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# Prevalence and risk factor analysis of feline haemoplasma infection in New Zealand domestic cats using a real-time PCR assay

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## Abstract

Haemotropic mycoplasmas (haemoplasmas) are small epierythrocytic bacteria that have the potential to cause severe, life-threatening haemolytic anaemia. The aim of the current study was to evaluate feline haemoplasma prevalence using real-time polymerase chain reaction (PCR) from a convenience sample of New Zealand domestic cats, including blood film examination and a risk factor analysis. DNA was extracted from 200 blood samples submitted to a diagnostic laboratory for routine haematology over a 12-month period. Species-specific real-time PCR assays identified 62 cats that were positive for haemoplasma DNA, giving an overall prevalence of 31%. Twelve of the positive cats had dual infections. The prevalence of the three feline haemoplasmas was 25% for '*Candidatus* Mycoplasma haemominutum', 7.5% for *Mycoplasma haemofelis* and 4.5% for '*Candidatus* Mycoplasma turicensis' (CMt). All samples were positive for an internal control (feline 28S rDNA) by real-time PCR. Sensitivity and specificity of blood smear examination for haemoplasma infection in this study was 9.7% and 97.8%, respectively. Retroviral infection was tested using the Idexx Snap Feline Triple test on all samples. Twenty cats (10%) were feline immunodeficiency virus (FIV) positive and 11 cats (5.5%) were feline leukaemia virus (FeLV) positive. Statistical comparisons, using multivariate logistic regression, indicated that positive FIV status, male gender and non-pedigree breed were significantly ($P$ <0.05) associated with haemoplasma infection, with odds ratios of 10.16, 5.04 and 3.03, respectively. The results of this study demonstrate the prevalence of the three main feline haemoplasma species in New Zealand for the first time, with prevalences correlating with previous overseas studies. This is the first report of CMt in New Zealand.

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## Introduction

Haemotropic mycoplasmas (haemoplasmas) are small, uncultivatable bacteria that parasitise erythrocytes of many domestic and wild animal species, including humans.[1,2] From the 20 or so species of haemoplasmas currently reported, three species are well recognised to infect cats.[3,4] Feline haemoplasma species vary in their pathogenicity[5] and can present with a wide spectrum of clinical disease, from severe, life-threatening haemolytic anaemia to non-symptomatic carrier states.[4] *Mycoplasma haemofelis* (Mhf) is widely regarded as the most pathogenic of the feline haemoplasmas, with both acute experimental and natural infection resulting in moderate-to-severe haemolytic anaemia.[4,6] *Candidatus* Mycoplasma haemominutum (CMhm) and *Candidatus* Mycoplasma turicensis (CMt) are regarded to have lower pathogenic potential, with infection resulting in both smaller reductions in packed cell volume and fewer clinically significant signs of anaemia.[7,8] Chronic carrier status with feline haemoplasma infections can also occur; such infections involving either CMhm or CMt can last months to years, even after appropriate antimicrobial therapy.[3,9]

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The mode of transmission for haemoplasma infection has been the focus of numerous studies over the last decade, but remains poorly understood.[10–12] Aggressive social contact between cats, such as fighting and biting with the exchange of blood, would appear to be the strongest candidate for infection transmission. Experimental studies in this area have transmitted infection by subcutaneous inoculation of haemoplasma infected blood.[13,14] Other modes have been investigated, including transmission through exchange of infected saliva via mutual grooming or sharing of food dishes[15] and transmission through haematophagous arthropod vectors, such as fleas or ticks;[16–18] however, direct evidence for these remains inconclusive.[11,19,20]

As haemoplasmas cannot be cultured in the laboratory, haemoplasma infection has been traditionally diagnosed by cytological examination of Romanowsky-stained blood smears.[3,21,22] Cytological diagnosis is still used as a rapid bedside test in clinical practice today, but is unreliable as a sole method of haemoplasma diagnosis owing to poor diagnostic sensitivity and specificity. This is owing, in part, to cyclical fluctuation and variability in organism copy numbers, and is exacerbated by time-dependent detachment from erythrocytes after sampling.[4,21,23,24] Polymerase chain reaction (PCR) was first used for the diagnosis of haemoplasma infection in 1998 and since then has become the diagnostic method of choice for both researchers and clinicians owing to its increased diagnostic sensitivity and specificity when compared with cytology.[25–27]

The prevalence of haemoplasma infection in cats has not been previously investigated in New Zealand, despite the first cytological confirmation of *Haemobartonella felis* in this country occurring more than three decades ago.[14] The aim of the current study was to determine the prevalence of feline haemoplasma infection, including blood smear examination and evaluating risk factors for infection in New Zealand cats.

## Materials and methods

### Case material

A convenience sample of 200 ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood samples, submitted to New Zealand Veterinary Pathology Limited, Palmerston North, over a 12-month period was collected. The samples were from cats with a variety of disease conditions and were from localities throughout the country. The samples were chosen at random and were not specifically selected for anaemia. For each sample, data were recorded for potential risk factors, and blood smears generated on the day of collection as part of routine haematology. The smears were stained with Leishman's stain (Fisher Scientific) and examined for evidence of haemoplasma infection using 1000× magnification. After initial storage for 5 days at 4°C, the samples were then held in long-term storage at −80°C, prior to DNA extraction.

### DNA extraction

Frozen EDTA blood samples were thawed at room temperature and vortexed gently before removing 100 µl for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's protocol. The DNA was eluted with 100 µl elution buffer, and DNA concentration quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA was stored at −80°C prior to performing real-time PCR.

### Primer and probe development

Sequences for feline 28S rDNA primers plus Mhf, CMhm and CMt species-specific primers and fluorescently-labelled sequence-specific probes have been reported previously.[25,28] Primers were manufactured by Invitrogen New Zealand, and probes by Biosearch Technologies.

### Real-time PCR

Real-time PCR assays were performed using a Rotor-Gene Q real-time PCR machine (Qiagen) and Rotor-Gene Probe PCR Master Mix. Each real-time PCR reaction consisted of 1 × Master Mix, 400 nM of each species-specific primer, 200 nM of each sequence-specific probe and 2.5 µl DNA, made up to a final volume of 12.5 µl with water. A separate reaction mix was set up for each species-specific primer/probe set. To confirm the presence of amplifiable DNA in the samples and the absence of PCR inhibitors, a feline 28S rDNA-specific primer and sequence specific probe was run in conjunction to the haemoplasma assays. A negative control (water) was included in each real-time PCR run to check for the presence of contaminants. DNA for each of the three haemoplasma species was used as a positive control for every real-time PCR run. Real-time PCR conditions consisted of an initial incubation step of 3 mins at 95°C, followed by 40 cycles of 95°C for 3 s (denaturation step) and 60°C for 10 s (combined annealing/extension step) during which fluorescence data were collected. Standard curves using 10-fold serial dilutions of DNA were generated for each haemoplasma species and feline 28S rDNA, to determine the efficiency (range 94–98%) and accuracy (correlation coefficient $R^2$, range 0.94–0.99) of the real-time PCR reactions. The haemoplasma species specificity of the primer/probe sets were confirmed using real-time PCR and triple cross-checking using positive control DNA for each species against each primer/probe set and a negative control (water). Positive control samples and a selection of samples positive during real-time PCR testing were analysed on a 1.5% (w/v) UltraPure Agarose gel (Invitrogen)

**Table 1** Real-time polymerase chain reaction (PCR) results for feline haemoplasma infection in a sample of 200 New Zealand domestic cats

| Real-time PCR result (n = 200) | Number of infected cats | Percentage (%) |
|---|---|---|
| Haemoplasma positive | 62 | 31 |
| CMhm positive | 50 | 25 |
| Mhf positive | 15 | 7.5 |
| CMt positive | 9 | 4.5 |
| CMhm alone | 38 | 19 |
| Mhf alone | 4 | 2 |
| CMt alone | 8 | 4 |
| CMhm + Mhf | 11 | 5.5 |
| CMhm + CMt | 1 | 0.5 |
| CMhm + Mhf + CMt | 0 | 0 |

CMhm = '*Candidatus* Mycoplasma haemominutum'; Mhf = *Mycoplasma haemofelis*; CMt = '*Candidatus* Mycoplasma turicensis'

containing ethidium bromide and visualised under ultraviolet light on a transilluminator.

### *Sequencing reaction*

Positive control samples and a selection of samples positive during real-time PCR testing were sequenced. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's protocol. Using respective species-specific primers, the amplicons were subjected to automatic dye-terminator cycle sequencing with BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing kit on either the ABI3730 or ABI3130xl Genetic Analyser (Applied Biosystems).

### *Feline immunodeficiency virus/feline leukaemia virus testing*

The Snap Feline Triple test (Idexx Laboratories) was used to assess retroviral infection status as per the manufacturer's instructions.

### *Statistical analysis*

Potential risk factors were evaluated using a multivariate logistic regression model and calculated using Minitab 15 statistical software (Minitab). Odds ratios (ORs) were calculated with a 95% confidence interval (CI). A $P$-value ≤ 0.05 was considered statistically significant. Potential risk factors evaluated in this study were feline immunodeficiency virus (FIV) status (negative vs positive), feline leukaemia virus (FeLV) status (negative vs positive), age (≤ 10 years vs > 10 years), breed (pedigree vs non-pedigree), presence of anaemia [haematocrit (HCT) ≤ 0.24 vs > 0.24], gender (male neutered vs female neutered) and seasonal variation (spring: September–November; summer: December–February; autumn: March–May; winter: June–August). Data were available for all 200 cats, apart from HCT, where one result was unavailable.

## Results

### *Haemoplasma real-time PCR results*

All reactions had efficiencies in excess of 94%. All positive and negative control reactions were positive and negative, respectively, and all 200 samples used in the study were positive for feline 28S rDNA, with $C_T$ values < 32. There was no cross-reactivity seen between the primer/probe sets and the non-target species, confirming the primer/probe sets were species-specific. When separated by agarose gel electrophoresis, the positive controls and selected positive samples each produced a single amplicon of expected size. A total of 200 cats was tested for haemoplasma infection by real-time PCR. Of these, 138 (69%) were negative and 62 (31%) cats were positive for haemoplasma DNA. A complete list of prevalence results can be found in Table 1. The amplicons obtained from real-time PCR of positive controls for Mhf, CMhm and CMt plus selected positive samples were sequenced. All sequences showed 100% identity to 16S ribosomal RNA for Mhf, CMhm and CMt, respectively.

### *Real-time PCR versus blood smear examination*

Of the 200 samples randomly selected for real-time PCR, nine had haemoplasma infection diagnosed on blood smear examination by a veterinary diagnostic laboratory. Of these samples, 6/9 were subsequently positive on real-time PCR. Of the six PCR positive samples, two were Mhf positive and four had Mhf/CMhm dual infections. Based on the real-time PCR results, the sensitivity and specificity of blood smear examination for haemoplasma infection in this study was 9.7% and 97.8%, respectively. The negative predictive value was 70.7% and positive predictive value was 66.7%.

### *Risk associations*

A multivariate logistic regression model was applied to data retrieved from laboratory submission forms and

**Table 2** Sample characteristics and statistics of 200 domestic cats testing positive or negative for feline haemoplasma by real-time polymerase chain reaction (PCR), including gender, breed, feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) infection status, age and anaemia status. Significant associations are shown in bold

| Variable (number of cats) | PCR negative (%) | PCR positive (%) | OR | 95% CI | *P* |
|---|---|---|---|---|---|
| Gender (n = 200) | | | | | |
| Male | 62 (31) | 48 (24) | 5.04 | 2.32–10.97 | **<0.001** |
| Female | 76 (38) | 14 (7) | | | |
| Breed (n = 200) | | | | | |
| Non-pedigree | 99 (49) | 55 (28) | 3.03 | 1.12–8.33 | **0.028** |
| Pedigree | 39 (19) | 7 (4) | | | |
| FeLV (n = 200) | | | | | |
| Positive | 6 (3) | 5 (3) | 1.75 | 0.68–4.48 | 0.247 |
| Negative | 132 (66) | 57 (28) | | | |
| FIV (n = 200) | | | | | |
| Positive | 4 (2) | 16 (8) | 10.16 | 2.88–35.89 | **<0.001** |
| Negative | 134 (67) | 46 (23) | | | |
| Age (n = 200) | | | | | |
| ≤10 years | 73 (36) | 23 (12) | 1.93 | 0.94–3.96 | 0.07 |
| >10 years | 65 (33) | 39 (19) | | | |
| Anaemia status (n = 199) | | | | | |
| Anaemic | 12 (6) | 7 (3) | 0.74 | 0.23–2.41 | 0.621 |
| Non-anaemic | 125 (63) | 55 (28) | | | |

OR = odds ratio; CI = confidence interval

clinicopathological tests, and included gender, breed, FIV and FeLV infection status, age and anaemia status (Table 2). Male gender, non-pedigree breed and FIV infection were all found to be significant risk factors in this study (Table 2). Age, anaemia and FeLV infection did not represent significant risk factors when evaluated against being PCR positive or negative for feline haemoplasma infection (Table 2). Season also did not represent a significant risk factor ($P$ = 0.765). Three subgroups were created to include all Mhf-infected cats, all CMhm-infected cats and all CMtc-infected cats, respectively, including singly and co-infected samples. These were also evaluated against the risk factors, with two further associations identified. A significant association between cats older than 10 years and CMhm infection was found ($P$ = 0.001) with an OR of 3.13 (95% CI 1.52–6.25), and anaemia was significantly associated with Mhf infection ($P$ = 0.018) with an OR of 0.21 (95% CI 0.06–0.70).

## Discussion

This is the first study to report the presence and prevalence of haemoplasma infection with the three main feline haemoplasma species in a population of New Zealand cats, using real-time PCR. Haemoplasma infections were found to be highly prevalent, with haemoplasma DNA identified in 62/200 (31%) cats sampled. In addition, this is the first report of CMt infection in New Zealand. The samples in the present study were derived from those submitted to a central New Zealand veterinary diagnostic laboratory for haematology testing, and are likely to be comprised of unhealthy (sick) pet cats, but not directly suspected of haemoplasma infection. The total prevalence rate was similar to recent studies from Japan (26.4%),[29] the USA (28.1%),[30] Germany (27.8%),[31] Canada (38.3%)[32] and Australia (38.8%).[8] A higher percentage of infected cats has been reported from separate studies in Japan (58.3%)[33] and Ontario, Canada (60%).[17] This may be explained by the studies having much narrower sampling criteria, sampling only stray cats (Japan) and cats suspected to have haemoplasma infection (Ontario, Canada). Comparably lower total prevalence (range 3.9–20.9%) has been reported in studies over the last decade in which samples were collected from both healthy and sick cats.[10,11,19,26,34–39]

Consistent with the majority of prevalence studies completed over the last decade (26/28 evaluated) CMhm was the most common haemoplasma species infecting cats in New Zealand. This would appear to reflect the efficient infection and replication with comparatively reduced virulence of this species.[29] Two notable studies did not follow this general trend. A report from Ontario, Canada, reported a strikingly high prevalence of Mhf infection (46.6%) amongst a small (n = 45) population of stray cats,[17] possibly reflecting the increased likelihood for transmission owing to aggressive behaviour and increased arthropod infestation in stray cats. A second study in South Africa described a remarkably high prevalence of CMt infection (26%)[8] with the study (similar to Fujihara et al[33]) notably selecting specifically for cases

suspicious of haemoplasma infection. Concurrent infections (with two or three feline haemoplasma species) have also been detected in the majority of prevalence studies that used quantitative real-time PCR, with overall co-infection rates ranging from 0.2 to 6.5% of cats.[8,19,25,26,30,34,37,39,40] Co-infection with multiple haemoplasma species suggests there is no immunological cross-protection between the three feline haemoplasma species.[8,41]

Haemoplasma infection was detected by light microscopy examination of blood smears in 6/62 real-time PCR haemoplasma-positive cases in the present study, and all six were positive for Mhf infection. Some studies have suggested that Mhf is twice the size of the other two feline haemoplasma species,[7,42] which may explain why it was more easily detected; however, a further nine Mhf and 47 other haemoplasma-infected blood samples were not identified by light microscopy. In previous prevalence studies, the sensitivity for cytological diagnosis has ranged from 0 to 37.5%.[21,26,31,34] The low sensitivity is likely owing to the cyclical fluctuations of acute Mhf infection, and the smaller size and lower copy numbers of CMhm and CMt infections.[5,27] Importantly, sensitivity can also be affected by haemoplasmas exhibiting a time-dependent detachment from erythrocytes, especially in blood stored in ETDA or heparin anticoagulant prior to a smear being made.[23,43]

Previous studies using quantitative DNA analysis found CMt had the lowest relative blood loads of the three species.[19,25] The relative low loads of CMt, plus its smaller size, may help explain why this species has yet to be identified by light microscopy on blood smears, despite two recent experimental studies focusing on this species.[9,42]

Interpretation and comparison of data from individual haemoplasma prevalence studies over the last decade remains difficult. However, trends in regard to the strongest risk factors associated with haemoplasma infection have emerged. Those most significantly and repeatedly associated with infection include male gender, non-pedigree status and FIV infection.[8,30,34,44] Other more loosely associated risk factors include older age, outdoor access, FeLV infection and a recent history of cat bite abscesses.[31,36–38] Consistent with the strongest trends, our study found that in haemoplasma PCR-positive cats, 80% were male, 89% were non-pedigree and these cats were 10 times more likely to be FIV-positive. The association with male sex and FIV seropositivity provides strong evidence for direct transmission of haemoplasma infection;[45] FIV infection is transmitted through bite wounds [46] and haemoplasma could be transmitted concurrently. However, it is also possible that FIV is associated with haemoplasma infection owing to the immunosuppressive effects that certain stages and strains of FIV infection induce.[29,47]

Consistent with previous epidemiological reports[30,34,37] this study found that overall haemoplasma infection was not significantly associated with anaemia ($P = 0.621$). However, samples with Mhf infection (but not CMhm or CMt) were significantly associated with anaemia ($P = 0.018$) which supports Mhf being considered the more pathogenic species.[19,48,49] Although not statistically significant, cats co-infected with both Mhf and CMhm were more likely to be older (average age: 12 years) and less likely to be anaemic (27% of samples) compared with cats with Mhf infection alone (average age: 3 years; 50% of samples anaemic). This could reflect a more prolonged or chronic clinical picture, as may be seen in a long-term carrier state commonly associated with CMhm infection.[19,38,50] This is further supported in our study as older age (cats older than 10 years) was significantly associated with CMhm infection. The apparent diametric age distribution between Mhf and CMhm infection could be explained, at least in part, by the increased pathogenicity of Mhf compared with CMhm.[41,51,52] Younger cats could be predisposed to infection with Mhf, having comparatively immature immune systems and also perhaps increased exposure due to aggressive social contact. Infection with CMhm is more likely to be found in older cats who have had an increased risk of exposure over time and produced a chronic asymptomatic carrier state.[30] A recent report[53] demonstrated a significantly higher acute phase response in Mhf-infected adult cats compared with CMhm, providing further strength to the pathogenic nature of Mhf.

The majority of domestic cats in New Zealand have outdoor access and could be exposed to disease transmission via vectors, such as fleas, especially in the summer and autumn months when flea populations are their highest.[54] New Zealand has limited potential for vector transmission via ticks as the only cattle tick present, *Haemaphysalis longicornis*, is an uncommon finding on companion animals outside the upper North Island.[55] Worldwide, vector-borne diseases have been shown to exhibit seasonal variation[37] and haemoplasma DNA has been amplified from fleas and ticks;[15–17] however, the current study did not find any association between haemoplasma infection and season. This may reflect either unusual seasonal characteristics that did not cause marked fluctuations in flea populations during this time period or, more likely, that blood-borne transmission via fleas is not the main route for haemoplasma transmission in New Zealand domestic cats.

The results of this convenience study should be interpreted with caution as it has selected for non-healthy cases. This may have resulted in a relatively higher prevalence rate compared with previous studies, which included a healthy sample population.[11,25,37] As a result, the present study may not reflect the general New Zealand cat population. Further research in this area

could include sampling healthy domestic cats from New Zealand to generate more accurate haemoplasma prevalence data regarding the general cat population.

## Conclusions

This is the first study to report prevalence rates of feline haemoplasma infection using real-time PCR in a population of New Zealand cats, with haemoplasma infection found to be highly prevalent. This is also the first report of CMt in New Zealand. Statistical comparisons, using multivariate logistic regression, indicated that positive FIV status, male gender and non-pedigree breed were significantly ($P$ <0.05) associated with haemoplasma infection. The results of this study demonstrate that New Zealand feline haemoplasma prevalence is similar to other overseas studies, with CMhm being the most common infection.

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