FULL PAPER *Clinical Pathology*

An Epidemiological Survey of Feline Hemoplasma Infection in Japan

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ABSTRACT. Hemoplasma (hemotropic mycoplasma) often causes hemolytic anemia in infected cats, especially those with immune suppression. An updated nationwide epidemiological survey of feline hemoplasmosis was conducted in Japan. Blood samples were collected from 1,770 outdoor-accessing cats from March to October 2008. The infections were molecularly detected by PCR analyses, which are able to distinguish *Mycoplasma haemofelis* (Mhf), '*Candidatus* M. haemominutum' (CMhm), and '*Candidatus* M. turicensis' (CMt) infections. Of the 1,770 cats, 468 cases (26.4%) revealed a single- or co-infection of feline hemoplasmas [Mhf alone, 42 cases (2.4%); CMhm alone, 280 cases (15.8%); CMt alone, 48 cases (2.7%); Mhf+CMhm, 28 cases (1.6%); Mhf+CMt, 6 cases (0.3%); CMhm+CMt, 50 cases (2.8%); Mhf+CMhm+CMt, 14 cases (0.8%)]. In addition, male gender, middle to old age, history of fight wounds, and feline immunodeficiency virus infection were shown to be risk factors for hemoplasma infection. Close attention must be paid to the acute onset of disease in feline practice because a prevalence of hemoplasma infection was detected even in clinically healthy cats.

KEY WORDS: epidemiological survey, feline, hemoplasma.

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Hemoplasma (hemotropic mycoplasma) is a Gram-negative bacterium without a cell wall that infects the cell surface of erythrocytes [16]. The infected host occasionally develops hemolytic anemia, thrombocytopenia, pyrexia, and jaundice [16]. Two strains (large and small, or Ohio and California) of the rickettsia *Haemobartonella felis* (*H. felis*) have been considered causal agents of feline hemobartonellosis [4, 5, 13, 16]. However, a recent study concerning taxonomic classification showed that *H. felis* is genetically more closely related to mycoplasma based on the sequence of the 16S rRNA gene [4]. Additionally, the two strains mentioned above have been reclassified as *Mycoplasma haemofelis* (Mhf) and '*Candidatus* M. haemominutum' (CMhm), respectively [4, 5, 13]. In 2004, the third feline hemoplasma was isolated in Switzerland and designated '*Candidatus* M. turicensis' (CMt) [25]. Currently, these three species of hemoplasmas are recognized as important pathogens in the field of feline practice. Mhf is suggested to be the most virulent species in cats [4, 23].

It has been suggested that both direct and indirect transmission routes exist among cats with feline hemoplasmosis [25]. However, a discrepancy, especially regarding potential oral infection, has been mentioned in several reports. Flint *et al.* successfully reproduced an oral hemoplasma infection in an experimental model, while a recent report by Museux *et al.* failed to do so [3, 12]. Alternatively, blood-sucking arthropods are suspected to be a vector for hemoplasmas; however, this has not yet been confirmed [19]. A high prevalence of hemoplasma and an acute onset of hemolytic anemia secondary to hemoplasma infection have been detected in older, male, outdoor-accessing cats [8, 25]. This suggests that direct transmission via fight wounds may be an important infection route for feline hemoplasmas. The transmission route of feline hemoplasma has not been completely ascertained and leaves much room for discussion.

The diagnosis of hemoplasma infection has historically been based on cytological findings from a blood smear specimen. However, the recent development of molecular-based methods has made it possible to detect hemoplasma infection with greater sensitivity and specificity as well as to perform epidemiological surveys [6, 9, 11, 15, 21, 22]. Prevalence of feline hemoplasma infection is described in previous reports as ranging from approximately 10 to 40% of cats in the United Kingdom, Switzerland, Australia, South Africa, Canada, and the United States [10, 18, 26, 27]. Epidemiological surveys for feline hemoplasma infection have also been performed in Japan. However, these studies were carried out with different inclusion criteria, and the study scale was limited [6, 9, 21]. Currently, no information is available regarding a large-scale epidemiological study of feline hemoplasma infection covering the entirety of Japan.

In the present study, we conducted a nationwide molecular epidemiological survey for feline hemoplasma infection—not only to determine prevalence, but to understand the clinical characteristics of hemoplasma-infected cats, risk factors for infection, and possible transmission routes.

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MATERIALS AND METHODS

Examined cats and sample collection: Blood samples were collected from 1,770 cats admitted to 47 private veterinary hospitals located in each of 47 prefectures in Japan from March to October 2008. Cats accessing the outdoors at least once a week were included; however, cats kept strictly indoors were excluded. Age, gender, chief complaints, and other clinical information for each cat were recorded at each hospital. The statuses of feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infections were screened by detection of anti-FIV antibody and FeLV p27 antigen in serum using a commercially available test kit (SNAP FeLV/FIV combo kit; IDEXX Laboratories Inc., Westbrook, ME, U.S.A.). The profiles of the examined cats are shown in Table 1.

Detection of hemoplasma-derived DNA by polymerase chain reaction (PCR): Total DNA was extracted from 200 μl of peripheral whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) with a final elution volume of 200 μl. Obtained DNA was then used as a template to amplify the DNA from three species of feline hemoplasmas and an internal control by performing PCRs.

To detect Mhf- and CMhm-derived DNA, one-step PCR amplification was performed using two forward and one reverse oligonucleotides, including OH-OK (Mhf-specific forward primer, 5'-ATGCCCCTCTGTGGGGGAT-AGCCG-3', nt 103–126, GenBank/EBML/DDBJ accession number U88563), CA-B2 (CMhm-specific forward primer, 5'-CTGGGAAACTAGAGCTTCGCGAGC-3', nt 176–199, U88564), and 00CR-r1 (common reverse primer, 5'-ATGGTATTGCTCCATCAGACTTTCG-3', nt 353–377) as reported by Watanabe *et al.* [21]. Reaction mixtures (50 μl) contained three primers (0.4 nM each), 2 μl of template DNA, *Taq* DNA polymerase (1.25 unit), and the reagents recommended by the manufacturer (Takara, Kyoto, Japan). The PCR reaction involved 35 cycles of denaturation (94°C, 45 sec), annealing (56.8°C, 45 sec), and polymerization (72°C, 45 sec).

The CMt PCR amplification was carried out based on a report by Peters *et al.* [15]. Briefly, the oligonucleotides CMt-F (5'-AGAGGCGAAGGCGAAAACT-3', nt 621–639, GenBank/EBML/DDBJ accession number DQ157150) and CMt-R (5'-CTACAACGCCGAAACACAAA-3', nt 739–758) were utilized in reactions. Reaction mixtures (50 μl) contained two primers (0.2 nM each), 2 μl of template DNA, *Taq* DNA polymerase (1.25 unit), and the reagents recommended by the manufacturer (Takara, Kyoto, Japan).

Table 1. Clinical profile, frequency and risk factors of hemoplasmas in 1,770 examined cats

		Number of cats (%)	Hemoplasma PCR		Univariate analysis			Multivariate analysis		
			Negative (%)	Positive (%)	Crude OR	95% CI	*P*	Adjusted OR	95% CI	*P*
Total		1,770 (100.0)	1,302 (73.6)	468 (26.4)	N/A*	N/A	N/A	N/A	N/A	N/A
Breed										
	Mixed breed	1,695 (95.8)	1,241 (73.2)	454 (26.8)	N/A	N/A	N/A	N/A	N/A	N/A
	American short hair	26 (1.5)	20 (76.9)	6 (23.1)						
	Persian	5 (0.3)	4 (80.0)	1 (20.0)						
	Other pure breeds	44 (2.5)	37 (84.1)	7 (15.9)						
Gender										
	Female	825 (46.6)	719 (87.2)	106 (12.8)	1.00			1.00		
	Male	939 (53.1)	578 (61.6)	361 (38.4)	4.20	3.29–5.35	<0.05	3.15	2.41–4.11	<0.05
	Unknown	6 (0.3)	5 (83.3)	1 (16.7)						
Age										
	<2 yrs	402 (22.7)	356 (88.6)	46 (11.4)	1.00			1.00		
	>2 yrs	1,368 (77.3)	946 (69.2)	422 (30.8)	3.42	2.46–4.74	<0.05	2.50	1.72–3.64	<0.05
Frequency of outdoor accessing										
	1–4 days per week	358 (20.2)	289 (80.7)	69 (19.3)	1.00			1.00		
	5–7 days per week	1,364 (77.1)	974 (71.4)	390 (28.6)	0.79	0.61–1.03	0.08	0.75	0.56–0.99	0.05
	Unknown frequency	48 (2.7)	39 (81.3)	9 (18.7)						
History of bite wound suffered in fighting										
	No	956 (54.0)	781 (81.7)	175 (18.3)	1.00			1.00		
	Yes	716 (40.5)	446 (62.3)	270 (37.7)	2.69	2.15–3.36	<0.05	1.43	1.11–1.85	<0.05
	Unknown	98 (5.5)	75 (76.5)	23 (23.5)						
Some sort of clinical signs										
	No	586 (33.1)	476 (81.2)	110 (18.8)	1.00			1.00		
	Yes	1,175 (66.4)	818 (69.6)	357 (30.4)	1.87	1.47–2.38	<0.05	1.03	0.77–1.37	0.83
	Unknown	9 (0.5)	8 (88.9)	1 (23.5)						
Serum anti-FIV antibody										
	Negative	1,360 (76.8)	1,102 (81.0)	258 (19.0)	1.00			1.00		
	Positive	410 (23.2)	200 (48.8)	210 (51.2)	4.55	3.59–5.77	<0.05	3.07	2.35–4.01	<0.05
Serum FeLV antigen										
	Negative	1,554 (87.8)	1,163 (74.8)	391 (25.2)	1.00			1.00		
	Positive	216 (12.2)	139 (64.4)	77 (35.6)	1.67	1.23–2.25	<0.05	1.36	0.95–1.93	0.09

*N/A, not applicable.

The PCR reactions involved 35 cycles of denaturation (95°C, 10 sec), annealing (58°C, 30 sec), and polymerization (72°C, 30 sec).

As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was also amplified in each sample using the primers cGS (5'-CTCATGACCACAGTCCATGC-3', nt 514–533 in AB038240) and cGR (5'-TGAGCTTGACAAAGTGGTCA-3', nt 925 to 906). The PCR reactions involved 25 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min), and polymerization (72°C, 3 min). Only the samples in which the G3PDH gene was successfully detected were included in this study. All PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. If DNA bands of the expected size (Mhf, 273 bp; CMhm, 202 bp; CMt, 138 bp) were obtained, those cases were judged as positive for each feline hemoplasma.

Sensitivity of PCR analyses and detection of co-infection: Before applying the above-mentioned PCR method to clinical surveys, the sensitivity of each PCR system was evaluated by determining its detection limit using a serially diluted plasmid template. DNA fragments of 16S rRNA genes derived from Mhf, CMhm, and CMt were inserted into plasmids (pCR2.1, Invitrogen, Calsbad, CA, U.S.A.). Serial 10-fold dilutions corresponding to 10^{3}–10^{-4} copies were prepared and used as templates. Three plasmids containing Mhf-, CMhm-, or CMt-derived DNA fragments were mixed and used for analyses to prepare for conceivable co-infection. In addition, genomic DNA obtained from healthy cats was added to the all-PCR reaction mixture to bring the analysis closer to the condition in clinical surveys. Triplicate analyses were performed for each dilution. Then, PCR products were electrophoresed and the minimum detection limits were determined.

Statistical analysis: The variables in the clinical factors associated with hemoplasma infection were assessed with crude and adjusted odds ratios (ORs) calculated by a logistic regression analysis within 95% confidence intervals (CIs). The geographical distribution pattern of hemoplasma was analyzed by the Kruskal-Wallis test. All statistical analyses were performed using StatView software (Hulink Inc., Tokyo, Japan) with a significance level of $P<0.05$.

RESULTS

Sensitivity of PCR amplifications: As shown in Fig. 1A, if the DNA derived from Mhf, CMhm, or CMt contained 10^{-2}, 10^{1}, or 10^{0} copies in one reaction mixture, respectively, then the PCR methods used in this study could detect them. These results show that the limits when blood samples are used are 5×10^{-3} copies per μl for Mhf, 5×10^{0} copies per μl for CMhm, and 5×10^{-1} copies per μl for CMt. Therefore, the two PCR methods used in this study possess enough sensitivity to apply our clinical epidemiological survey to hemoplasma infection in cats.

In addition, the detection efficacy was also analyzed using mixed templates. The representative data are shown in Fig. 1B. All possible combinations of co-infections were efficiently amplified and differentiated by using two PCR methods. If the concentration of each plasmid was changed, the efficacy of PCR amplification for each hemoplasma-derived gene was comparable to those in the analyses using a single plasmid as mentioned above. Thus, even if the clinical cases were infected with multiple species of hemoplasma, our PCR system could detect and differentiate them.

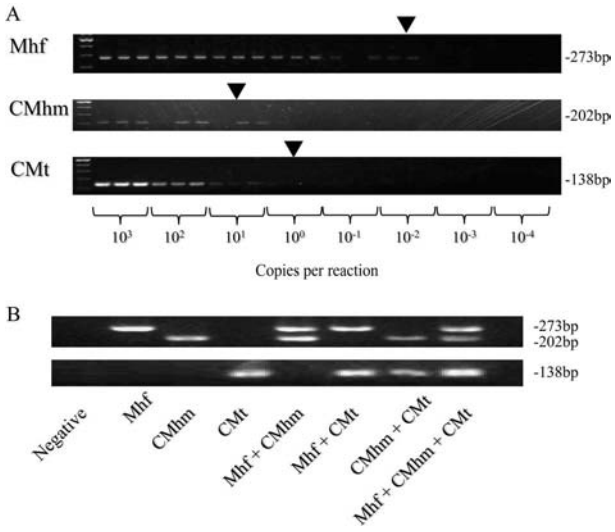


Fig. 1. Sensitivity and specificity of the PCR system used in this study. (A) Agarose gel electrophoresis of PCR products derived from reactions specific for the amplification of Mhf, CMhm, or CMt using diluted plasmids as a template. (B) Confirmation of efficient and accurate PCR amplification for the possible co-infections.

Epidemiological survey for hemoplasma infections: The G3PDH gene was successfully detected in blood samples derived from 1,770 cats; thus, all samples were used for the molecular epidemiological survey. DNA fragments derived from Mhf, CMhm, and/or CMt were detected in 468 cases (26.4%) (Table 1). The infection rate for each species was 5.1% (90 cases), 21.0% (372 cases), and 6.7% (118 cases) for Mhf, CMhm, and CMt, respectively (Fig. 2). Co-infection was observed in 98 cases (5.5%) with Mhf+CMhm evident in 28 cases (1.6%), Mhf+CMt in six cases (0.3%), and dual CMhm+CMt infection apparent in 50 cases (2.8%). All three species were present in 14 cases (0.8%) (Fig. 2).

Risk factors and distribution of feline hemoplasmas: Next, we analyzed the clinical characteristics of hemoplasma-infected cats and risk factors for hemoplasma infection (Table 1). In gender, male cats constituted approximately 77% of all hemoplasma-infected cats and exhibited a greater risk of infection than females (OR=3.15, 95% CI=2.41–4.11, $P<0.05$). In age, the prevalence of hemoplasma in adult cats (>2 years old) was 30.8% (422 of 1,368 cats), while that in kittens and juvenile cats (<2 years old) was 11.4% (46 of 402 cats). The adult cats also showed a significantly greater risk for hemoplasma infection

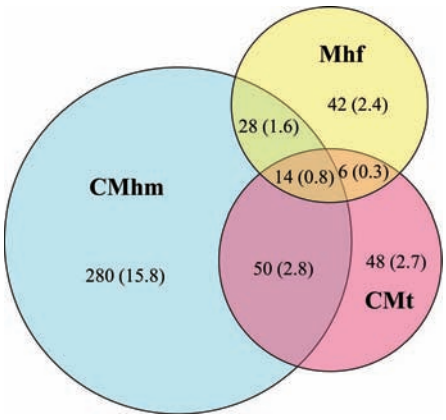
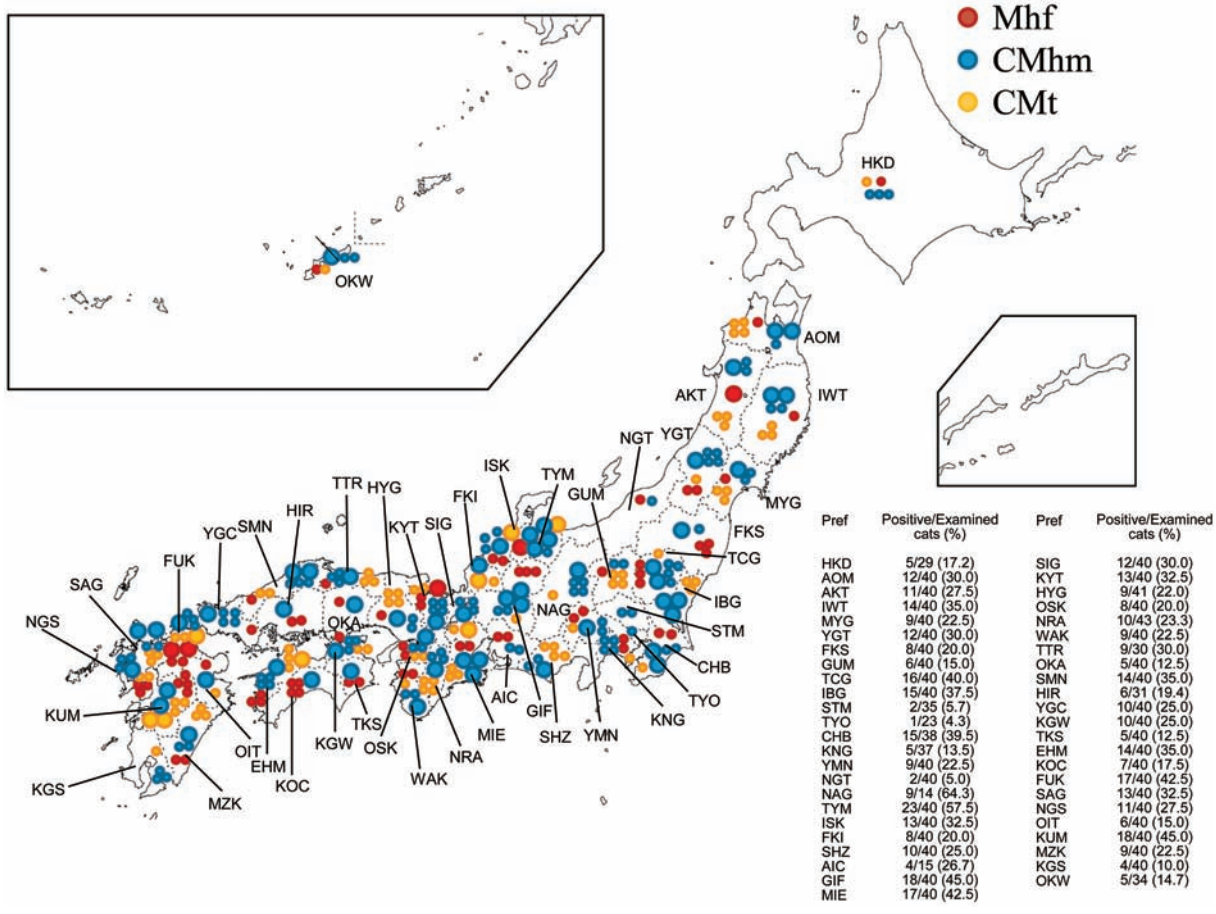


Fig. 2. Number of cases infected with feline hemoplasmas. Figure in parentheses indicates a percentage of infected cases in 1,770 examined cats. Each circle shows the Mhf, CMhm, or CMt-infected cats, and areas of overlap indicate a double or triple co-infection of feline hemoplasmas.

(OR=2.50, 95% CI=1.72–3.64, $P<0.05$). A greater amount of outdoor access was not related to the risk of infection (OR=0.75, 95% CI=0.56–0.99, $P=0.05$). The infection rate in 716 cats with a history of fight wounds was 37.7% (270 cases), significantly higher (OR=1.43, 95% CI=1.11–1.85, $P<0.05$) than the 18.3% (175 cases) in 956 cats without that history. Hemoplasma was detected in 30.4% (357 of 1,175 cases) and 18.8% (110 of 586 cases) of cats with and without any clinical signs, respectively. Documented clinical signs included anorexia, emaciation, eye or nasal discharge, pyrexia, skin lesions, anemia, chronic kidney disease, difficulty in micturition, and respiratory distress. Crude OR in cats with any clinical sign was significantly higher than those in cats without signs (OR=1.87, 95% CI=1.47–2.38, $P<0.05$); however, the adjusted OR did not significantly differ (OR=1.03, 95% CI=0.77–1.37, $P= 0.83$). The statuses of FIV and FeLV infections were evaluated in all 1,770 cats, and the overall prevalences of both viruses were 23.2 and 12.1%, respectively. FIV-infected cats exhibited higher hemoplasma infection rates (51.2%) than cats without infection (19.0%) (OR=4.48, 95% CI=3.54–5.68, $P<0.05$). No significant association between FeLV and hemoplasma



Pref	Positive/Examined cats (%)	Pref	Positive/Examined cats (%)
HKD	5/29 (17.2)	SIG	12/40 (30.0)
AOM	12/40 (30.0)	KYT	13/40 (32.5)
AKT	11/40 (27.5)	HYG	9/41 (22.0)
IWT	14/40 (35.0)	OSK	8/40 (20.0)
MYG	9/40 (22.5)	NRA	10/43 (23.3)
YGT	12/40 (30.0)	WAK	9/40 (22.5)
FKS	8/40 (20.0)	TTR	9/30 (30.0)
GUM	6/40 (15.0)	OKA	5/40 (12.5)
TCG	16/40 (40.0)	SMN	14/40 (35.0)
IBG	15/40 (37.5)	HIR	6/31 (19.4)
STM	2/35 (5.7)	YGC	10/40 (25.0)
TYO	1/23 (4.3)	KGW	10/40 (25.0)
CHB	15/38 (39.5)	TKS	5/40 (12.5)
KNG	5/37 (13.5)	EHM	14/40 (35.0)
YMN	9/40 (22.5)	KOC	7/40 (17.5)
NGT	2/40 (5.0)	FUK	17/40 (42.5)
NAG	9/14 (64.3)	SAG	13/40 (32.5)
TYM	23/40 (57.5)	NGS	11/40 (27.5)
ISK	13/40 (32.5)	OIT	6/40 (15.0)
FKI	8/40 (20.0)	KUM	18/40 (45.0)
SHZ	10/40 (25.0)	MZK	9/40 (22.5)
AIC	4/15 (26.7)	KGS	4/40 (10.0)
GIF	18/40 (45.0)	OKW	5/34 (14.7)
MIE	17/40 (42.5)		

Fig. 3. Geographic distribution of three species of hemoplasma detected in 468 cases. The large and small dots represent five and one hemoplasma-infected cat(s), respectively. Red, Mhf; Green, CMhm; Yellow, CMt. A three-letter code was assigned to each prefecture, and examined case numbers in each prefecture were indicated by the results of hemoplasma PCR status.

infection was detected in this study (adjusted OR=1.36, 95% CI=0.95–1.93, P=0.09), although the crude OR in FeLV-positive cats was significantly higher than that in negative cats (OR=1.67, 95% CI=1.23–2.25, P<0.05). Furthermore, the geographical distribution pattern of the three species of feline hemoplasma was evaluated; however, no fixed pattern was observed (P=0.77) (Fig. 3). All three species of hemoplasma were distributed in Japan.

DISCUSSION

To our knowledge, this is the first report of a nationwide epidemiological survey for feline hemoplasmosis. Our results indicated that 26.4% of examined cats were infected with one or more species of hemoplasma, and that there were 5.1, 21.0, and 6.7% of Mhf-, CMhm-, and CMt-infected cats, respectively. These infection rates are generally lower than results from previous reports in Japan [6, 9]. PCR systems used in this study seem to possess enough sensitivity, comparable with that in previous studies, although more accurate results could be obtained if both real-time and conventional PCR analyses could be performed [15, 17, 21]. Accordingly, the low infection rate observed in this study may have been due to the difference in cat population and numbers. The clinical profile of cats in the previous study was unclear; however, one possible reason is the difference in inclusion criteria of cats (e.g., age, gender, or cats' living environment) in the study. The cats examined in previous studies were selected based on the presence of clinical signs and suspicion of hemoplasma infection [6, 9, 21]. Therefore, it is conceivable that there was a bias toward elevation of the infection rate. In addition, a study scale may have influenced the difference between previous results and ours [6, 9, 21]. Previous studies included a smaller number of cats; therefore, our results seem to show a more accurate infection rate.

It is also difficult to directly compare the results of previous epidemiological surveys in foreign countries with ours due to the differences in case selection [10, 15, 18, 26, 27]. However, the infection rates of the three species of hemoplasma found in the present study were similar to those in a previous study from Australia, while they were approximately two- to three-fold higher than those from the UK and Switzerland [26, 27]. Although we cannot conclude which major factor has the most decisive impact on the prevalence of hemoplasmosis due to a lack of information about the life circumstances of cats examined in previous studies, the trend in prevalence of these three species of feline hemoplasma was common in all studies. Our and previous studies showed that cats infected with CMhm were more frequently observed than those with Mhf or CMt. This might suggest that CMhm infects and multiplies in cats more efficiently than Mhf and CMt. In addition, the higher prevalence of CMhm compared with the other two species of hemoplasma may be due to the lower virulence of this species, which may produce a prosperous coexistence between cats and the microbe [2, 4, 5, 7].

The present study clarified some clinical characteristics and risk factors of feline hemoplasma infections based on the findings obtained from more than a thousand cases. Infections were more frequently observed in older cats as opposed to juvenile ones. This finding suggests that many cats may have been infected with hemoplasma posteriorly rather than experiencing transplacental or vertical transmissions. In addition, male cats were shown to be at greater risk of infection. These two parameters resulted in a greater opportunity for contact with other cats. A third parameter, a history of fighting, is also recognized as an important factor in the risk of hemoplasma infection. Therefore, a horizontal and direct transmission appears to be the major infection route of feline hemoplasmas. The findings on retrovirus infection, especially FIV infection, also seem to support this. FIV is mainly transmitted through bite wounds inflicted during fighting, in terms of saliva-to-blood transmission [14]. Hemoplasma may be transmitted with FIV via such a route. However, there is another possible explanation for the high frequency of hemoplasma and FIV co-infections. FIV infection often causes immune suppression in infected cats [1, 20]. In such cases, multiplication of hemoplasma may be enhanced and more likely to be detected by PCR; however, immunological status was not determined in the cats examined in the present study. Unexpectedly, FeLV infection was not statistically associated with hemoplasma infection, unlike the findings in previous studies [8, 16, 24]. The exact reason is unknown; however, a significant association might have been observed if we could have analyzed the relationship between the acute onset of hemoplasma infection and FeLV status.

Frequent outdoor access and the presence of clinical signs were not recognized as risk factors, either. A major difference between our and previous studies is an inclusion criteria of the previously mentioned cases [8, 16, 24]. All cats in our study had a history of outdoor access, and we evaluated its frequency. Therefore, if we could have analyzed all cats admitted to the hospital, a significantly higher prevalence of hemoplasma might have been observed in outdoor-accessing cats. An important finding is that no significant predisposition to hemoplasma infection was observed between clinically ill and healthy cats. This finding suggests potential hemoplasma infection and the possibility of future acute onset of disease, even in healthy cats.

In addition to the inclusion criteria for sample collection confined to outdoor-accessing cats, the limitation of this study was that we could not determine the relationship between hemoplasma infection status and the presence of clinical symptoms, especially when anemia was caused by hemoplasma. Hematological examination was not performed in every case because the cats were presented not only for diagnosis and treatment of disease, but for prophylaxis and vaccination. If we had evaluated hematological parameters in the examined cats, we would be able to discuss the pathogenicity of hemoplasma species and the risk factors for acute onset of hemoplasmosis. Furthermore, we do not know the parasitic history of blood-sucking arthro-

pods (e.g., fleas and ticks) in cats. Blood-sucking arthropods may be a vector for hemoplasmas. This point is also important in understanding the details of the transmission route of feline hemoplasmas.

In the present study, we performed a nationwide epidemiological survey of three species of feline hemoplasmas and clarified their prevalence in Japan. Risk factors for hemoplasma infection, including male gender, frequency of outdoor access, history of bite wounds, and retrovirus infections, were also discussed. Furthermore, we strongly suggested that the major transmission route of feline hemoplasmas was a horizontal and direct, and/or possibly via blood-sucking arthropods. It may be necessary for veterinary clinicians to be alert for possible acute onsets of hemoplasmosis, even in the clinically healthy cats, because feline hemoplasmas are widespread throughout Japan.

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