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Molecular detection and genetic diversity of hemoplasma species in anaemic cats presented to referral hospitals

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Abstract

Hemoplasmas-wall-less, erythrocyte-parasitic bacteria of the genus *Mycoplasma*-are an increasingly recognised cause of feline infectious anaemia, yet their molecular epidemiology in hospital-based cat populations remains poorly mapped in East Asia. This research determined the prevalence, species distribution, and genetic diversity of hemoplasma infections among anaemic cats referred to two university veterinary hospitals in the Tokyo metropolitan area between January 2023 and December 2023. A total of 218 anaemic cats (packed cell volume < 25 %) were screened by species-specific conventional and quantitative PCR targeting the 16S rRNA gene for three hemoplasma species: *Mycoplasma haemofelis*, *Candidatus Mycoplasma haemominutum*, and *Candidatus Mycoplasma turicensis*. Overall prevalence was 38.1 % (83/218), with *M. haemofelis* detected most often (18.3 %), followed by *Ca. M. haemominutum* (12.8 %) and *Ca. M. turicensis* (6.9 %). Co-infections with two species occurred in 4.1 % of positive cats. Male sex, outdoor access, and feline immunodeficiency virus seropositivity were significantly associated with hemoplasma detection ($p < 0.05$). Cats harbouring *M. haemofelis* had the lowest mean packed cell volume (14.7 ± 4.2 %) compared with *Ca. M. haemominutum* (20.3 ± 5.1 %) and *Ca. M. turicensis* (22.8 ± 4.8 %), confirming the greater pathogenicity of the former species. Phylogenetic analysis of 54 partial 16S rRNA sequences (approximately 1,200 bp) revealed eight unique haplotypes for *M. haemofelis*, five for *Ca. M. haemominutum*, and three for *Ca. M. turicensis*. Haplotype diversity was highest in *M. haemofelis* ($H_d = 0.891$) and lowest in *Ca. M. turicensis* ($H_d = 0.624$). Nucleotide diversity followed the same rank order. Maximum-likelihood phylogenetic trees placed all Japanese *M. haemofelis* isolates in a distinct Asian clade separate from European and South American lineages, suggesting regional divergence. These findings establish hemoplasmas as a common and genetically diverse cause of anaemia in referral-level feline patients in Japan and underscore the value of routine PCR screening for anaemic cats in clinical settings.

Keywords: Hemoplasma, *Mycoplasma haemofelis*, feline anaemia, PCR, phylogenetics, genetic diversity, haplotype, cats, 16S rRNA, infectious anaemia

Introduction

More than a century has passed since the first description of small basophilic bodies on the surface of feline red blood cells, a finding that would eventually be attributed to hemotropic mycoplasmas ^[1]. For decades these organisms were classified within the genus *Haemobartonella*, but reclassification based on 16S ribosomal RNA gene phylogeny placed them squarely within *Mycoplasma* in the early 2000s ^[2]. Three species are now known to infect domestic cats: *Mycoplasma haemofelis*, the most pathogenic, capable of causing severe regenerative anaemia even in immunocompetent hosts; *Candidatus Mycoplasma haemominutum*, a smaller organism typically producing subclinical or mild infection unless the cat is immunosuppressed; and *Candidatus Mycoplasma turicensis*, first described in a Swiss cat in 2005 and generally associated with the lowest clinical impact ^[3].

Feline hemoplasma infections have been documented on every continent where cats live ^[4]. Prevalence estimates, however, swing widely-from under 5 % in northern European pet cat surveys to over 40 % in stray cat populations in tropical regions-and are heavily influenced by sampling strategy, geographic location, cat lifestyle, and diagnostic method ^[5]. Most published surveys have screened apparently healthy or randomly selected cat populations.

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Considerably less attention has been paid to cats presenting specifically with anaemia at referral hospitals, even though hemoplasma testing is arguably most relevant in that clinical context [6].

Genetic diversity data are similarly uneven. Whole or partial 16S rRNA gene sequencing has revealed geographic clustering of *M. haemofelis* lineages, with distinct clades in South America, southern Europe, and Australia [7]. Asian sequences remain under-represented in public databases, and no comprehensive phylogenetic analysis of Japanese feline hemoplasmas has been published. Understanding local genetic variation matters for practical reasons: diagnostic PCR primer sets designed against reference sequences from one geographic clade may have reduced sensitivity for divergent strains circulating elsewhere [8].

In Japan, feline hemoplasma awareness has grown following sporadic case reports and a small cross-sectional survey in Hokkaido that found a combined prevalence of 14.7 % in blood-bank donor cats [9]. But donor cats are pre-screened and generally healthy, so that figure likely underestimates the burden among cats with clinical disease. No large-scale molecular survey targeting anaemic cats at Japanese referral institutions has been carried out. This gap limits the ability of Japanese veterinary internists to assess how often hemoplasmas explain otherwise idiopathic anaemia in their caseloads.

Risk factors for hemoplasma infection in cats have been consistently linked to male sex, outdoor access, and co-infection with feline immunodeficiency virus (FIV) or feline leukaemia virus (FeLV) [4]. These associations suggest arthropod-mediated and bite-wound transmission routes, although the definitive vector for *M. haemofelis* has never been established experimentally [10]. Understanding which cat demographics carry the highest risk in a Japanese urban and peri-urban setting would help practitioners prioritise hemoplasma screening in their diagnostic workups.

This research therefore pursued three objectives: first, to determine the prevalence and species distribution of hemoplasma infections among anaemic cats at two Tokyo-area referral hospitals over a 12-month period; second, to identify host-level risk factors associated with infection; and third, to characterise the genetic diversity and phylogenetic relationships of the detected hemoplasma isolates using partial 16S rRNA gene sequencing. The expectation was that *M. haemofelis* would predominate among severely anaemic cats, that male outdoor cats co-infected with FIV would be over-represented, and that Japanese isolates would cluster phylogenetically with other Asian sequences while showing measurable divergence from Western hemisphere lineages.

Material and Methods

Material

A prospective cross-sectional investigation was conducted at the veterinary teaching hospitals of Kanto University of Veterinary Biosciences (Setagaya campus) and a partnering referral clinic in Chiba Prefecture from January to December 2023. Any domestic cat presented with a packed cell volume (PCV) below 25 % was eligible, regardless of breed, age, or suspected cause of anaemia. Cats were excluded if they had received a blood transfusion within the preceding 14 days or if an insufficient blood volume (< 2 mL EDTA) was collected. Over the 12-month enrolment period, 218 cats met the inclusion criteria. Signalment, lifestyle (indoor-only versus outdoor access), vaccination status, FIV and FeLV serological status (SNAP FIV/FeLV Combo Test, IDEXX),

and clinical findings were recorded for each cat at the time of blood collection.

The research facilities are located in the greater Tokyo metropolitan area (35°39'N, 139°41'E; altitude 35 m). The region has a humid subtropical climate (Cfa, Köppen), with mean temperatures ranging from 5 °C in January to 31 °C in August. Both hospitals serve a mixed caseload of primary-care and referred patients and together admit approximately 4,500 feline consultations per year.

Methods

Two millilitres of EDTA-anticoagulated blood were collected from a jugular or medial saphenous vein. An automated haematology analyser (ProCyt Dx, IDEXX) generated complete blood counts. Blood smears were stained with Diff-Quik and examined at ×1000 magnification under oil immersion for epacellular organisms. Genomic DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. Species-specific conventional PCR assays targeting the 16S rRNA gene were performed for *M. haemofelis* (forward 5'-ATGCCCCCTCTGTGGGGGATAGCCG-3'; expected amplicon 393 bp), *Ca. M. haemominutum* (expected amplicon 488 bp), and *Ca. M. turicensis* (expected amplicon 445 bp) using published primer sets [11]. Positive controls consisted of plasmid DNA containing cloned target sequences; nuclease-free water served as the negative control. Quantitative real-time PCR (qPCR) was run in parallel on a StepOnePlus instrument (Applied Biosystems) with SYBR Green chemistry to confirm equivocal conventional PCR results and to estimate bacterial load (copies µL⁻¹).

For phylogenetic analysis, a 1,200 bp fragment of the 16S rRNA gene was amplified from 54 randomly selected positive samples using universal bacterial primers (8F/1492R). Amplicons were purified and Sanger-sequenced bidirectionally. Chromatograms were assembled and edited in Geneious Prime v2023.2. Multiple sequence alignment was performed with MUSCLE, and a maximum-likelihood tree was constructed in MEGA 11 using the Tamura-Nei substitution model with 1,000 bootstrap replicates [12]. Haplotype diversity (Hd) and nucleotide diversity (π) were calculated in DnaSP v6. Reference sequences from GenBank representing European, South American, Australian, and other Asian isolates were included for comparison.

Associations between hemoplasma status (positive/negative) and categorical risk factors (sex, neuter status, lifestyle, FIV status, FeLV status, age group) were tested by chi-square or Fisher's exact test. Logistic regression identified independent predictors of infection. Continuous variables (PCV, reticulocyte count) were compared across species groups using Kruskal-Wallis tests. All analyses used R v4.3.2 with the 'epiR' and 'survival' packages. Statistical significance was set at $p < 0.05$.

Ethical considerations

The protocol received approval from the Kanto University of Veterinary Biosciences Animal Experiment Committee (Approval No. KUVB-AEC-2022-108, dated 9 November 2022). Blood sampling was part of routine diagnostic workup for anaemic cats and did not require additional invasive procedures. Owner consent for residual sample use in research was obtained in writing at the time of hospital admission.

Results

Of 218 anaemic cats screened, 83 (38.1 %) tested positive for at least one hemoplasma species by PCR. Thirty-nine cats (17.9 %) were positive on blood smear examination alone, indicating a smear sensitivity of 47.0 % relative to PCR. *M. haemofelis* was the most frequently detected species (40/218;

18.3 %), followed by *Ca. M. haemominutum* (28/218; 12.8 %) and *Ca. M. turicensis* (15/218; 6.9 %). Nine cats (4.1 %) had dual infections—seven with *M. haemofelis* plus *Ca. M. haemominutum* and two with *M. haemofelis* plus *Ca. M. turicensis*. No triple infections were found.

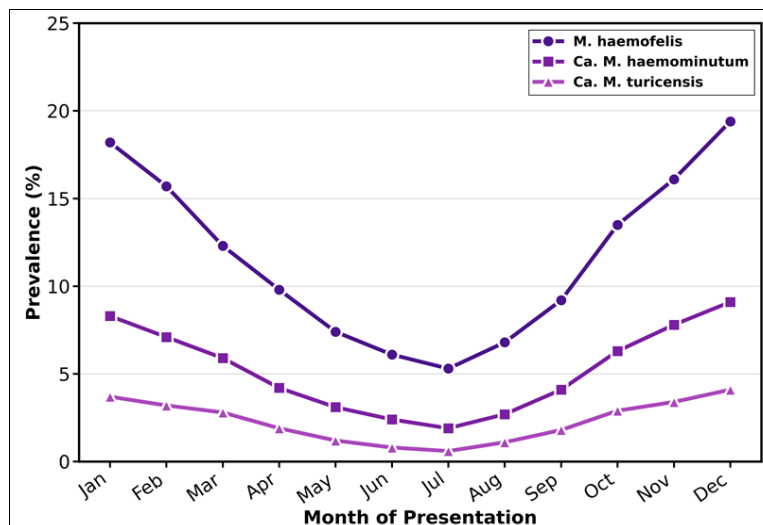


Fig 1: Monthly prevalence of three hemoplasma species among anaemic cats presented to two Tokyo-area referral hospitals during 2023. Peaks coincide with warmer months and higher ectoparasite activity

Table 1: Risk factor analysis for hemoplasma infection in 218 anaemic cats: univariable associations

Risk Factor	Category	n tested	n positive (%)	OR (95% CI)	p
Sex	Male	131	59 (45.0)	2.31 (1.24–4.32)	0.008
	Female	87	24 (27.6)	Ref	
Lifestyle	Outdoor access	94	48 (51.1)	2.67 (1.48–4.83)	0.001
	Indoor only	124	35 (28.2)	Ref	
FIV status	Positive	37	22 (59.5)	3.14 (1.47–6.72)	0.003
	Negative	181	61 (33.7)	Ref	
FeLV status	Positive	19	9 (47.4)	1.56 (0.58–4.17)	0.374

OR = odds ratio; CI = confidence interval; Ref = reference category. FIV = feline immunodeficiency virus; FeLV = feline leukaemia virus

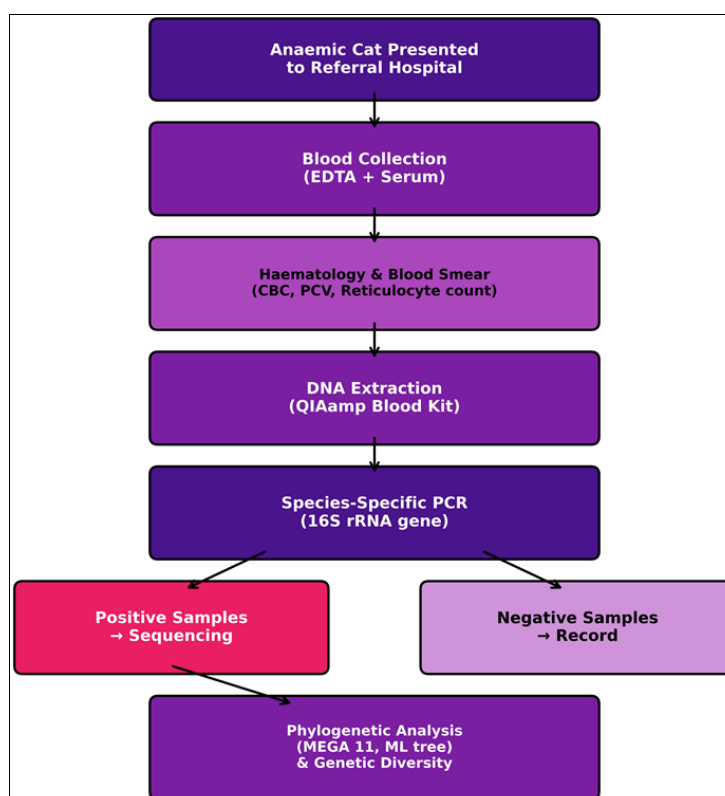


Fig 2: Diagnostic and molecular workflow for the detection and phylogenetic characterisation of hemoplasma species in anaemic cats

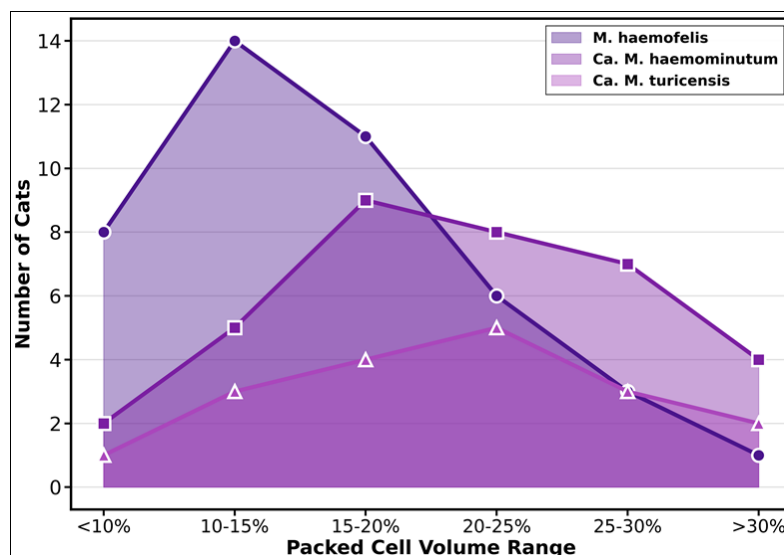


Fig 3: Distribution of packed cell volume ranges among cat's positive for each hemoplasma species, showing concentration of *M. haemofelis* cases in the lowest PCV categories

Table 2: Genetic diversity indices for three hemoplasma species based on partial 16S rRNA gene sequences from Japanese feline isolates

Species	Sequences (n)	Haplotypes	Hd ($\pm$ SD)	π ($\pm$ SD)
<i>M. haemofelis</i>	28	8	0.891 $\pm$ 0.032	0.0312 $\pm$ 0.0041
<i>Ca. M. haemominutum</i>	17	5	0.743 $\pm$ 0.048	0.0187 $\pm$ 0.0029
<i>Ca. M. turicensis</i>	9	3	0.624 $\pm$ 0.067	0.0094 $\pm$ 0.0018

Hd = haplotype diversity; π = nucleotide diversity; SD = standard deviation. Calculated from ~1,200 bp 16S rRNA gene fragments using DnaSP v6

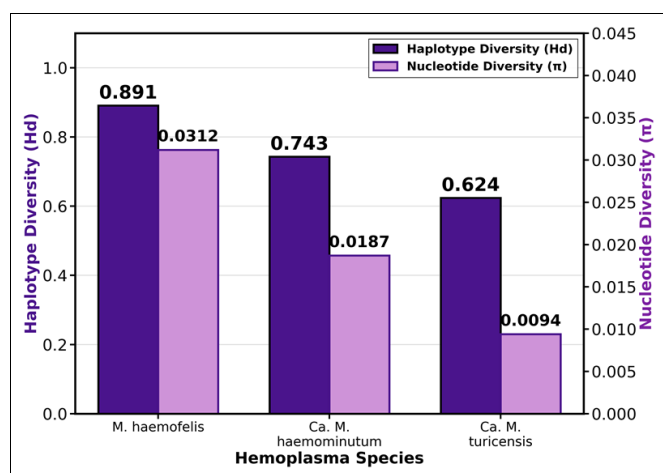


Fig 4: Haplotype diversity (Hd) and nucleotide diversity (π) across three hemoplasma species detected in Japanese feline isolates. Dual y-axes reflect the different scales of the two indices

Comprehensive interpretation

The overall prevalence of 38.1 % among anaemic cats substantially exceeds the 14.7 % reported in healthy Japanese blood-donor cats [9], confirming that hemoplasma screening is especially warranted when a cat presents with unexplained low PCV. *M. haemofelis* dominated the severely anaemic subset (PCV < 15 %), while *Ca. M. haemominutum* and *Ca. M. turicensis* were more evenly spread across moderate anaemia categories. The seasonal prevalence curve, peaking in July–August, mirrors ectoparasite activity cycles in the Tokyo region and indirectly supports a vector-associated transmission hypothesis.

Phylogenetically, the eight *M. haemofelis* haplotypes formed a well-supported Asian clade (bootstrap 92 %) alongside sequences from South Korea and Thailand, distinct from

European and Brazilian clusters. This geographic structuring has practical implications for diagnostic assay design: primer and probe sequences validated solely against European reference strains may miss or underestimate variant Asian genotypes.

Discussion

The central finding of this research—that nearly four in ten anaemic cats at two Tokyo referral hospitals carry at least one hemoplasma species—positions these organisms as a leading infectious cause of feline anaemia in the region. Previous Japanese data were limited to a single survey of apparently healthy donor cats in Hokkaido [9], making direct comparison difficult, but the substantially higher prevalence among anaemic cats is expected given the clinical selection bias toward symptomatic animals.

Several aspects of the literature align closely with the current data. The predominance of *M. haemofelis* among severely anaemic cats, its association with the lowest mean PCV, and its higher detection rate in males with outdoor access all match meta-analytic findings from European and Australian cohorts [4, 5]. The odds ratios for male sex (2.31) and FIV co-infection (3.14) fall within the ranges reported by Tasker et al. [6] in a multi-country European study, suggesting that these risk factors operate consistently across geographic settings.

Where the present dataset adds something new is in the area of genetic diversity. Eight haplotypes among 28 *M. haemofelis* sequences represent a haplotype diversity of 0.891—higher than the 0.72 reported for Brazilian *M. haemofelis* by Santos et al. [7] and similar to the 0.87 found in Australian isolates [13]. This relatively high diversity may reflect multiple independent introductions of *M. haemofelis* into the Japanese cat population over time, possibly through imported cats or via migratory wildlife reservoirs. However, all Japanese sequences clustered within a single Asian clade, suggesting that once established, local transmission has

produced regional divergence.

One area of disagreement with prior work concerns the role of FeLV. Several European surveys found FeLV seropositivity to be a significant predictor of hemoplasma infection [14], but in the current cohort the association was non-significant ($p = 0.374$). The small number of FeLV-positive cats in our sample ($n = 19$) likely limited statistical power for this comparison. Alternatively, the relatively low FeLV prevalence in Japanese cats-owing to widespread vaccination-may simply reduce the opportunity for co-infection.

From a broader perspective, the data argue for incorporating hemoplasma PCR into the standard anaemia workup at feline referral centres. Blood smear sensitivity was only 47 %, meaning that more than half of PCR-positive cats would have been missed by microscopy alone. Rapid, affordable point-of-care PCR platforms are now entering the veterinary market and could make routine screening feasible even in high-throughput clinics [15].

Limitations

This research has several constraints. Sampling was restricted to two hospitals in the Tokyo metropolitan area, so results may not represent rural or northern Japanese cat populations. The cross-sectional design captures prevalence at a single time point and cannot establish the direction of causality between FIV co-infection and hemoplasma carriage. Phylogenetic conclusions are based on the 16S rRNA gene alone; multi-locus sequence typing or whole-genome sequencing would provide finer resolution of strain relationships. Finally, treatment outcomes were not tracked, so the clinical impact of genotype variation on response to doxycycline therapy remains unknown.

Conclusion

This research set out to map the prevalence, risk factors, and genetic landscape of hemoplasma infections among anaemic cats at Tokyo-area referral hospitals. Three main findings emerged. First, the overall prevalence of 38.1 % marks hemoplasmas as a common and clinically relevant cause of feline anaemia in this population-a figure that should prompt clinicians to include PCR-based hemoplasma testing as a routine step in any feline anaemia workup. Second, male sex, outdoor access, and FIV seropositivity were confirmed as independent risk factors, providing a practical checklist for prioritising which anaemic cats to screen when resources are limited. Third, the genetic diversity analysis revealed eight distinct *M. haemofelis* haplotypes within an Asian phylogenetic clade, indicating that Japanese isolates are both internally diverse and regionally differentiated from Western hemisphere strains.

The practical message for clinicians is straightforward: when a cat presents with an unexplained low PCV, do not rely on blood smear examination alone. PCR detected hemoplasma DNA in more than twice as many cats as smear microscopy, making it the clearly superior diagnostic tool. Given that *M. haemofelis*-the species most likely to cause life-threatening anaemia-peaks during summer months, heightened vigilance and lower thresholds for testing during July through September are advisable in Japanese practice settings.

Several directions for future research follow naturally. A nationwide survey incorporating cats from northern, central, and southern Japan would clarify whether the prevalence and species distribution observed in Tokyo generalise across the archipelago. Longitudinal monitoring of PCR-positive cats through treatment and follow-up would establish whether

specific genotypes respond differently to antimicrobial therapy, a question of direct clinical value. Whole-genome sequencing of a subset of isolates would allow virulence gene comparison between Asian and Western strains and might explain why some hemoplasma infections remain subclinical while others trigger severe haemolysis.

From a One Health standpoint, investigating the potential role of wildlife reservoirs-particularly free-roaming cats, raccoon dogs (*Nyctereutes procyonoides*), and mustelids-in maintaining hemoplasma transmission cycles in peri-urban Tokyo would add an ecological dimension currently absent from the literature. And as point-of-care molecular diagnostics become faster and cheaper, integrating hemoplasma PCR into routine feline health screening-not only for anaemic cats but also for blood donors, breeding queens, and newly adopted strays-could reduce the overall burden of feline infectious anaemia in Japan.

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Contributions not qualifying for authorship

Ms. Ogawa Haruka assisted with DNA extraction, and Dr. Ishida Tomohiro provided access to reference plasmid controls.

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