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Use of Conventional and Real-Time Polymerase Chain Reaction to Determine the Epidemiology of Hemoplasma Infections in Anemic and Nonanemic Cats

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Background: The goals of this study were to develop and apply conventional (c) and real-time TaqMan polymerase chain reaction (PCR) assays for *Mycoplasma haemofelis* (*Mhf*), '*Candidatus* Mycoplasma haematoparvum' (*Mhp*), and '*Candidatus* Mycoplasma haemominutum' (*Mhm*) to blood samples of cats to determine the epidemiology of these infections in cats.

Hypothesis: Cats are infected with > 2 hemoplasma species, and organism load correlates with disease induced by these organisms.

Animals: Blood samples from 263 anemic and nonanemic cats were used.

Methods: A retrospective study was conducted.

Results: Forty-seven (18%) samples were positive. Three samples (1%) yielded 170 base pair cPCR products, 1 of which was positive for *Mhf* using real-time PCR. Forty-four samples (17%) yielded 193 base pair cPCR products, 40 of which were positive for *Mhm* using real-time PCR. Organism loads ranged from 375×10^6/mL to 6.9×10^6/mL of blood. Sequencing of cPCR products from samples testing negative using real-time PCR identified 2 *Mhp*-like sequences, 1 *Mhm*-like sequence, and 1 sequence resembling '*Candidatus* Mycoplasma turicensis.' Cats infected with *Mhm* were less likely to be anemic than uninfected cats. Older age, outdoor exposure, feline immunodeficiency virus (FIV) seropositivity, cutaneous squamous cell carcinoma (SCC), and stomatitis were associated with *Mhm* infection. Cats from the Sacramento Valley were more often infected with *Mhm* than cats from the San Francisco bay area.

Conclusions and Clinical Importance: Cats may be infected with 4 hemoplasma species. The association between *Mhm* infection, FIV, and SCC may reflect outdoor roaming status of infected cats. The clustered distribution of infection suggests an arthropod vector in transmission.

Key words: Cat; Divergence; Haemobartonella; Infectious; Quantitative.

Three different species of epierythrocytic mycoplasmas (hemoplasmas) are known to infect cats. Infection with *Mycoplasma haemofelis* (*Mhf*) has been associated with severe hemolytic anemia in immunocompetent cats. In contrast, infection with '*Candidatus* Mycoplasma haemominutum' (*Mhm*), a smaller organism, has not yet been associated with disease in immunocompetent cats.[1–3] However, cats coinfected with *Mhm* and feline leukemia virus (FeLV) are more likely to develop clinically relevant anemia than are cats infected with *Mhm* alone, and there is some evidence of acceleration of the development of myeloproliferative disease in cats concurrently infected with FeLV.[4] Recently, an additional hemoplasma, '*Candidatus* Mycoplasma turicensis' (*Mtc*), was detected in Swiss cats.[5,6] Sequencing and phylogenetic analysis of the 16S rRNA gene of this organism revealed that it was more closely related to *Mycoplasma coccoides* than to other hemoplasmas in cats, and inoculation of specific-pathogen free (SPF) cats with *Mtc* resulted in anemia.

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The authors' laboratory has identified a new small hemoplasma, '*Candidatus* Mycoplasma haematoparvum' (*Mhp*), which was detected in a splenectomized dog with hemic neoplasia.[7] This organism is more closely related to *Mhm* than *Mycoplasma haemocanis*. Whether this organism infects cats is unknown.

The advent of polymerase chain reaction (PCR) technology has improved the ability to detect hemoplasmas, which have never been successfully cultured. A number of studies using conventional PCR (cPCR) have employed an assay that differentiates between *Mhm* and *Mhf* on the basis of amplicon length after agarose gel electrophoresis.[2,8] However, at the time of the cPCR assay development, *Mhp* and *Mtc* had not yet been described, and this assay would not be expected to distinguish between *Mhm* and *Mhp* or *Mhf* and *Mtc*. In addition, it cannot be used to quantify organism burden in infected cats. To overcome these problems, the authors developed specific real-time TaqMan PCR assays for *Mhf*, *Mhp*, and *Mhm*.

The goals of this study were (1) to compare the results of a conventional hemoplasma PCR assay[2] to those of newly designed real-time TaqMan assays for *Mhf*, *Mhp*, and *Mhm* when applied to blood samples collected from cats presenting to the University of California, Davis, Veterinary Medical Teaching Hospital (VMTH); (2) to use the results to determine the prevalence of these infections in cats and to determine whether any correlation exists between infection, organism burden, and clinical presentation, and (3) to determine whether other hemoplasmas might exist in North American cats by sequencing amplicons generated from samples testing positive using cPCR and negative using real-time PCR.

Materials and Methods

Selection Criteria

EDTA anticoagulated blood samples were obtained during a 1-year period (June 2002 to May 2003) from 263 cats presented to the VMTH and from whom CBCs were taken. These consisted of 135 cats in which anemia (hematocrit <26%) was present based on analysis of the CBC, and 128 cats in which the hematocrit on the CBC was ≥26%. Samples from anemic and nonanemic cats were collected concomitantly. Samples were stored at 4°C until processed within 1–2 weeks of sample collection.

For each cat, hematologic variables on the CBC and the presence of organisms consistent with hemoplasmas were recorded as documented in the CBC performed at the time of sampling. Approximately 12–18 months after sample collection, the electronic database of the VMTH was searched for each patient, and the city of residence, age, sex, breed, environment, number of cats in the household, recent administration of antimicrobials, and ultimate clinical diagnosis relating to the visit at which testing for hemoplasmas was performed were recorded. Cats were categorized as being purebred or non-purebred, indoor and outdoor or indoor only, and from households containing <3 or ≥3 cats. The cities of residence were grouped into those from the San Francisco bay area (including Marin, Sonoma, Napa, Solano, Contra Costa, Alameda, Santa Clara, Santa Cruz, San Mateo and San Francisco counties), the Sacramento Valley area (including Yolo and Sacramento counties), the eastern Sacramento and Sierra Nevada foothills area (including Placer, Amador, and El Dorado counties), the central valley north of Sacramento county, northwestern California (including Humboldt and Del Norte counties), the central valley south of Sacramento county (including the San Joaquin, Stanislaus, Merced, and Fresno counties), the Lake Tahoe area, and southern California.

Conventional PCR

DNA was extracted from 200 μL of blood as previously described,[7] with a final elution volume of 200 μL. cPCR was performed as previously described.[2] Positive controls consisted of infected blood samples from cats that had been experimentally infected with *Mhf* and *Mhm*. Negative controls included both negative thermocycling controls, whereby sample DNA in the reaction mixture was replaced with purified DNase-free, RNase-free water and negative extraction controls, whereby blood from a healthy adult dog that had previously tested negative using cPCR for hemoplasmas was subjected to the extraction and thermocycling process to ensure that contamination did not occur during the extraction process. Samples were analyzed on a 2.4% agarose gel. Samples yielding products of approximately 170 base pairs (bp) were considered positive for a large hemoplasma (such as *Mhf* or *Mtc*), and samples yielding products of approximately 193 bp were considered positive for a small hemoplasma (such as *Mhm* or *Mhp*). Because *Mhm* and *Mhf* can be difficult to differentiate based on their size[3,8] and because the size of *Mtc* is unknown,[5] the terms large and small were arbitrarily replaced with Group I and Group II, respectively.

Real-time PCR Assays

Three separate real-time PCR assays were developed for detection of the 16S rRNA gene of *Mhm*, *Mhf*, and *Mhp*. TaqMan PCR was carried out using a 7700 ABI PRISM SDS instrument and a commercially available Universal Mastermix[a] containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 5 mM $MgCl_2$; 300 μM each of dATP, dCTP, and dGTP; 600 μM dUTP; 0.25 U uracile *N*-glycosylase (UNG); and 0.625 U of AmpliTaq Gold DNA polymerase per reaction, 400 nM of each primer, 80 nM of fluorogenic TaqMan probe, and 5 μL of diluted template. Amplification was performed using the default cycling parameters of the manufacturer's real-time PCR platform: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Data collection and processing were performed in an attached computer using ABI Sequence Detection System software.

The assay for *Mhf* used forward and reverse oligonucleotides previously published for conventional hemoplasma PCR,[2] as well as a specific probe that should not bind to amplicons generated from *Mhm* or *Mhp*. An additional set of forward and reverse oligonucleotides was used to detect *Mhm* and *Mhp*, together with specific probes that enabled differentiation between these 2 organisms based on differences in the sequence between the 2 oligonucleotides (Table 1).

The full-length 16S rRNA gene of *Mhp*, a 1,022-bp fragment of the 16S rRNA gene of *Mhm*, and a 995-bp fragment of the 16S rRNA gene of *Mhf* were amplified, cloned into commercial plasmids, and sequenced as previously described.[9,10] The partial sequences amplified for *Mhm* and *Mhf* included the region of the target sequence for all 3 real-time PCR assays.

Each of the 3 plasmids was used to construct plasmid standard curves for assay validation. Plasmid copy numbers were determined using spectrophotometry. Serial 10-fold dilutions of purified plasmid were diluted in carrier DNA.[b] Dilutions were performed 3 times, and each dilution was analyzed in triplicate. Analytical specificity was determined by testing the plasmids containing *Mhf*, *Mhm*, and *Mhp* DNA with each assay, as well as DNA extracted from uninfected SPF cats; cats that were naturally and experimentally infected with *Mhf*, *Mhm* or both; and a dog that was naturally infected with *Mhp*. The infection status of these patients was ascertained based on the results of cPCR and sequencing of the PCR products. All real-time TaqMan PCR assays were validated by running standard curves on positive controls to calculate PCR amplification efficiencies, where doubling of the amount of DNA at each cycle is equivalent to 100% efficiency. The standard curve generated using a plasmid positive control was compared with that generated after extraction of genomic DNA from whole blood. Intra-assay and interassay precision were measured by triplicate analysis of aliquotted DNA samples on the same or on separate plates, and coefficients of variation were calculated.

A TaqMan PCR system specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cats as previously described[11] was used to test for the integrity of extracted genomic DNA. Positive controls for each species and negative controls were included in each run. Quantification of the number of copies of organism DNA present in each sample was performed using the standard curve generated by serial dilutions of plasmid DNA template containing known copy numbers of either *Mhf* or *Mhm* and expressed as copy numbers of DNA template per milliliter of blood.[10] In addition, the real-time PCR system targeting the GAPDH pseudogene was used to quantify nucleated blood cells of cats by extrapolating the GAPDH cycle threshold value to a standard curve generated with genomic DNA extracted from known numbers of white blood cells of cats. The hemoplasma load and the number of nucleated cells then were combined into a unit hemoplasma load per million nucleated cells as previously described.[12,13]

The results of real-time PCR were compared to those using cPCR. When the results of real-time PCR and cPCR did not agree, both assays were repeated. For the purpose of epidemiologic analysis, results were considered positive only if 2 or 3 of the 4 assays performed on each discordant sample were positive. PCR products from samples with repeatedly discordant results were sequenced using Prism dye-terminator chemistry and an ABI 377 DNA sequencer.[c]

Table 1. Sequence of PCR primers and TaqMan probes.

Target	Primer	Sequence (5′→3′)	Length	Probe	Probe[a] sequence (5′→3′)
Feline GAPDH	GAPDH.94f	GCCGTGGAATTTGCCGT	81	GAPDH.118p	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA
	GAPDH.174r	GCCATCAATGACCCCTTCAT			
Canine GAPDH	cGAPDH.395f	GATGGGCGTGAACCATGAG	131	cGAPDH.427p	CCCTCAAGATTGTCAGCAATGCCTCCT
	cGAPDH.525r	TCATGAGGCCCTCCACGAT			
Mhp	Mhp-84f	GGCGAACGGGCGAGTAA	164	CMhp-192p	CCTTCGGGAGCCCCGCGC
	Mhp-247r	ACCCCACCAACAACCtAATAGGA			
Mhm	Mhm-68f	GGCGAACGGGCGAGTAA	168	CMhm-177p	TGGGAAACTAGAGCTTCGCGAGCAG
	Mhm-235r	ACCCCACCAACAACCTAATAGGA			
Mhf	Mhf-355f	ACGAAAGTCTGATGGAGCAATA	194	Mf-418p	TACGAGGGATAATTATGATAGTACTTCGTGA
	Mhf-548r	ACGCCCAATAATCCGRATAAT			

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *Mhp*, '*Candidatus* Mycoplasma haematoparvum'; *Mhm*, '*Candidatus* Mycoplasma haemominutum'; *Mhf*, *Mycoplasma haemofelis*.
[a] TaqMan probes were labeled with 6-FAM at the 3′ and TAMRA at the 5′ end.

Statistical Analysis

The unpaired *t*-test was used to detect differences in continuous variables between groups. Equality of variance between groups was examined using an F test. Two-tailed chi-squared analysis (with Yates' correction when 1 or more frequencies were <20%) was used to detect group differences using frequency data. The relationship between organism burden and hematologic variables and between organism burden per milliliter of blood and organism burden per million nucleated cells was tested using the Spearman rank correlation test. Significance was defined as $P < .05$. All analyses were performed using standard statistical software.[d] Where appropriate, results are expressed as mean ± standard deviation.

Results

Case Characteristics

The mean age of the patient population was 10.2 ± 5 years (n = 259). The sex was known for 262 cats: 110 were females (101 neutered, 9 intact), and 152 were males (145 neutered, 7 intact). Twenty-seven cats were purebred, and 236 cats were nonpurebred. All cats were ill except for 2 cats, which were being evaluated as potential blood or kidney donors. All except 5 cats were from California; 4 cats were from Nevada, and 1 was from Pennsylvania. A total of 102 cats were from the San Francisco bay area, 115 cats were from the Sacramento Valley area, 13 cats were from eastern Sacramento and the foothills, 14 cats were from the southcentral valley, 7 cats were from southern California, 3 cats were from the northcentral valley, 3 cats were from northwestern California, and 1 cat was from Lake Tahoe.

PCR Results

The limit of quantitation for the *Mhp*, *Mhm*, and *Mhf* real-time TaqMan PCR assays was 10 copies of the standard plasmid. This was consistent and highly reproducible. Amplification of the plasmid standard dilutions over 8 orders of magnitude revealed linearity across the entire range. The amplification efficiencies and coefficients of variation for detection of *Mhp, Mhm*, and *Mhf* are shown in Table 2. Amplification efficiency determined on dilution series of DNA extracted from whole blood samples differed less than 5% when compared with the plasmid standard curve, enabling accurate calculation of genomic hemoplasma DNA load in clinical samples from the standard curve generated with the plasmids. No cross reactions were detected between assays when performed on clinical and plasmid samples containing 16S rDNA from each hemoplasma species (Table 3).

GAPDH of cats was amplified successfully from all samples using real-time PCR. A comparison of the organism load per milliliter of blood with the organism load per million nucleated cells gave a very high correlation ($P < .0001$, $r_s = 0.95$, 95% CI 0.90–0.98).

Forty-four cats (17%) initially tested positive using real-time PCR, including 5 cats that were positive for *Mhf* and 39 cats that were positive for *Mhm*. Forty-five cats (17%) initially tested positive using cPCR, including 3 cats that were positive for Group I hemoplasmas and

Table 2. Amplification efficiencies, interassay, and intra-assay coefficients of variation for real-time TaqMan PCR assays for detection of *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum,' and '*Candidatus* Mycoplasma haematoparvum.'

	Mhp	*Mhm*	*Mhf*
Amplification efficiency using genomic DNA	97.1%	100.2%	97.2%
Amplification efficiency using plasmid DNA	95.7%	95.9%	95.2%
Interassay CV			
Based on CT value	0.6%	1.2%	1.8%
Based on absolute value[a]	5.4%	9.4%	14.6%
Intra-assay CV			
Based on CT value	0.5%	0.2%	0.3%
Based on absolute value	8.8%	2.8%	5.5%

Mhp, '*Candidatus* Mycoplasma haematoparvum'; *Mhm*, '*Candidatus* Mycoplasma haemominutum'; *Mhf*, *Mycoplasma haemofelis*; CV, coefficient of variation; CT, cycle threshold.

[a] After conversion of CT values to copy numbers through extrapolation to the standard curve.

42 cats that were positive for Group II hemoplasmas. Thirty-four of the cats were positive using both cPCR and real-time PCR, and 208 cats were negative using both assays. The sensitivity and specificity of the combined real-time PCR assay results compared with cPCR were 76% and 95%, respectively.

Discordant results were noted in 21 (8%) samples. Ten samples initially were positive using real-time PCR but negative using cPCR. When retested, 4 of these 10 samples tested positive for *Mhm* using both assays. Hemoplasma copy numbers in these 4 samples were 585, 2.4×10^5, 3.3×10^5, and 5.2×10^5/mL, respectively.

Table 3. Specificity of TaqMan assays for canine and feline hemoplasmas when tested against DNA from closely related hemoplasma strains.

	TaqMan assay CT values			
DNA source	*Mhm*	*Mhp*	*Mhf*	GAPDH[a]
Mhm cloned 16S rDNA	12.89	—	—	—
Mhf cloned 16S rDNA	—	—	12.76	—
Mhp cloned 16S rDNA	—	12.75	—	—
Mhm infected VMTH cat	25.69	—	—	25.33
Mhf infected VMTH cat	—	—	26.03	28.38
Mhp infected VMTH dog	—	24.56	—	19.04
SPF cat infected with *Mhm*	14.77	—	—	27.99
SPF cat infected with *Mhf*	—	—	25.88	27.33
Uninfected SPF cat	—	—	—	25.68

CT, Cycle threshold. CT values <40 are considered positive; *Mhm*, '*Candidatus* Mycoplasma haemominutum'; *Mhp*, '*Candidatus* Mycoplasma haematoparvum'; *Mhf*, *Mycoplasma haemofelis*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SPF, specific pathogen free.

[a] A cat-specific GAPDH TaqMan assay was used for blood samples in cats; a canine specific GAPDH TaqMan assay was used for the canine blood sample.

The remaining 6 samples (with copy numbers of 260, 270, 275, 328, 330, and 1,248/mL on initial analysis) subsequently tested negative using both assays and were classified as negative. Two of these 6 samples initially were positive for *Mhm* using real-time PCR, and 4 were positive for *Mhf*. The other 11 of the 21 discordant samples initially were positive using cPCR but negative using real-time PCR. Two of these 11 samples were negative using both assays on repeated analysis, and 2 were positive for *Mhm*, with copy numbers of 4,260 and 2.9×10^4/mL. One sample became negative using cPCR and positive using real-time PCR for *Mhm* with a copy number of 702/mL. In the remaining 6 samples, the results of cPCR were repeatedly positive despite repeatedly negative real-time PCR results. After retesting of discordant results, the sensitivity and specificity of the combined real-time PCR assay results compared with cPCR were 87% and 99.5%, respectively.

Using the combined results of conventional and real-time PCR after retesting, 47 cats (18%) were considered positive for hemoplasma DNA. All of these cats had positive cPCR results either on initial or subsequent runs. The results of cPCR indicated that 3 of these cats (1%) tested positive for Group I hemoplasmas, and 44 of these cats (17%) tested positive for Group II hemoplasmas. Coinfections with multiple hemoplasma species were not identified using any assay. One additional cat that tested negative using all PCR assays was diagnosed with hemoplasmosis 2 weeks before presentation based on clinical findings and the results of cytologic examination of blood smears, but was being treated with doxycycline at the time of sample collection. This cat also had FeLV-associated erythroleukemia.

Of the 3 samples testing positive for Group I hemoplasmas, 1 sample was positive using real-time PCR for *Mhf*. The hemoplasma copy number in this sample was 5.6×10^5/mL. The remaining 2 samples were repeatedly negative using real-time PCR. Sufficient DNA was amplified from 1 of these 2 samples to permit direct sequence analysis. The DNA sequence of this PCR product had 98% homology with that of *Mtc* (GenBank accession number AY831867).

Of the 44 samples testing positive for Group II hemoplasmas, 40 (15%) tested positive using real-time PCR for *Mhm*, and no samples tested positive for *Mhp*. The hemoplasma copy number in samples testing positive for *Mhm* using real-time PCR ranged from 375×10^6 to 6.9×10^6 ($7.3 \times 10^5 \pm 1.6 \times 10^6$)/mL. Sufficient DNA was amplified from 3 of the remaining 4 samples using cPCR to permit direct sequence analysis. The DNA sequence of 1 product had 99% homology with that of *Mhm* (*Mhm*-like) (GenBank accession number AY297712), and the sequence of the remaining 2 products had 99% homology with that of *Mhp* (*Mhp*-like) (Genbank accession number AY532390). Sequencing of a 224-bp fragment from the *Mhm*-like isolate that included the probe and primer binding regions revealed 100% identity to a variant strain of *Mhm* (Genbank accession number AY297712), which was not expected to be detected using the assay reported here because of divergence in the region targeted by the *Mhm* probe.

Table 4. Results of CBC analysis in 41 cats testing positive for infection with '*Candidatus* Mycoplasma haemominutum' and 215 cats testing negative for hemoplasmas using conventional and real-time PCR.

Variable	PCR test result	Number	Mean ± SD	Range	No. (%) with low values	No. (%) with high values	Reference range
Hematocrit (%)	Positive	41	31 ± 10	14–59	15 (37)	1 (2)	26–50
	Negative	215	27 ± 8[b]	10–59	115 (44)[c]	1 (0.005)	
Reticulocyte count[a]	Positive	41	33,650 ± 18,950	3,900–75,900	1 (2)	5 (12)	7,000–60,000
	Negative	213	37,130 ± 51,450	2,200–505,400	17 (8)	28 (13)	
MCV (fL)	Positive	41	46.8 ± 5.3	38.2–68.6	5 (12)	4 (10)	42–53
	Negative	215	46.7 ± 5.1	34.6–64.9	22 (10)	31 (14)	
MCHC (g/dL)	Positive	41	32.4 ± 1.0	30.4–34.2	0	8 (20)	30–33.5
	Negative	215	32.4 ± 1.3	27.2–36.5	8 (4)	30 (14)	
RDW (%)	Positive	41	16.5 ± 1.8	13.6–22.9	1 (2)	6 (15)	14–18
	Negative	214	17.1 ± 2.5	13.5–27.7	5 (2)	60 (28)	
Total white cells[a]	Positive	41	13,240 ± 9,859	700–46,480	1 (2)	12 (29)	4,500–14,000
	Negative	215	12,820 ± 9,619	910–75,630	16 (7)	70 (33)	
Segmented neutrophils[a]	Positive	41	10,180 ± 9,008	294–43,230	1 (2)	17 (41)	2,000–9,000
	Negative	212	10,020 ± 8,662	309–71,090	5 (2)	85 (40)	
Band neutrophils[a]	Positive	41	313 ± 687	0–2,974	NA	14 (34)	0–rare
	Negative	212	195 ± 561	0–4,176	NA	58 (27)	
Monocytes[a]	Positive	41	598 ± 1,154	0–6,816	1 (2)	8 (20)	50–600
	Negative	212	415 ± 565	0–6,300	5 (2)	41 (19)	
Lymphocytes[a]	Positive	41	1,708 ± 1,179	143–5,036	10 (24)	0 (0)	1,000–7,000
	Negative	212	1,749 ± 2,350	0–28,740	80 (38)	4 (2)	
Eosinophils[a]	Positive	41	364 ± 57	0–1,774	13 (32)	1 (2)	150–1,100
	Negative	212	399 ± 41	0–5236	81 (38)	17 (8)	
Platelets[a]	Positive	31	329,000 ± 176,000	4,000–708,000	6 (15)	5 (12)	180,000–500,000
	Negative	178	306,800 ± 182,300	19,000–1,170,000	41 (23)	15 (8)	

PCR, polymerase chain reaction; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

[a] Cells/μL.

[b] Statistically significant with $P < .005$.

[c] Statistically significant with $P < .05$.

Organisms were not noted using cytology in any blood smear. All 3 cats infected with Group I hemoplasmas were anemic. One cat had lymphosarcoma and was receiving chemotherapy, 1 had FeLV-related myeloproliferative disease, and the remaining cat was being treated for a nasal squamous cell carcinoma (SCC). The results of the CBC in cats testing positive for *Mhm* and cats testing negative for hemoplasmas are shown in Table 4. Anemia was more frequent in cats testing negative than in cats testing positive for *Mhm* (115/215 [44%] versus 15/41 [37%], OR = 2.0, 95% CI = 1.0–4.0, $P = .047$), and the mean hematocrit was greater in the cats testing positive than in cats testing negative ($P = .003$). When cats with chronic renal failure were removed from the analysis, this difference still was significant ($P = .011$).

Examination of the correlation between *Mhm* copy number and each hematologic variable shown in Table 4 disclosed no correlation between organism burden and any hematologic variable.

Apart from hematocrit, there was no difference in the mean value of any hematologic variable between (1) cats testing positive for *Mhm* and (2) cats testing negative for hemoplasmas. The proportions of cats in each of these 2 groups with hematologic values greater or less than the reference range was not different for any of the variables except hematocrit.

A comparison of the distribution of values for each hematologic variable between cats testing positive for *Mhm* and cats testing negative for hemoplasmas indicated significant differences for reticulocyte count ($P < .001$); red cell distribution width (RDW) ($P = .019$); and nucleated red blood cell ($P < .001$), lymphocyte ($P < .001$), eosinophil ($P < .001$), and monocyte ($P < .001$) counts. Moderate to marked reticulocytosis, anisocytosis, normoblastosis, lymphocytosis, and eosinophilia were not present in cats testing positive (Fig 1). The reticulocyte response was similarly blunted in anemic cats testing positive for *Mhm* when compared with anemic cats testing negative for hemoplasmas ($P < .001$).

Several variables were analyzed for an association with *Mhm* infection (Table 5). Factors associated with infection were outdoor access (OR = 3.4, 95% CI = 1.4–8.5, $P = .006$), feline immunodeficiency virus (FIV) infection (OR = 6.8, 95% CI = 1.7–26.3, $P = .007$), SCC

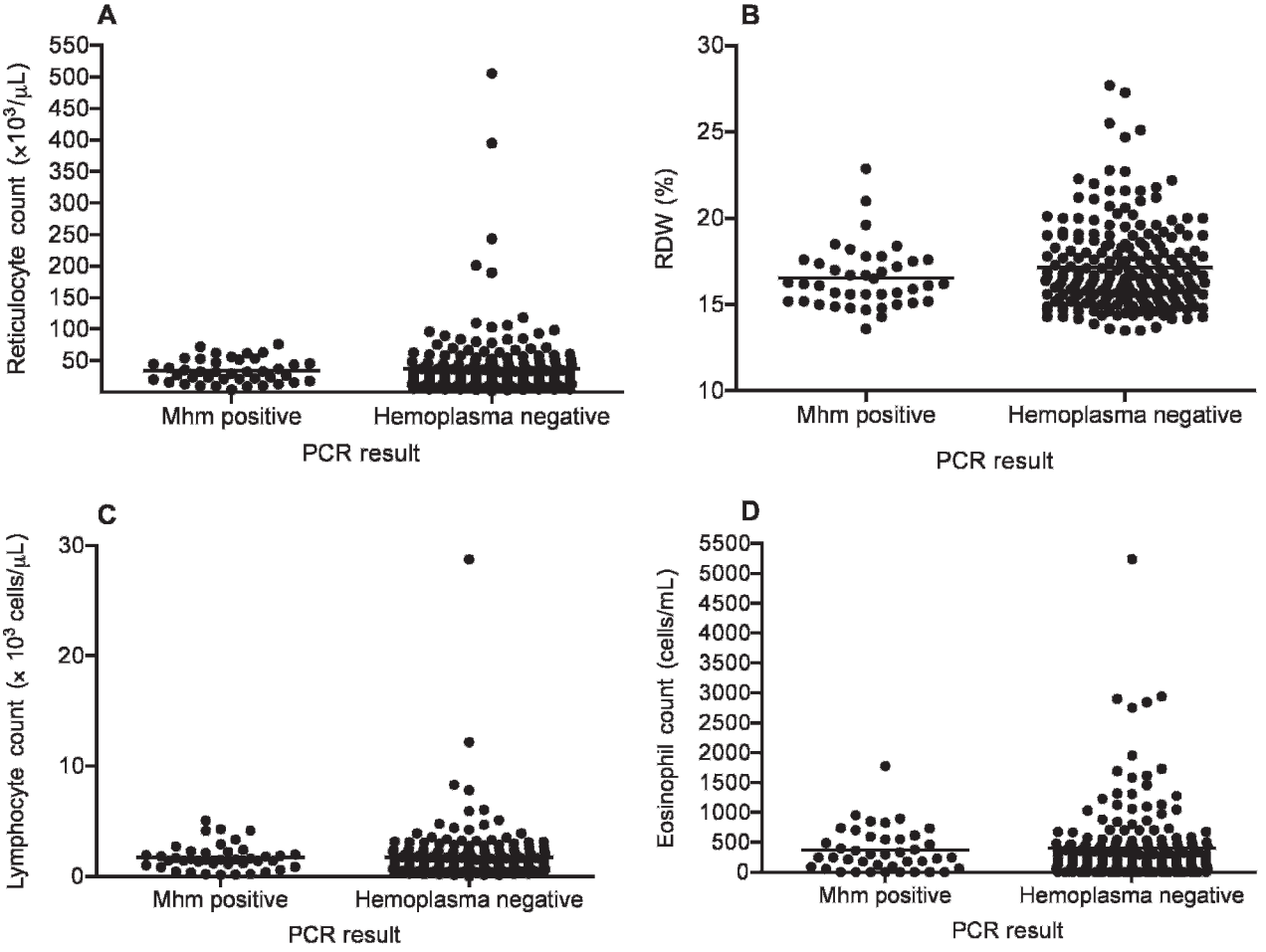

Fig 1. Distribution of selected hematologic variables in cats testing positive using PCR for '*Candidatus* Mycoplasma haemominutum' versus those testing negative for hemoplasmas. **(A)** Reticulocyte count. **(B)** Red cell distribution width. **(C)** Lymphocyte count. **(D)** Eosinophil count.

of the pinna or planum nasale (OR = 5.3, 95% CI = 1.3–22.0, $P = .041$), and stomatitis (OR = 7.8, 95% CI: 1.3–48.1, $P = .047$). Although there was no association between *Mhm* and FeLV infection, when all cats testing positive for hemoplasmas were considered, there was a significant association with FeLV infection ($P < .001$, OR = 44, CI = 2.3–824.6). The association between *Mhm* infection and stomatitis represented an interaction between FIV and stomatitis, as it disappeared when the FIV-positive stomatitis cases were removed from the analysis. The mean age of cats testing positive (11.7 ± 0.7, n = 41) was higher than that of cats testing negative (10.0 ± 0.4, n = 212) ($P = .040$). A total of 9/102 (8%) cats from the bay area were positive for *Mhm*. This was significantly different from the prevalence in the Sacramento Valley (22/111 [21%], OR = 0.4, 95% CI = 0.2–0.9, $P < .02$). The proportion of cats with access to the outdoors in these 2 areas was identical (48/89 versus 39/72, $P = .976$). The prevalence of infection in the southcentral valley, eastern Sacramento/the foothills, southern California, the northcentral valley and northwestern California was 1/12 (14%), 4/13 (31%), 1/7, 1/3, and 0/3, respectively.

Discussion

This study has identified the presence of 4 hemoplasma species in North American cats. In addition to *Mhf* and *Mhm*, 1 cat was infected with a *Mtc*-like hemoplasma, and 2 cats were infected with an *Mhp*-like organism. The prevalence of infection was 16% for *Mhm*, 0.7% for the *Mhp*-like organism, 0.4% for *Mhf*, and 0.4% for *Mtc*. These prevalence data approximate those found in Colorado and the United Kingdom,[2,8] and are slightly lower than the 8% and 22% reported for Group I and II hemoplasmas, respectively, in 92 cats from Alabama, Texas, and Maryland.[14] A recent study of Swiss cats using real-time PCR found a lower prevalence of *Mhm* (8.5%), and the prevalence of *Mhf* and *Mtc* were 0.5% and 1%, respectively.[6] The assay used in that study did not differentiate between *Mhm* and *Mhp*.

Mtc was first identified as a result of discrepant PCR results.[5] The infected cat in the current study was coinfected with FeLV and presented with traumatic wounds and moderate nonregenerative anemia. *Mhp* was initially detected in a splenectomized dog with hemic neoplasia.[7] None of the blood samples in this study were positive for *Mhp* using real-time PCR, but

Table 5. Association between clinical variables and positive results using conventional and real-time PCR for '*Candidatus* Mycoplasma haemominutum'.

Factor	Proportion of positive cats (%)	Proportion of negative cats (%)	*P* value	Odds ratio	95% Confidence interval
Nonpurebred cats	38/41 (93)	192/215 (89)	.511	1.5	0.4–5.3
>2 cats in the household	18/28 (64)	73/146 (50)	.166	1.8	0.8–4.1
Male sex	29/41 (71)	120/214 (56)	.081	1.9	0.9–3.9
Indoor only status	7/27 (26)	90/169 (53)	.008	0.3	0.1–0.8
Recent antibiotic administration	9/41 (22)	47/215 (22)	1.0	1.0	0.4–2.3
Recent enrofloxacin or doxycycline administration	2/41 (5)	15/215 (7)	.879	0.7	0.1–3.1
Recent chemotherapeutic drug administration	9/41 (22)	59/215 (27)	.466	0.7	0.3–1.7
Pancreatitis	2/41 (5)	13/215 (6)	.943	0.8	0.2–3.7
Diabetes mellitus	2/41 (5)	14/215 (7)	.965	0.7	0.2–3.4
Neurologic disease	3/41 (7)	10/215 (5)	.746	1.6	0.4–6.1
Noncutaneous SCC	0/41 (0)	4/215 (2)	.847	0.6	0.03–10.7
Myelodysplasia	1/41 (2)	1/215 (0.5)	.728	5.4	0.3–87.4
Inflammatory hepatopathy	2/41 (5)	9/215 (4)	.826	1.2	0.2–5.6
Hemangiosarcoma	1/41 (2)	1/215 (0.5)	.728	5.4	0.3–87.3
Inflammatory airway disease	1/41 (2)	6/215 (3)	.692	0.9	0.1–7.4
Lymphoma	4/41 (10)	34/215 (16)	.447	0.6	0.2–1.7
Vaccine-associated fibrosarcoma	2/41 (5)	10/215 (5)	.734	1.1	0.2–5.0
Acute renal failure	0/41 (0)	5/215 (2)	.711	0.5	0.03–7.9
Fever of unknown origin	0/41 (0)	5/215 (2)	.759	0.3	0.01–4.6
Immune-mediated hemolytic anemia	0/41 (0)	5/215 (2)	.711	0.5	0.03–8.5
Hypertrophic cardiomyopathy	5/41 (12)	15/215 (7)	.410	1.9	0.6–5.4
FeLV seropositivity	1/41 (2)	0/215 (0)	.353	16.0	0.6–399
Hyperthyroidism	7/41 (17)	20/215 (9)	.227	2.0	0.8–5.1
Urolithiasis	1/41 (2)	18/215 (8)	.316	0.3	0.04–2.1
Uveitis	2/41 (5)	2/215 (1)	.238	5.5	0.7–40.0
Upper respiratory disease	0/41 (0)	12/215 (6)	.252	0.2	0.01–3.4
Idiopathic nonregenerative anemia	3/41 (7)	4/215 (2)	.150	4.2	0.9–19.4
Inflammatory bowel disease	0/41 (0)	14/215 (7)	.192	0.2	0.01–2.9
Chronic renal failure	6/41 (15)	60/215 (28)	.113	0.4	0.2–1.1
Stomatitis	3/41 (7)	2/215 (1)	.036	8.4	1.4–52.0
SCC of the pinna or planum nasale	4/41 (10)	4/215 (2)	.030	5.7	1.4–23.8
FIV seropositivity	5/41 (12)	4/215 (2)	.005	7.3	1.9–28.6

SCC, squamous cell carcinoma; FIV, feline immunodeficiency virus.

the sequence of cPCR products from 2 cats suggested infection with a related organism. One cat had mild anemia and an intestinal stricture, and the other had lymphosarcoma without anemia. In addition, 1 cat tested positive using cPCR and sequencing for *Mhm*, yet was negative using real-time PCR for *Mhm*. The target sequences for the *Mhm* and *Mhp* real-time assays are located upstream of the target sequence for cPCR. Sequence divergence in this region resulted in failure of real-time PCR to detect the *Mhm*-like organism, and this also may have been the reason for failure of the *Mhp* assay to detect the *Mhp*-like organism. Sequencing efforts of the entire 16S rRNA genes of the *Mtc*-like and *Mhp*-like hemoplasmas currently are ongoing in this laboratory. We also are designing a less specific assay for *Mhm* that is capable of detecting both *Mhm* and the *Mhm*-like organism.

In this study, the hemoplasma load was determined both per volume of blood and after normalization to GAPDH. Normalization to an internal control helps to overcome variation in load resulting from variation in DNA extraction efficiency, which can vary considerably based on variables such as total cell numbers, protein content, or lipid content. Because use of an internal control that is dependent on nucleated cell numbers could confound the statistical methods used in this study, and the correlation between the 2 methods was very high, we reported the organism load per milliliter of blood for the purposes of this study.

The sensitivity and specificity of the combined real-time assays compared with cPCR was 76% and 95%, respectively. With retesting of discordant results, this increased to 87% and 99.5%, respectively. In the 6 samples that tested positive initially using real-time PCR only, but subsequently were negative using both assays, the hemoplasma copy number was relatively low (< 1300/mL). These may have represented true positives but were at the limit of assay quantification (<10 copies per reaction). The same was true for 1 sample that had different results using both assays on reanalysis. In contrast, only 2/33 samples testing persistently positive using both assays contained copy numbers between 300/mL and 1,500/mL. The reason why cPCR initially was positive and subsequently became negative in 4 cats testing repeatedly positive using real-time PCR is unclear. Only 1 sample contained relatively low numbers of hemoplasma genome copies. Positive controls were routinely included in each run, and the same DNA

extract was used for both assays. Consequently, technical errors during sample handling and formulation of the PCR mastermix seemed an unlikely explanation. Sample loading errors also might have contributed to discrepant results. Similarly, real-time PCR initially was negative but became positive with high copy numbers in 2 samples subsequently classified positive.

The low sensitivity of real-time PCR compared with cPCR might be explained by sequence variation within infecting hemoplasmas. Inclusion of a TaqMan probe in real-time PCR assays increases assay specificity, with a concomitant decrease in sensitivity for detection of divergent hemoplasma strains. Negative results using specific TaqMan assays therefore should be interpreted with caution in cats suspected to have hemoplasmosis, and the use of cPCR should be considered to rule out the possibility of infection with a hemoplasma species not detected by the real-time PCR panel, such as *Mtc*. Conversely, in the absence of sequencing of the cPCR product, cPCR cannot predict the infecting hemoplasma species.

Coinfections with multiple hemoplasma species were not detected in this study using conventional or real-time PCR. This is in contrast to previous studies, where the overall prevalence of coinfections was 0.2–2% of cats.[2,6,8] In the only study using real-time PCR, 6/9 cats infected with *Mtc* were coinfected with *Mhm*, and 11% of cats infected with *Mhm* were concurrently infected with *Mtc* or *Mhf*.[6] Because differentiation of *Mhp* and *Mhm* in this study was based on the probe sequence alone, the presence of *Mhp* in low concentrations may have been undetected because of primer consumption in the amplification of *Mhm* DNA, if the latter was more abundant. However, interference between amplification of *Mhf* and *Mhm* was not expected based on results of validation using plasmid mixtures and in cats coinfected with *Mhf* and *Mhm*.[10] We recently have detected coinfections with *Mhf* and *Mhm* in 2% of 310 anemic cats in California using the same assays (Sykes et al, unpublished data). Geographic differences also may explain a difference in prevalence of coinfections. Further refinement in the ability of real-time PCR to detect coinfections will require development of assays with both primers and probes that target species-specific portions of the genome.

As in previous studies,[2,8] there was no association between anemia and *Mhm* infection in this study. In fact, the mean hematocrit was higher in cats testing positive than in cats testing negative. Some of the cats testing positive for *Mhm* presented with moderate anemia. The presence of concurrent illness precluded determination as to whether infection with *Mhm* may have contributed to anemia in these cats, and treatment with antimicrobials effective against hemoplasmas generally was not initiated because the testing was not performed at the time of presentation. The magnitude of the erythrocyte regenerative response appeared blunted in cats testing positive for *Mhm*, and positive cats were less likely to display moderate to marked lymphocytosis and eosinophilia than cats testing negative for hemoplasmas. Obvious confounding variables such as chemotherapy or concurrent FeLV infection could not explain this phenomenon, although unidentified confounders may have been present. In light of these findings as well as those demonstrating accelerated neoplastic transformation of bone marrow elements by FeLV in cats concurrently infected with *Mhm*,[4] further studies of the effect of *Mhm* infection on the bone marrow may be warranted.

In contrast to results of an epidemiologic study performed in Switzerland,[6] the results of this study suggested an association between retroviral infections and hemoplasma infection. Although FeLV and *Mhm* infection were not associated in this study, when all cats testing positive for hemoplasmas were considered, there was a significant association with FeLV infection, as noted by others.[15] The association with FIV may reflect the tendency of infected cats to have access to the outdoors, an inability of FIV-infected cats to eliminate the infection, a common route of transmission through biting, or some combination of these factors. Only 1 of the 5 FIV coinfected cats had anemia, and infection with FIV did not appear to enhance the pathogenicity of *Mhm*, as documented in a recent study of experimentally infected cats.[16] Interestingly, male cats were not predisposed to infection in the current study, which contrasts with the results of some studies[6,8,15,17–19] but not others.[20]

Other variables found to be associated with *Mhm* infection in this study were older age, stomatitis, and cutaneous SCC. The associations between infection as determined using PCR, older age, and outdoor exposure have been recognized in other studies performed in Switzerland and the United Kingdom.[6,8] The association with stomatitis represented an interaction between FIV and stomatitis, as it disappeared when the FIV-positive stomatitis cases were removed from the analysis. The association with cutaneous SCC also may reflect outdoor exposure. In contrast to the Swiss study, which noted an association between renal insufficiency and *Mhm* infection,[6] the authors found no association with chronic renal failure.

The prevalence of infection with *Mhm* infection in cats in the central Sacramento Valley differed from that in cats from the San Francisco bay area, and this was not because of differences in outdoor exposure between the 2 locations. The clustered distribution of infection adds weight to suggestions of a possible arthropod vector. The distribution of cases does not match that of tick-borne companion animal infectious diseases in California.[21] Fleas have been suggested as a possible mode of transmission of hemoplasmas.[14,22] Subjectively, veterinarians feel that the prevalence of flea infestation in the bay area may in fact be higher than that in the Sacramento Valley (P. J. Ihrke, personal communication), but to the authors' knowledge, this has not been well studied. Certain species of mosquito tend to cluster in the Sacramento Valley region and may be involved in transmission. Data regarding arthropod exposure were not reliably included in the medical records of the cats in this study, and examination of a relationship between arthropod exposure and hemoplasma infection was not attempted.

Although a retrospective review of the medical records used in this study may have limited the availability of information regarding medication history, signalment, environment, retrovirus status, and diagnosis for each cat, the delay between sample collection and review of the records permitted access to a finalized clinical diagnosis for each cat after further diagnostic testing, and, in some cases, the results of postmortem examination. Other limitations of this study were the low numbers of cats in each disease category, the fact that only univariate analysis was performed, and the fact that many cats were affected with multiple disease processes, were receiving antimicrobial or immunosuppressive therapy, or both.

In conclusion, cats in North America appear to be infected with at least 4 different hemoplasma species. cPCR was more sensitive than the combined use of these 3 real-time PCR assays, probably because of sequence variation among infecting organisms. Cats infected with *Mhm* were less likely to be anemic than uninfected cats. A possible suppressive effect of the organism on the bone marrow requires further investigation. Older age, outdoor exposure, FIV infection, cutaneous SCC, and stomatitis were associated with *Mhm* infection. The geographic clustering of cases in the Sacramento valley was supportive of a possible arthropod vector.

Footnotes

[a] Universal mastermix, Applied Biosystems, Inc, Foster City, CA
[b] Calf thymus DNA, Invitrogen, Carlsbad, CA
[c] ABI 377 DNA sequencer, Davis Sequencing, Inc, Davis, CA
[d] GraphPad Prism version 4.00, San Diego, CA

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