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Polymerase chain reaction survey of feline haemoplasma infections in Greece

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The aim of this study was to use real-time polymerase chain reaction assays to determine the prevalence of three haemoplasma species in cats from Greece and to evaluate possible associations between haemoplasma infection and age, gender, feline immunodeficiency virus/feline leukaemia virus (FIV/FeLV) status and packed cell volume (PCV). Ninety-seven cats (24 ill anaemic, 55 ill non-anaemic, 18 healthy non-anaemic) were included in the study. Twenty cats (20.6%) were haemoplasma positive; seven cats were infected only with *Mycoplasma haemofelis*, 10 were infected only with '*Candidatus* Mycoplasma haemominutum' and three were co-infected with *M haemofelis* and '*Candidatus* M haemominutum'. '*Candidatus* Mycoplasma turicensis' was not detected. Haemoplasma infection was associated with older age ($P = 0.019$). *M haemofelis* infection tended to be more common in anaemic cats ($P = 0.058$). No association between gender and haemoplasma infection, or haemoplasma relative copy number and PCV, was detected. Retroviral infection rates were very low with only one FeLV proviral positive cat found.

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The haemoplasmas are infectious haemotropic mycoplasmas that can result in haemolytic anaemia. Three feline haemoplasma species have been identified: *Mycoplasma haemofelis*,[1] '*Candidatus* Mycoplasma haemominutum'[2] and '*Candidatus* Mycoplasma turicensis'.[3] A fourth species, '*Candidatus* Mycoplasma haematoparvum-like' has been recently identified in the United States.[4] These haemoplasma species differ in pathogenicity. *M haemofelis* can act as a primary pathogen and can cause severe haemolytic anaemia in the absence of other pathogens[5,6] whereas '*Candidatus* M haemominutum' usually needs co-infection with another haemoplasma, or immune compromise, to cause disease,[7–9] although mild to moderate anaemia has been described in the absence of any cofactors.[8,10] '*Candidatus* M turicensis' is generally mildly pathogenic[11] and is believed to require co-factors to cause disease,[12] although haemolytic anaemia has been reported.[3] '*Candidatus* M haematoparvum-like' infection has not been fully characterised and has been investigated in only two studies,[4,13] where it was detected in only two cats in one of these studies.[4]

Polymerase chain reaction (PCR) assays are now the diagnostic method of choice to detect haemoplasma infection, and PCR-based prevalence studies have been performed worldwide.[12–25] In these studies the prevalence of *M haemofelis* has ranged from 1.5%[22] to 30%[19] whilst that of '*Candidatus* M haemominutum' has ranged from 10%[22] to 57.1%.[18] The prevalence of '*Candidatus* M turicensis' is generally low (1.3%[22] to 10%)[21] however, occasionally high rates have been reported (27% in South Africa).[12]

The aim of our prospective study was to use real-time PCR assays to determine the prevalence of *M haemofelis*, '*Candidatus* M haemominutum' and '*Candidatus* turicensis' DNA in the blood of a convenience sample of cats from Greece, and to evaluate possible associations between haemoplasma infection and age, gender, feline immunodeficiency virus/feline leukaemia virus (FIV/FeLV) status and packed cell volume (PCV).

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Materials and methods

Surplus ethylene-diaminetetraacetic acid (EDTA)-anticoagulated blood (minimum 0.2 ml) remaining after routine haematology from feline samples submitted to the Diagnostic Laboratory, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece between March 2008 and January 2009, was used in this study. The samples were derived from sick cats that required haematological evaluation as part of a diagnostic profile, or from apparently clinically healthy cats undergoing a pre-anaesthetic screen. Following recruitment, samples were allocated into one of three groups: group A comprised ill anaemic (PCV $< 30\%$) cats, group B ill non-anaemic (PCV $\geq 30\%$) cats and group C clinically healthy non-anaemic cats. Blood samples were stored at -80°C before shipping on ice in batches of 15–27 samples to the Diagnostic Laboratories, University of Bristol, UK for PCR analysis. Data were collected from each cat's case record regarding age, gender, FIV/FeLV serological test results (Snap Combo Plus, Idexx, Petline SA, Greece) (where available) and PCV.

Haemoplasma PCR testing was performed using three previously described real-time PCR assays.[24] Briefly, these quantitative PCR assays use primers and TaqMan probes designed against published 16S rDNA sequences for *M haemofelis*, '*Candidatus* M haemominutum' and '*Candidatus* M turicensis'. Each PCR also includes amplification of an internal control (feline 28S rDNA) to screen for an absence or reduction of host DNA or the presence of PCR inhibitors; cases would only be included in the study if their 28S rDNA threshold cycle (*Ct*) values were ≤ 32 following PCR analysis, indicating the presence of amplifiable DNA in the sample. Positive and negative control samples were included in each PCR run. To allow comparisons to be made between cases, samples that were real-time PCR haemoplasma positive had their relative haemoplasma copy numbers calculated, as described previously.[26] This was undertaken by assigning a *Ct* value of 40 to correspond to one haemoplasma copy number per PCR, and taking into account the known PCR reaction efficiencies (99.1% for *M haemofelis*, 93% for '*Candidatus* M haemominutum' and 91.3% for '*Candidatus* M turicensis')[24] for the PCR assays used.

FeLV PCR testing was also performed on all samples as described previously,[27] using a real-time PCR assay that detects and quantifies FeLV proviral DNA, as well as concurrent amplification of feline 28S rDNA as an internal control. Positive and negative control samples were included in each PCR run.

For statistical analysis haemoplasma prevalence was compared between groups and for the different potential risk factors using the χ^2 test for categorical variables or the Mann–Whitney U test for continuous variables (after assessing for normality using a Kolmogorov–Smirnov test). Correlation between PCV and relative haemoplasma copy number was measured by the Spearman rank test. Cats that were co-infected with both species of haemoplasma were excluded from the correlation analysis. Statistical Package for the Social Sciences (12.0 SPSS Inc, Chicago, USA) was used for statistical analysis and a $P < 0.05$ was considered significant.

Results

A total of 97 samples were collected for the study: 24 in group A (ill, anaemic), 55 in group B (ill, non-anaemic), and 18 in group C (healthy, non-anaemic). All samples were positive for feline 28S rDNA with *Ct* values of <32. Positive and negative control samples were appropriately positive and negative in all PCR runs. Haemoplasma PCR results are summarised in Table 1. Overall, 20/97 (20.6%) cats were positive for one or more haemoplasma species. Seven cats (7.2%) were positive only for *M haemofelis*, 10 cats (10.3%) positive only for '*Candidatus* M haemominutum' and three cats (3.1%) were infected with both *M haemofelis* and '*Candidatus* M haemominutum'. '*Candidatus* M turicensis' was not detected in any of the samples.

There was no significant difference in haemoplasma infection rates [overall ($P = 0.147$), for *M haemofelis* alone ($P = 0.104$) or for '*Candidatus* M haemominutum alone ($P = 0.549$)] between groups A, B and C. No significant difference in haemoplasma infection was found between anaemic and non-anaemic cats when overall haemoplasma ($P = 0.112$) and '*Candidatus* M haemominutum' infection rates ($P = 0.891$) were compared but *M haemofelis* infection tended to be more common in anaemic cats ($P = 0.058$). No significant difference in haemoplasma infection [overall ($P = 0.118$), for *M haemofelis* alone ($P = 0.130$) or for '*Candidatus* M haemominutum alone ($P = 0.314$)] was detected between healthy and ill (both anaemic and non-anaemic) cats.

The relative haemoplasma copy numbers of all the haemoplasma-infected cats are shown in Table 2 and

Table 1. Prevalence of the feline haemoplasmas in Greece.

	N	Overall haemoplasma prevalence	*M haemofelis* infection alone	'*Candidatus* M haemominutum' infection alone	Co-infected with both *M haemofelis* and '*Candidatus* M haemominutum'
All cats	97	20 (20.6%)	7 (7.2%)	10 (10.3%)	3 (3.1%)
Group A (ill anaemic)	24	8 (33.3%)	5 (20.8%)	3 (12.5%)	0
Group B (ill non-anaemic)	55	11 (20.0%)	2 (3.6%)	6 (10.9%)	3 (5.5%)
Group C (healthy non-anaemic)	18	1 (5.6%)	0	1 (5.6%)	0

Table 2. Haemoplasma relative copy numbers in the 20 samples yielding real-time PCR positive haemoplasma results.

PCV (%)	*M haemofelis* relative copy number per PCR	'*Candidatus* M haemominutum' relative copy number per PCR	Age (years)	Sex
36.5	4.2×10^0	1.6×10^1	9	M
26.9	5.2×10^0	–	13	M
28.1	5.2×10^0	–	3	M
25.1	1.3×10^2	–	6.5	F
44.9	3.3×10^2	7.2×10^3	8	M
31.7	6.9×10^2	–	7	M
32.4	1.6×10^3	4.8×10^4	7	M
18.1	1.1×10^5	–	12	M
35.9	2.0×10^5	–	1	F
12.5	3.4×10^7	–	4	M
23.5	–	1.1×10^0	4	M
12.2	–	6.3×10^0	12	M
34.4	–	4.5×10^3	14	F
44.6	–	6.7×10^3	12	M
10.5	–	1.7×10^4	5	F
36.9	–	1.8×10^4	13.5	F
30.5	–	2.1×10^4	7	F
38.2	–	2.7×10^4	12	F
42.3	–	1.8×10^5	13	M
30.4	–	6.7×10^5	1	M

PCV values indicative of anaemia (<30%) are highlighted in bold. M = male, F = female.

ranged from 1.1×10^0 to 3.4×10^7 per PCR. There was no correlation between PCV and the relative haemoplasma copy numbers for either haemoplasma species (*M haemofelis* spearman rank correlation coefficient (r_s) = −0.23; $P = 0.613$; '*Candidatus* M haemominutum' $r_s = 0.36$; $P = 0.310$).

The haemoplasma-infected cats were significantly older (median 8.2 years (range 1−14 years)) ($P = 0.019$) than the haemoplasma negative cats (median 5.0 years (range 0.4−16.5 years)). No significant difference ($P = 0.157$) in gender was found between the haemoplasma-infected cats (65.0% male) and the haemoplasma negative cats (63.6% male). FeLV and FIV serological testing was only carried out in 13 cases; 5/20 haemoplasma positive and 8/77 haemoplasma negative cats. All 13 FIV serological tests were negative, whilst only 1/13 FeLV serological tests was positive; this cat was haemoplasma PCR and FeLV provirus PCR negative. Only 1/97 cats (which did not have FIV/FeLV serological testing performed) was FeLV provirus PCR positive, and this cat was also infected with '*Candidatus* M haemominutum'. Positive and negative control samples for the FeLV PCR were appropriately positive and negative in all PCR runs.

Discussion

This is the first study to document the presence of *M haemofelis* and '*Candidatus* M haemominutum' in cats in Greece. The overall haemoplasma infection prevalence of 20.6% is similar to two recent European studies from Germany (27.1%)[23] and Italy (18.6%).[25] However, lower prevalences have been reported in other parts of Europe; 14.0% in the United Kingdom[24] and 11.2% in Switzerland.[22] The 10.3% *M haemofelis* prevalence found in the current study is slightly higher than in the studies from Germany, Italy, UK and Switzerland, whereas the 13.4% '*Candidatus* M haemominutum' prevalence is slightly lower or similar to those reported in these studies.

In agreement with other studies,[13–25] we demonstrated haemoplasma infection in both anaemic and non-anaemic cats. No association between anaemia and haemoplasma infection was found, although an association between *M haemofelis* infection and anaemia approached significance. It may be that a larger number of samples would have enabled significance to be reached. As *M haemofelis* can act as a primary pathogen to induce anaemia, an association between anaemia and its presence makes sense biologically, although the existence of chronic asymptomatic carriers may confound such an association. The majority of previous studies have failed to demonstrate an association between anaemia and haemoplasma infection[4,17,21–23,25] however, one study[14] reported an association between anaemia and infection with *Haemobartonella felis-OH* (now *M haemofelis*) as well as with *H felis-OH* and *Haemobartonella felis-CA* (now '*Candidatus* M haemominutum') co-infections.

All of these studies, including ours, have used convenience sampling for data collection but differences do exist in the type (proportions of healthy or anaemic

cats for example) and numbers of cats sampled. In addition, these studies have employed different PCR assays which will differ in sensitivity and specificity. Such differences have to be taken into account when comparing results from various studies.

The results of our study in Greece are consistent with the previous observation of a higher prevalence of haemoplasma infection in countries with warmer climates.[12,15,16,20,21] Additionally, a study in northern Italy found higher rates of haemoplasma infection in summer months when compared to autumn.[25] The suggested cause of this phenomenon is the higher number and species of blood sucking arthropods that are found in warmer climates compared to colder climates.[12] However, the geographical variation in the prevalence of arthropods is not dependent only on climatic differences but other factors such as humidity. It has been suggested that haemoplasmas are transmitted by arthropod vectors[13,28,29] although this has not yet been proven for natural transmission.

There was no gender predisposition identified for haemoplasma infection in the current study. This is in contrast to several studies that have found a higher risk of infection in male cats,[12,13,16,17,22] which is thought to reflect a possible route of cat-to-cat transmission via outdoor access and/or fighting. A previous study on retroviral infection in pet cats from the same region of Greece[30] reported that most cats lived in single cat households and did not have any outdoor access. Although we were unable to collect this type of information during the current study, all the cats sampled were pets and it is likely that their environment was similar to that reported in the retroviral study (personal communication, Z Polizopoulou), with most cats having very limited outdoor access. A lack of outdoor access and fighting as a potential means of haemoplasma transmission could have contributed to the absence of a male predisposition in this population of cats.

The haemoplasma positive cats in our study were significantly older than the negative cats, which is in agreement with previous studies.[17,22,23] This age association is believed to be multifactorial. It is known that once infected, cats do not always reliably eliminate the organism[6] and longer lived cats are also more likely to have an increased chance of exposure to haemoplasmas.

Our study did not identify a relationship between relative haemoplasma copy number and PCV for either haemoplasma species, consistent with a previous study in naturally infected cats that also reported no relationship.[22] However, other studies have reported significant inverse correlations between *Ct* value and haematocrit for *M haemofelis*, but not for '*Candidatus* M haemominutum'.[20,21] It is interesting to note that in the current study, the *M haemofelis*-infected cat with the lowest PCV (12.5%) had the highest relative *M haemofelis* copy number (3.4×10^7 per PCR). It may be speculated that this cat was suffering from acute haemoplasmosis resulting in anaemia, but in the majority of samples there was no association between PCV and relative copy number, as evidenced by the lack of significant correlation. This may be because many of the *M haemofelis*-infected cats in the current study were asymptomatic carrier cats with chronic infection, and therefore less likely to be associated with anaemia which is most commonly seen during acute infection.[5,6,22] Despite the low number of cats infected with *M haemofelis*, there was a trend for *M haemofelis* infection to be associated with anaemia.

Previous studies have found that FIV and/or FeLV infections are risk factors for haemoplasma infection.[13,16,23,25] In this study the only PCR FeLV positive cat was infected with '*Candidatus* M haemominutum', but it was not anaemic (PCV 30.5%). The single positive FeLV serological test was later found to be negative by PCR. This discordant result is thought likely to reflect a false positive result, possibly due to the presence of a cross reacting antigen in the sample serum or the use of an anti-mouse antibody in the test.[27] All positive and negative controls generated appropriate results on PCR, and although the given specificity of the Idexx Snap combo FeLV test is high (98.2%; 95% Confidence Limits (CL) 94.5–99.6%),[31] a recent study has demonstrated that when independently evaluated the specificity of some in-house retrovirus testing systems is often much lower than the manufacturers' estimations.[32] The low rate of retrovirus infection in our study is in agreement with a previous study published from the same region of Greece, where overall rates of infection with FIV and FeLV were only 3.5% and 1.5%, respectively.[30]

'*Candidatus* M turicensis' was not detected in any of the cats sampled in this study. Previous European studies have generally reported low rates of '*Candidatus* M turicensis' infection[22–25] although higher rates have been found in Australia[21] and South Africa.[3] It is possible that the number of cats surveyed in this study was insufficient to detect the presence of '*Candidatus* M turicensis' in Greece.

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