analysis. . Each risk factor was considered statistically significant when the probability value was less than 0.05 ($p < 0.05$).

Results

Microscopic examination

Only 985 blood smears were examined and analyzed since some were uninterpretable due to background debris. Organisms were found as either single or paired dots or chains based on microscopic examination. In total, 115 (11.68%) samples showed the presence of feline hemoplasmas; however further speciation was not possible.

External parasites

In total, 575 specimens of external parasites were collected from the stray cats, consisting of 88.35% (508/575) fleas and 5.74% (33/575) other species such as ticks, lice and mange. Based on their morphology, the fleas were identified as *Ctenocephalides felis*, *C. felis orientis* and *Xenopsylla cheopis*. Likewise, the mites and lice were identified as *Notoedres cati* and *Felicola subrostratus*, respectively. However, the mange species were not identified. In general, there was one type of ectoparasite per cat. However, co-infestations per cat were also recorded of *N. cati* and *F. subrostratus, C. felis* and *C. felis orientis, F. subrostratus* and *C. felis, and N. cati* and *C. felis*.

Polymerase chain reaction amplification, sequencing and phylogenetic analysis

The PCR-RFLPs showed initial amplification of 170 bp and 193 bp PCR products that were referred to as *Mhf* and *CMhm*, respectively (Fig. 1A). Based on the PCR-RFLPs, the PCR products of *Mhf* showed an undigested band (170 bp), while the PCR products of *CMhm* showed 2 digested bands (76 bp and 117 bp), as expected (Fig. 1B). The conventional PCR for *CMt* was successfully amplified as approximately 1,230 bp (Fig. 1C). The homology of 15 sequences of the 16S rRNA gene obtained was in the range 97.63–99.98% to the reference sequences.

Phylogenetic analysis showed three distinguishable clades: *CMhm* (clade 1), *CMt* (clade 2) and *Mhf* (clade 3). *Mycoplasma wenyonii, 'Candidatus* Mycoplasma haemolamae', *M. pneumoniae* and *M. suis* were less closely related (Fig. 2).

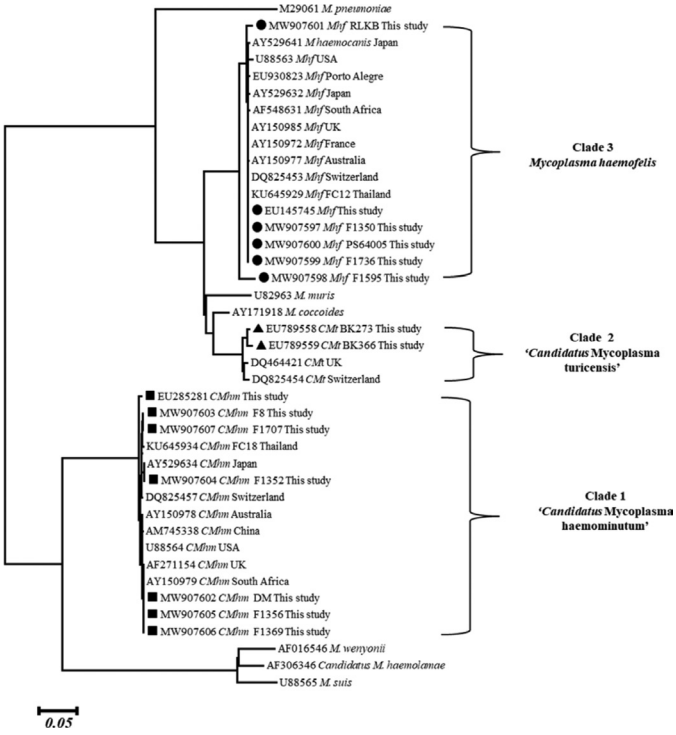

Fig. 3 Phylogenetic analysis based on 16S rRNA partial sequences of *Mhf*, *CMhm* and *CMt* Thailand sequences, other country isolates and related organisms generated using CLUSTAL X (2.0) and MEGA 6 software, where sequences derived from the current study are marked as (●) for *Mhf*, (■) for *CMhm* and (▲) for *CMt*

Prevalence of feline hemoplasmas in stray cats in Bangkok

Out of the 1,488 stray cats, 46% (685/1,488) were infected with at least one species of feline hemoplasma. The prevalence levels of *Mhf*, *CMhm* and *CMt* were 16.06% (239/1,488), 24.53% (365/1,488) and 1.55% (23/1,488), respectively. Dual infection with *Mhf*/*CMhm*, *Mhf*/*CMt* and *CMhm*/*CMt* was seen in 1.41% (21/1,488), 0.87% (13/1,488) and 1.41% (21/1,488) of cats, respectively, and triple infection with all three feline hemoplasma species (*Mhf*/*CMhm*/*CMt*) was observed in 0.20% (3/1,488) (Fig. 3). *Mhf* was found in 47 (94%) of the 50 districts, predominantly in the 6 districts of Lat Krabang, Bang Khun Thain, Beung Kum, Huai Kwang, Bang Plat and Bang Kho Leam (Fig. 4A). CMhm was found in all 50 districts but predominantly in Bang Bon, Phasi Charoen and Thung Kru districts (Fig. 4B). CMt was found in 17 (34%) of the 50 districts, predominantly in Din Daeng (Fig. 4C). Mixed infections were found in 28 (56%) districts, predominantly inBang Plat, Chom Thong, Rat Burana, Yan Nawa, Klong Toei, Prawet and Bang Kapi (Fig. 4D).

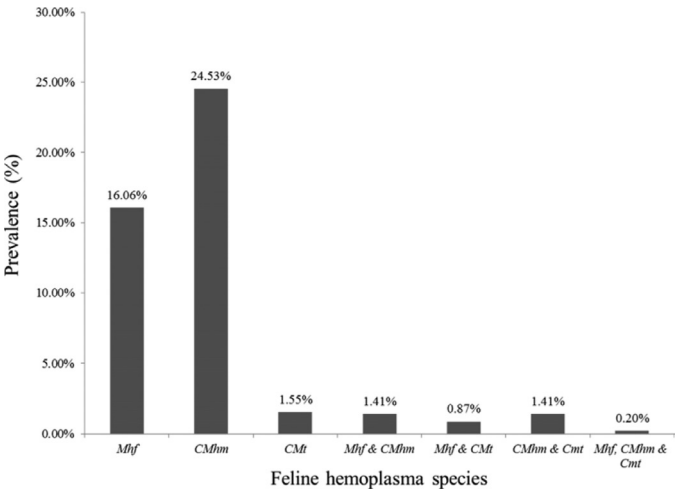

Fig. 2 Prevalence of feline hemoplasma infections in stray cats in Bangkok, Thailand

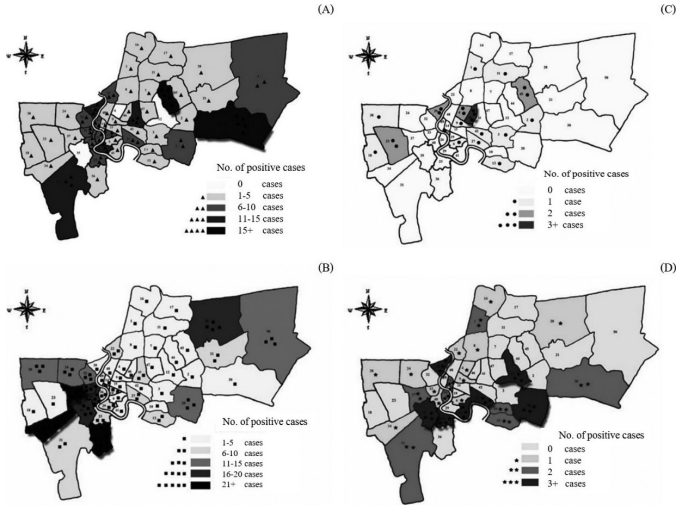

Fig. 4 Levels of feline hemoplasma infection in cats sampled in 50 districts of Bangkok metropolitan area: (A) *Mycoplasma haemofelis* infection; (B) '*Candidatus* Mycoplasma haemominutum' infection; (C) '*Candidatus* Mycoplasma turicensis' infection; (D) mixed (dual or triple) hemoplasma infection

Risk factors associated with feline hemoplasmas infection of stray cats in Bangkok

Risk factors were categorized as two groups of sex (male and female), three age groups (< 2, >2–4 and > 4 yrs), three groups of external parasites (no infestation, fleas and mixed (fleas and others), three groups of environmental conditions (good, fair and poor) and four groups of number of cats per monastery (unclear, low, medium and high). The number of animals of each group were shown in Table 1.

Based on the population of cats per monastery, *Mhf* infection was significantly higher (18.40%) in moderately crowded populations of cats (167/908; $\chi2$ = 17.05, degrees of freedom, df = 3, p = 0.0007). *CMhm* infection was significantly higher (35%) in older cats (> 4 years of age; 79/226; $\chi2$=16.40, df = 2, p = 0.0003). Furthermore, *CMt* infection was significantly higher (3.4%) in male cats (19/563; $\chi2$ = 19.90, df = 1, p = 0.0000) and in cats with fleas (2.9%; 14/480; $\chi2$ = 9.11, df =2, p = 0.0000).

Interestingly, feline hemoplasma dual infection (a mix of two pathogens) was significantly higher (1.6%) in male cats (9/563; $\chi2$ = 18.51, df = 1, p = 0.0000) and old cats (> 4 yr; 4%; 9/226; $\chi2$ =15.78, df = 3, p = 0.0004). In addition, triple infection (a mix of *Mhf*, *CMhm* and *CMt*) was significantly higher (0.5%) in male cats (3/563; $\chi2$ = 4.94, df =1, p = 0.0263) as shown in Table 1.

Discussion

This research investigated the prevalence of hemoplasma infection with the three major feline hemoplasma species in a population of stray cats in Bangkok, Thailand using PCR. Hemoplasma infections were highly prevalent, with 46% (685/1,488) of blood samples testing positive from 439 monasteries among 50 districts in Bangkok. The sampled cats were less cared for and roamed in the monasteries. The current level was comparably higher than in previous studies in Thailand that reported hemoplasma infections with a prevalence of 22.9% in 153 domestic cats (Assarasakorn et al., 2012), 2.7% in 75 wild felids (Suksai et al., 2010) and 38.05% in 473 semi-domesticated cats (Do et al., 2020). In the current study, 29.4% (129/439) of monasteries were infected, clearly demonstrating that some monasteries might be of concern as endemic areas of hemoplasma infections in Thailand. The health management of these animals in monasteries was not up to the standard of normal pet care because these animals had no identified owners and congregated in overcrowded populations. Ectoparasite control is a real problem that is made more complicated by the vector-borne transmission among stray animals.

Table 1 Prevalence and risk factor analysis of feline hemoplasma infection in cats collected from Bangkok, Thailand

Parameter	Number of tested cats	Polymerase chain reaction positive/prevalence												Mixture of 3 species
		Mhf		*CMhm*		*CMt*		*Mhf & CMhm*		*Mhf &CMt*		*CMhm & CMt*		
Sex		χ^2=0.91, df=1, p=0.3389		χ^2=0.26, df=1, p=0.6105		χ^2=19.90, df=1, p=0		χ^2=18.51, df=1, p=0						χ^2=4.94, df=1, p=0.0263
Male	563	97/	17.20%	134/	23.80%	19/	3.40%	8/	1.40%	9/	1.60%	19/	1.60%	3/ 0.50%
Female	925	142/	15.40%	231/	25.00%	4/	0.40%	13/	1.40%	4/	0.40%	2/	0.20%	0/ 0.00%
Age groups		χ^2=1.81, df=2, p=0.4045		χ^2=16.40, df=2, p=0.0003		χ^2=3.71, df=2, p=0.1563		χ^2=15.78, df=3, p=0.0004						χ^2=1.95, df=2, p=0.3774
≤ 2	541	78/	14.40%	116/	21.40%	4/	0.70%	6/	1.10%	1/	0.20%	2/	0.40%	0/ 0.00%
> 2 ≤ 4	721	121/	16.80%	170/	23.60%	14/	1.90%	11/	1.50%	8/	1.10%	10/	1.40%	2/ 0.30%
> 4	226	40/	17.70%	79/	35.00%	5/	2.20%	4/	1.80%	4/	1.80%	9/	4.00%	1/ 0.40%
External parasites		χ^2=3.38, df=2, p=0.1848		χ^2=8.74, df=2, p=0.0127		χ^2=9.11, df=2, p=0.0105		χ^2=3.05, df=2, p=0.2178						χ^2=1.65, df=2, p=0.4372
No infestation	955	164/	17.20%	257/	26.90%	8/	0.80%	12/	1.30%	7/	0.70%	12/	1.30%	1/ 0.10%
Fleas	480	70/	14.60%	95/	19.80%	14/	2.90%	7/	1.50%	6/	1.30%	7/	1.50%	2/ 0.40%
Mixed (fleas and others)*	53	5/	9.40%	13/	24.50%	1/	1.90%	2/	3.80%	0/	0.00%	2/	3.80%	0/ 0.00%
Environmental conditions		χ^2=1.97, df=2, p=0.3734		χ^2=5.10, df=2, p=0.0781		χ^2=0.29, df=2, p=0.8633		χ^2=19.56, df=2, p=0.0001						χ^2=0.72, df=2, p=0.6984
Good	844	135/	16.00%	189/	22.40%	14/	1.70%	9/	1.10%	8/	0.90%	13/	1.50%	1/ 0.10%
Fair	634	104/	16.40%	174/	27.40%	9/	1.40%	11/	1.70%	3/	0.50%	8/	1.30%	2/ 0.30%
Poor	10	0/	0.00%	2/	20.00%	0/	0.00%	1/	10.00%	2/	20.00%	0/	0.00%	0/ 0.00%
Number of cats per monastery		χ^2=17.05, df=3, p=0.0007		χ^2=4.47, df=3, p=0.215		χ^2=5.12, df=3, p=0.1634		χ^2=5.3594, df=3, p=0.1473						χ^2=5.21, df=3, p=0.1568
Unclear	119	6/	5.00%	35/	29.40%	4/	3.40%	4/	3.40%	0/	0.00%	1/	0.80%	1/ 0.80%
Low	142	25/	17.60%	41/	28.90%	0/	0.00%	5/	3.50%	0/	0.00%	2/	1.40%	1/ 0.70%
Medium	908	167/	18.40%	220/	24.20%	13/	1.40%	11/	1.20%	10/	1.10%	17/	1.90%	1/ 0.10%
High	319	41/	12.90%	69/	21.60%	6/	1.90%	1/	0.30%	3/	0.90%	1/	0.30%	0/ 0.00%
Total	1,488	239/	16.06%	365/	24.53%	23/	1.55%	21/	1.41%	13/	0.87%	21/	1.41%	3/ 0.20%

Df = degrees of freedom; * = mixture of external parasites (flea, ticks, lice and mange).

The *Mhf* sequence derived from the current study was similar to *Mhf* from other countries (Rikihisa et al., 1997; Tasker et al., 2003; Inokuma et al., 2004; Willi et al., 2005; Dos-Santos et al., 2008; Barrs et al., 2010; Spada et al., 2014). Additionally, the *CMt* sequence derived from the current study was closely related with *M. coccoides* and *M. muris* (Fig. 2) which are found in rodents (Neimark et al., 2005).

Consistent with most prevalence studies worldwide, *CMhm* (24.53%, 365/1,488) was the most common hemoplasma species infecting cats in Thailand compared to as high as 10.3% (Korea; Yu et al., 2007), 15.3% (Australia; Barrs et al., 2010) and 22.3% (Italy; Spada et al., 2014). Concurrent infections with two or three feline hemoplasma species were detected in the current study (0.2–1.41%) compared to other co-infection rates in the range 0.2–6.5% (Jenkins et al., 2016).

Sequencing of the 16S rRNA gene of hemoplasmas in Thailand was similar to sequences from the USA (Rikihisa et al., 1997), the UK (Tasker et al., 2003), Brazil (Dos-Santos et al., 2008), Australia (Barrs et al., 2010), Switzerland (Willi et al., 2005), South Africa, France (Tasker et al., 2003), Italy (Spada et al., 2014), Japan (Inokuma et al., 2004) and as previously reported in Thailand (Suksai et al., 2010). Phylogenetic analysis clearly distinguished the three clades *Mhf*, *CMhm* and *CMt*. Notably, *Mhf* from the current study was also similar to the 16S rRNA gene of *M. haemocanis* (*Mhc*) from Japan, while *CMt* was in the same clade as *M. muris* and *M. coccoides*, which are rodent hemoplasmas. However, there was no clear evidence of host sharing of these two pathogens and future study is needed to clarify any such host sharing (Birkenheuer et al., 2002). In addition, the phylogeny of the 16S rRNA and rnpB genes alone was not able to differentiate these two pathogens (Peter et al., 2008) but phylogenetic comparisons based on the average nucleotide identity and tetranucleotide signature suggest that *Mhf* and *Mhc* are different species of mycoplasmas (Nascimento et al., 2012). The Thai *CMt* isolate was similar to those from the UK and Switzerland (Willi et al., 2005). It could be that cats might become infected from their prey; however further studies are required.

In the current study, risk factor analysis indicated that ectoparasites infestation was a significant factor associated with hemoplasma infections ($p = 0.0127$). This may have been due to outdoor activity and the lack of ectoparasite controls in the stray cats, which resulted in tick, louse and flea infestations. In addition, the cats (and observed stray dogs) in the current study shared the same habitat in the monastery. Therefore, it was not unexpected that many ectoparasite were detected in the stray cats in this study. Blood-sucking ectoparasites could be one of the potential vectors for feline hemoplasmas transmission. However, further study is required to identify the potential vector for hemoplasma transmission. On the other hand, the interpretation and comparison of data from hemoplasma studies in stray cats indicated that there were various factors associated with feline hemoplasmas (Willi et al., 2006). Hence, the presence of fleas was evidently associated with hemoplasma infections in cats, since 55.9% of fleas collected from cats had *CMhm* DNA (Barrs et al., 2010).

The results of the current study should be interpreted and analyzed with care since the sample was taken from cats having less than standard health care, which might have resulted in a higher prevalence rate compared with housed cats. Consequently, the current study might not represent the real prevalence of hemoplasmas in Thailand. Further study involving housed cats would provide a more accurate estimate of the prevalence of hemoplasmas.

Ethics Statements

All authors certify that the practices used on all animals in the present work met the International Guiding Principles for Biomedical Research Involving Animals.

Conflict of Interest

The authors declare that there are no conflict of interests.

Acknowledgment

This study was supported by a research grant from the Graduate School, Kasetsart University, Bangkok, Thailand and partially supported by the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand.

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