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Abstract

Objectives The objectives of this study were to estimate the prevalence of feline haemoplasma infections in Northern Serbia, identify potential risk factors and perform molecular subtyping of feline immunodeficiency virus (FIV).

Methods PCR analysis for feline haemoplasmas was performed on surplus EDTA blood samples from 373 cats from the Belgrade region, Serbia. An ELISA was used to determine the prevalence of feline leukaemia virus (FeLV) and FIV; PCR was performed on a subpopulation of these cats. FIV subtyping was performed using PCR.

Results Within this population, 64/373 cats (17.2%) were infected with one or more haemoplasma species. *Mycoplasma haemofelis* was detected in 20/373 cats (5.4%), ‘*Candidatus Mycoplasma haemominutum*’ in 47/373 cats (12.6%) and ‘*Candidatus Mycoplasma turicensis*’ in 23/373 cats (6.2%). Coinfections were observed in 21/373 cats (5.6%). Based on ELISA serological retroviral testing, 4/310 cats (1.3%) were infected with FeLV, whereas 78/331 (23.6%) were infected with FIV. Multivariable analysis identified significant associations between haemoplasma infection and anaemia (anaemic/non-anaemic, odds ratio [OR] 2.7, 95% confidence interval [CI] 1.04–7.1; $P = 0.041$), male gender (male/female, OR 4.5, 95% CI 2.22–9.03; $P < 0.0005$), outdoor access (yes/no, OR 5.2, 95% CI 2.28–11.92; $P < 0.0005$), non-pedigree breed (non-pedigree/pedigree, OR 5.5, 95% CI 1.24–24.84; $P = 0.025$) and FIV seropositive status (positive/negative, OR 2.4, 95% CI 1.21–4.83; $P = 0.012$). PCR analysis of the FIV ELISA-positive samples revealed clade D as being the most prevalent.

Conclusions and relevance All three known species of feline haemoplasma were detected, confirming their presence in Serbia; ‘*Candidatus Mycoplasma haemominutum*’ was the most prevalent. We found a high prevalence of FIV-infected cats and FIV clade D was most prevalent.

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Introduction

Haemotropic mycoplasmas (haemoplasmas) are small, unculturable cell-wall-free bacteria that attach to the red blood cell surface of many domestic and wild animal species, and humans.¹ Three feline haemoplasma species have been described so far: *Mycoplasma haemofelis* (Mhf) 'Candidatus Mycoplasma haemominutum' (CMhm) and 'Candidatus Mycoplasma turicensis' (CMt).

Mhf is the most pathogenic of the three haemoplasma species and acute infection often results in haemolytic anaemia, although some prevalence studies failed to show an association between Mhf and anaemia.^{2,3} Most infections with CMhm and CMt are not associated with anaemia or other clinical abnormalities, although they may play a pathogenic role in cats with concurrent disease, in particular coinfection with retroviruses, or immunosuppression.^{4,5}

The natural route of transmission of the feline haemoplasmas has not yet been determined. It has been suggested that horizontal transmission through arthropod vectors,^{6,7} aggressive interaction with blood transmission such as during cat bites^{8,9} and blood transfusions,³ as well as vertical transmission from queen to kittens,^{10,11} may play a role, but further studies are required.

There have been several studies investigating the prevalence of feline haemoplasmas, as well as potential risk factors for infection, in various countries worldwide. Such studies have added relevance since the development of PCR assays, which are considered the diagnostic method of choice. Studies performed across Europe among healthy and unhealthy cats have identified prevalence rates ranging from 12–43.4%.^{2,12–20}

The aims of this study were to: (1) determine the prevalence of Mhf, CMhm and CMt infections using PCR in cats from Serbia; (2) investigate potential associations between feline haemoplasma infections and risk factors such as sex, age, indoor/outdoor status, presence of ectoparasites, season, health status, anaemia and retroviral infections; (3) determine the prevalence of feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) using ELISAs in cats from Serbia, followed by PCR in selected cases, as noted below; and to (4) identify the different clades of FIV present in Serbian cats using PCR.

Materials and methods

Case material

Blood samples were collected from 373 cats living in Belgrade and the surrounding wider region of Northern Serbia (Vojvodina). The cats were presented to various veterinary clinics between February 2011 and November 2012, and the samples were obtained either from cats that were described as sick based on the presence of clinical signs (eg, vomiting, hyporexia/anorexia, dyspnoea, oral lesions and dysuria, among others), or from cats

described as clinically healthy, mostly presenting for a pre-anaesthetic screen or health check-up, as determined by the veterinarian that collected the blood samples. For each cat, information regarding breed, sex, age, weight, outdoor/indoor status and presence of ectoparasites was recorded.

Blood (0.5 ml) was collected from all cats into EDTA tubes for haematological analysis and real-time quantitative (q)PCR testing. An additional 0.2–0.5 ml of blood was placed into plain tubes for serological testing.

Haematological analysis was performed within 1 h of blood collection using one of three different automated analysers (Abacus Junior Vet [Vetlab Supplies], AcT Diff [Beckman Coulter], Sysmex XS-500i [Sysmex]), depending on the practice where the cats were seen. Cats with haemoglobin concentrations <80 g/l were diagnosed as anaemic.

Surplus whole blood and serum samples were stored at –20°C before being transported on dry ice to the Diagnostic Laboratories, Langford Vets, University of Bristol, UK, for molecular and serological testing.

Haemoplasma qPCR assays

The DNA was extracted from 100 µl EDTA blood using a commercial kit and following the manufacturer's instructions (Macherey–Nagel Nucleospin Blood Kit).

The DNA was eluted with 100 µl BE elution buffer provided with the kit and stored at –20°C prior to analysis. Real-time qPCR assays were performed for Mhf, CMhm and CMt as described previously.²¹

An internal control assay (feline 28S rRNA gene) was run in multiplex with each haemoplasma species assay to confirm the presence of amplifiable DNA in each sample and a threshold cycle (Ct) cut-off value of <30 was used to indicate adequate amplifiable DNA. Positive controls (species-specific DNA from known haemoplasma-positive cats) and a negative control (water) were included in each qPCR.

FIV/FeLV ELISA and PCR assays

Serological testing for FIV (ELISA; PetChek FIV Antibody Test [IDEXX]) and FeLV (ELISA; PetChek FeLV Antigen Test [IDEXX]) was performed on available sera for the detection of specific antibodies to FIV gag (p24) and or/env (gp40) and p27 FeLV antigen, following the manufacturer's instructions.

For the FIV/FeLV PCR assays the extracted nucleic acid samples described above were used. The PCR tests for FeLV and/or FIV were performed on all cats that tested positive by ELISA and from those that were not tested owing to insufficient or unavailable serum samples. The FeLV provirus qPCR analysis was performed as described previously,²² and positive (DNA from known FeLV-positive cats) and negative (water) controls were included in each qPCR.

For the FIV PCR assay the extracted nucleic acid samples were first transported on dry ice to the IDEXX laboratory in Ludwigsburg (Germany) for synthesis of cDNA. Then the cDNA was transported to the IDEXX laboratory in Sacramento (CA, USA) for FIV PCR testing using a commercially available assay (FIV RealPCR; IDEXX). The latter is a PCR (and reverse-transcriptase PCR) assay for detection of FIV provirus and virus targeting a conserved region of the *gag* gene, while FIV clade was determined in infected cats using clade-specific primer pairs for clades A, B, C, D and F^{23,24} (reported sensitivity and specificity for FIV RealPCR™ is 94% and 94% respectively).^{23,54}

Statistical analysis

Data were recorded into Microsoft Office Excel 2007 and then imported into SPSS (IBM) for statistical analysis. Descriptive statistics were obtained for the continuous variables (age, haemoglobin) and the following categorical variables: presence or absence of anaemia, sex, outdoor access, breed (pedigree/non-pedigree), presence of ectoparasites, health status (healthy/ill, defined by a veterinarian at presentation as specified above), season of sampling, as well as FeLV and FIV ELISA serology status. For assessment of seasonal variation, the months were grouped as follows: December, January and February (winter); March, April and May (spring); June, July and August (summer); September, October and November (autumn). The association of haemoplasma status and categorical variables was examined by univariate analysis using the Pearson χ^2 test or Fisher's exact test. Odds ratios (OR) for predictor variables and haemoplasma-positive status were also calculated. For the continuous variables, after assessing for normality of distribution (using a Kolmogorov-Smirnov test), their association to haemoplasma status (positive/negative) was examined using a Student's *t*-test for normally distributed data and the

Mann-Whitney U-test for non-normally distributed data. Binary logistic regression was performed to adjust for confounding factors for the outcome. Variables with *P* values <0.2 on the univariable analysis were considered to have a trend towards significance and were included in the multivariable model. Associations were considered statistically significant when *P* <0.05.

Results

Cats

Samples from 373 cats were collected and included in the study. The age of the cats ranged from 2 months to 22 years (median 4 years). Male and female cats represented 44.2% (*n* = 165/373) and 55.8% (*n* = 208/373) of the population, respectively. The number of non-pedigree cats (*n* = 280/373; 75.1%) was higher than that of pedigree cats (*n* = 93/373; 24.9%). Information regarding outdoor access was available for 372 cats of which 182 (48.9%) had outdoor access and 190 (51.1%) did not. The presence or absence of ectoparasites was specified in the records of 368 cats of which 79 (21.5%) stated the presence of ectoparasites. Information regarding health status was available for 372 cats of which 107 (28.8%) were classified as ill and 265 (71.2%) as healthy. Of the 373 cats, 37 were defined as anaemic (9.9%) and 336 as non-anaemic (90.1%). Eighty-one cats (21.7%) were sampled in the summer, 77 (20.6%) in the autumn, 27 (7.2%) in the winter and 188 (50.4%) in the spring.

Haemoplasma qPCR results

All of the DNA extraction and qPCR-positive and negative controls performed, as well as the internal control qPCRs, were appropriately positive (with adequate DNA amounts for the internal control) or negative. Sixty-four of the 373 cats (17.2%) were positive for one or more feline haemoplasma species; Mhf was detected in 20 (5.4%), CMhm in 47 (12.6%) and CMt in 23 (6.2%) cats. Coinfections were identified in 21 cats (5.6%) (Table 1).

Table 1 Real-time quantitative PCR (qPCR) results for feline haemoplasma infection in 373 cats from Serbia

qPCR result (<i>n</i> = 373)	Number of infected cats	%
Any haemoplasma positive	64	17.2
Mhf positive	20	5.4
CMhm positive	47	12.6
CMt positive	23	6.2
Mhf positive only	3	0.8
CMhm positive only	31	8.3
CMt positive only	9	2.4
Mhf and CMhm dual positive	7	1.9
Mhf and CMt dual positive	5	1.3
CMhm and CMt dual positive	4	1.1
CMhm, Mhf and CMt triple positive	5	1.3

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

FIV and FeLV ELISA and PCR results

The ELISA for FIV was performed on samples from 331/373 cats as sera were not available for the remaining 42 cats. When the available serum was adequate to perform only a single ELISA test, FIV ELISA was prioritised over FeLV ELISA. Seventy-eight (23.6%) of these 331 cats were FIV antibody positive on ELISA. In total, samples from 120 cats (78 that tested FIV antibody positive on ELISA and 42 for which sera were not available) were tested for FIV proviral DNA; 36 of these tested positive (25 were FIV antibody positive on ELISA and 11 had unknown FIV antibody status on ELISA). Subsequent clade designation of the 36 FIV PCR-positive cats identified 24 to be infected with clade D (66.7%), nine with clade F (25%), one with clade A (2.8%), one with clade B

(2.8%) and one with a clade for which designation was not possible (2.8%).

For FeLV, ELISA was performed on samples from 310/373 cats, as sera were not available for the remaining 63 cats. Four of the 310 cats (1.3%) were FeLV p27 antigen ELISA-positive. FeLV PCR analysis was performed on 67 samples (four were FeLV ELISA p27 positive and 63 had unknown FeLV ELISA p27 status), of which eight (11.9%) tested FeLV provirus positive (one was FeLV ELISA p27 positive and seven had unknown FeLV ELISA p27 status).

Statistical analysis

Univariable analysis revealed *P* values of <0.2 for all nine categorical variables (Table 2) and the two continuous variables (Table 3) with any haemoplasma species

Table 2 Univariable relationships between categorical variables and any haemoplasma species-positive status in cats from Serbia; real-time quantitative PCR testing*

Variable	PCR		OR	95% CI	<i>P</i> value (Pearson χ^2 or Fisher's exact test†)
	Negative (% of total)	Positive (% of total)			
Anaemia (n = 373)					
Anaemic	25/373 (6.7)	12/373 (3.2)	2.60	1.24–5.54	0.009‡
Non-anaemic	284/373 (76.1)	52/373 (13.9)			
Sex (n = 373)					
Male	122/373 (32.7)	42/373 (11.3)	2.90	1.66–5.14	<0.0005
Female	187/373 (50.1)	22/373 (5.9)			
Outdoor access (n = 372)					
Yes	132/372 (35.5)	50/372 (13.4)	5.16	2.69–9.88	<0.0005
No	177/372 (47.6)	13/372 (3.5)			
Breed (n = 373)					
Non-pedigree	218/373 (58.4)	62/373 (16.6)	12.94	3.10–54.0	<0.0005
Pedigree	91/373 (24.4)	2/373 (0.5)			
Presence of ectoparasites (n = 368)					
Yes	61/368 (16.6)	18/368 (4.9)	1.64	0.89–3.04	0.112
No	245/368 (66.6)	44/368 (12.0)			
Health status (n = 372)					
Ill	80/372 (21.5)	27/372 (7.3)	2.08	1.19–3.36	0.009
Healthy	228/372 (61.3)	37/372 (9.9)			
Season (n = 373)					0.038
Summer (June–August)	61/373 (16.4)	20/373 (5.4)	1.85	1.02–3.36	0.042
Autumn (September–November)	70/373 (18.8)	7/373 (1.9)	0.42	1.03–1.23	0.035
Winter (December–February)	20/373 (5.4)	7/373 (1.9)	1.78	0.72–4.39	0.210
Spring (March–May)	158/373 (42.4)	30/373 (8.0)	0.843	0.49–1.45	0.535
FeLV status (ELISA) (n = 310)					
Positive	2/310 (0.6)	2/310 (0.6)	5.25	0.72–38.13	0.128
Negative	257/310 (82.9)	49/310 (15.8)			
FIV status (ELISA) (n = 331)					
Positive	54/331 (16.3)	24/331 (7.3)	3.18	1.73–5.86	<0.0005
Negative	222/331 (67.1)	31/331 (9.4)			

*Any haemoplasma species-positive status indicates positivity in at least one of the following haemoplasma PCRs: *Mycoplasma haemofelis*, 'Candidatus Mycoplasma haemominutum', 'Candidatus Mycoplasma turicensis'

†Fisher's exact test used when one or more expected cell frequencies were <5

‡Variables with a *P* value of <0.05 are shown in bold

OR = odds ratio; CI in = confidence interval; FeLV = feline leukaemia virus; FIV = feline immunodeficiency virus

Table 3 Univariable relationships between continuous variables and any haemoplasma species (AHS)-positive status in cats from Serbia*

Variable	AHS-positive status (%)	Median	Minimum–maximum	<i>P</i> value
Age (years)	Positive 64/373 (17.1)	5	1–16	0.053
	Negative 309/373 (82.8)	4	0.17–20	
Haemoglobin (g/l)	Positive 64/373 (17.1)	108.2	37–167	0.162
	Negative 309/373 (82.8)	113.6	33–170	

*Any haemoplasma species-positive status indicates positivity in at least one of the following haemoplasma PCRs: *Mycoplasma haemofelis*, 'Candidatus Mycoplasma haemominutum' and 'Candidatus Mycoplasma turicensis'

Table 4 Final significant multivariable relationships between predictor variables and any haemoplasma species-positive status in cats from Serbia*

Variable	Adjusted OR	95% CI for OR	<i>P</i> value
Anaemia (anaemic vs non-anaemic)	2.72	1.04–7.10	0.041
Sex (male vs female)	4.48	2.22–9.03	<0.0005
Outdoor access (yes vs no)	5.22	2.28–11.92	<0.0005
Breed (non-pedigree vs pedigree)	5.55	1.24–24.81	0.025
FIV ELISA status (positive vs negative)	2.42	1.21–4.83	0.012

*Any haemoplasma species-positive status indicates positivity in at least one of the following haemoplasma qPCRs: *Mycoplasma haemofelis*, 'Candidatus Mycoplasma haemominutum' and 'Candidatus Mycoplasma turicensis'

OR = odds ratio; CI = confidence interval; FIV = feline immunodeficiency virus

(AHS)-positive status, which was defined as positivity in at least one of the three haemoplasma qPCRs: Mhf, CMhm and CMt. Significant associations ($P < 0.05$), and thus significant risk factors, were identified between AHS-positive status and seven categorical variables. Five of these seven variables maintained significance following multivariable analysis: anaemia, male sex, outdoor access, non-pedigree breed and FIV ELISA-positive status (Table 4). Associations with the remaining two categorical variables (presence of ectoparasites and FeLV serology status) were not found to be significant with AHS-positive status. Of the two continuous variables (age and haemoglobin), age was not found to be significantly associated with AHS-positive status. For haemoglobin, a statistically significant association for AHS-positive status was not found either, despite the above reported association found with anaemia; however, this had a trend towards significance.

A similar statistical analysis was also performed for the cats infected only with CMhm ($n = 31$), as this number of animals was considered adequate for statistical analysis. Such analysis was not possible for the cats positive only for Mhf ($n = 3$) or CMt ($n = 9$) as the number of infected animals was low. Univariable analysis revealed *P* values of < 0.2 for 7/11 variables (Tables 5 and 6) with PCR positivity only for CMhm. Significant associations ($P < 0.05$), and thus significant risk factors, were identified between CMhm only infection and five categorical variables and older age. Following multivariable analysis, three variables maintained significance: outdoor access, FIV ELISA-positive status and older age (Table 7).

Univariable analysis revealed significant associations between AHS infection and anaemia, as well as dual or triple haemoplasma coinfections and anaemia (Table 8).

Discussion

To the best of our knowledge, this is the first study to investigate the prevalence of haemoplasmas in cats in Serbia and identify the different FIV clades in the feline population of this country. The amplification of haemoplasma DNA from the population sampled confirms the presence of all three known haemoplasma species, as well as coinfections with two or three of these species. Haemoplasma infection was present in both healthy and ill cats within the population studied.

Some of the characteristics of the cats in our population, such as age, sex and breed, were similar to those in other published European studies,^{2,13,15} although direct comparisons are difficult.

Overall, the prevalence of feline haemoplasma infections found in this study was not substantially different to other European studies,^{2,12–18,20,25}

CMhm was the most prevalent haemoplasma species (12.6%) in the Serbian cat population, similar to other reports in Europe (range 9.5–41.6%).^{2,12–15,17,20,25,26} Most previous studies reported CMt as the least prevalent feline haemoplasma species,^{13,16,17,20,25} but in the current study the prevalence of CMt infection was greater than that of Mhf.

The final multivariable logistic regression model for AHS infection revealed that male, non-pedigree cats that had outdoor access and that were anaemic or FIV

Table 5 Univariable relationships between categorical variables and only 'Candidatus Mycoplasma haemominutum' species-positive status in cats from Serbia

Variable	qPCR		OR	95% CI	P value (Pearson χ^2 test or Fisher's exact test*)
	Negative (% of total)	Positive (% of total)			
Anaemia (n = 373)					
Anaemic	33/373 (8.8)	4/373 (1.1)	1.39	0.46–4.21	0.531*
Non-anaemic	309/373 (82.8)	27/373 (7.2)			
Gender (n = 373)					
Male	146/373 (39.1)	18/373 (4.8)	1.86	0.88–3.91	0.099
Female	196/373 (52.5)	13/373 (3.5)			
Outdoor exposure (n = 372)					
Yes	159/372 (42.7)	23/372 (6.2)	3.29	1.43–7.56	0.003†
No	182/372 (48.9)	8/373 (2.1)			
Breed (n = 373)					
Non-pedigree	250/373 (67.0)	30/373 (8.0)	11.04	1.48–82.12	0.004
Pedigree	92/373 (24.7)	1/373 (0.3)			
Presence of ectoparasites (n = 368)					
Yes	74/368 (20.1)	5/368 (1.4)	0.68	0.25–1.84	0.449
No	263/368 (71.5)	26/368 (7.1)			
Health status (n = 372)					
Ill	92/372 (24.7)	15/372 (4.0)	2.54	1.21–5.34	0.012
Healthy	249/372 (66.9)	16/372 (4.3)			
Season (n = 373)					0.049
Summer (June–August)	71/373 (19.0)	10/373 (2.7)	1.82	0.82–4.03	0.137
Autumn (September–November)	74/373 (19.8)	3/373 (0.8)	0.39	0.12–1.31	0.115
Winter (December–February)	22/373 (5.9)	5/373 (1.3)	2.80	0.98–7.99	0.061*
Spring (March–May)	175/373 (46.9)	13/373 (3.5)	0.69	0.33–1.45	0.325
FeLV status (ELISA) (n = 310)					
Positive	3/310 (1.0)	1/310 (0.3)	4.10	0.41–41.02	0.277*
Negative	283/310 (91.3)	23/310 (7.4)			
FIV status (ELISA) (n = 331)					
Positive	62/331 (18.7)	16/331 (4.8)	5.68	2.51–12.85	<0.0005
Negative	242/331 (73.1)	11/331 (3.3)			

*Fisher's exact test used when one or more expected cell frequencies were <5

†Variables with a P value <0.05 are shown in bold

qPCR = quantitative PCR; OR = odds ratio; CI = confidence interval; FeLV = feline leukaemia virus; FIV = feline immunodeficiency

Table 6 Univariable relationships between continuous variables and only 'Candidatus Mycoplasma haemominutum' (CMhm) species-positive status in cats from Serbia

Variable	Only CMhm species qPCR-positive status (%)	Median	Minimum–maximum	P value
Age (years)	Positive 31/373 (8.3) Negative 342/373 (91.7)	6.5 4	2–16 0.17–20	0.002*
Haemoglobin (g/l)	Positive 31/373 (8.3) Negative 342/373 (91.7)	110.7 112.9	64–160 33–170	0.647

*Variables with a P value of <0.05 are shown in bold

qPCR = quantitative PCR

ELISA- positive were more likely to be infected with AHS. Furthermore, outdoor access, positive FIV ELISA and age were identified as risk factors for cats infected with only CMhm.

The increased likelihood of male cats being infected with AHS over female cats has been frequently reported in other studies.^{2,3,27–29} Similarly, non-pedigree status and outdoor access are also commonly identified as risks

Table 7 Final significant multivariable relationships between predictor variables and only 'Candidatus Mycoplasma haemominutum' (CMhm) species quantitative PCR-positive status in cats from Serbia

Variable	Adjusted OR	95% CI	P value
Outdoor access (yes vs no)	5.54	1.84–16.70	0.002
FIV ELISA status (positive vs negative)	3.98	1.67–9.47	0.002
Age (years)*	1.19	1.08–1.33	0.001

*Odds ratio (OR) for a 1 year increase in age
CI = confidence interval; FIV = feline immunodeficiency virus

Table 8 Univariable relationships between anaemia and various haemoplasma species quantitative PCR-positive status in cats from Serbia

	P value (Pearson χ^2 test or Fisher's exact test*)
Any haemoplasma species positive†	0.009*
Only Mhf positive	1*
Only CMhm positive	0.562
Only CMt positive	0.608*
Haemoplasma coinfections (dual or triple)	<0.0005

*Fisher's exact test used when one or more expected cell frequencies were <5

†Any haemoplasma species-positive status indicates positivity in at least one of the following haemoplasma PCRs: *Mycoplasma haemofelis* (Mhf), 'Candidatus Mycoplasma haemominutum' (CMhm) and 'Candidatus Mycoplasma turicensis' (CMt)

‡Variables with a P value <0.05 are shown in bold

factors,^{2,3,29,30} which is in agreement with our findings. The higher prevalence of haemoplasmosis in male, non-pedigree cats with outdoor access supports the suggestion of transmission through aggressive interactions,⁸ as these cats are more likely to be involved in fighting and biting. The concurrent association with FIV seropositivity further supports this, as FIV is mostly transmitted through bites from persistently infected cats,³¹ and it is possible that these two infectious diseases are transmitted concurrently or through similar routes. Furthermore, FIV infection may play a role in haemoplasma infection owing to its immunosuppressive effects.³²

We identified that the cats infected only with CMhm were significantly older (median 6.5 years) than those that were not (median 4 years), which is in agreement with previous studies,^{2,26} possibly owing to the increased likelihood of exposure to the organism with age,² and also the fact that CMhm is not reliably eliminated after infection.^{33–35}

In our study an association between anaemia and AHS infection was identified ($P = 0.041$) on multivariable analysis, with haemoplasma coinfections being significantly ($P < 0.0005$) associated with anaemia using univariable analysis. No significance was identified between anaemia and single infections with any of the haemoplasma species. Although some studies have similarly reported an association between anaemia and haemoplasma infection,^{36,37} often this is not demonstrated.^{15,28,38,39} For Mhf and CMt this may be somewhat affected by the low numbers of

cats that had infection with only one of these species. Furthermore, it has been suggested that anaemia is induced mainly in acute Mhf infections,^{3,4} and clinical signs may not be present in chronically infected animals. In the infected cats comprising our study population, the stage of infection was not known, but it is possible that these were chronically infected with Mhf. For CMhm and CMt it is generally acknowledged that these are often detected in non-anaemic cats.⁴ However, it is possible that in our population coinfections with two or three haemoplasma species played a role in the development of anaemia, as has been previously suggested.⁴⁰

Associations between haemoplasma infection and FIV,^{13,15,25,38,39,41–43} or, less commonly, FeLV,^{26,42} have been identified in several studies suggesting that cats infected with these retroviruses are at an increased risk for haemoplasma infection. However, another study did not identify retroviral-positive status as a risk factor.³ It is considered that the immunosuppressive effect of retroviruses is likely the cause of this association, although the possible common route of infection may also play a role. This study demonstrated a significant association of haemoplasma infection with FIV ELISA positivity, but not with FeLV. It is possible that the very low number of FeLV-positive cats in this population affected the outcome.

To the best of our knowledge this is the first study to generate information regarding the FIV clades in Serbia using PCR assays. In our population a high

seroprevalence of FIV-infected cats was identified (23.6% [n = 78/331] on ELISA) and on the provirus PCR tested samples 30% (n = 36/120) were positive, whereas FeLV seroprevalence was found to be low (1.3% [n = 4/310]). A 2014 study on cats also from the Northern region of Serbia (from the area of Belgrade, and also of Novi Sad in Vojvodina, covering regions similar to this study) identified similar FIV (22%; n = 13/60) and FeLV (0/60 cats) prevalence using the rapid test SNAP Combo.⁴⁴ It is interesting that clade D was prevalent in our study population, as clade D FIV viruses seem to have arisen from Japan.^{31,45,46} The so-far described worldwide distribution of FIV suggests that clades A, B and C are present in the USA,⁴⁷ whereas in Europe clades A and B virus isolates are mainly found.⁴⁸ Clade A is more common in Northern European countries, whereas in Southern and Central Europe clade B is the most frequently reported FIV clade;⁴⁹ in our population clade B was found in one cat only. This could reflect that in the Balkan region FIV clades vary significantly compared with central Europe, but further studies in other Balkan countries are needed to establish this.

The FIV qPCR assays used at Diagnostic Laboratories, Langford Vets, are designed to detect clade A isolates, which is the predominant clade reported in the UK,⁵⁰ so PCR analyses of the Serbian samples were performed at IDEXX where the qPCRs employed can detect other FIV clades. We acknowledge that there were discrepancies between the PCR- and ELISA-positive samples for both FIV and FeLV. Only 25/78 FIV ELISA-positive samples were found to be positive for FIV provirus on PCR analysis, which may reflect false positive results on ELISA, which have been previously reported with similar ELISA tests;^{23,51} the presence of other cross-reacting antibodies in the sample serum has been suggested as one possible explanation.^{51,52} Furthermore, in our study population, of the 78 cats that tested positive for FIV on ELISA, six were aged 6 months or younger, so it is possible that some of these tested positive as a result of the detection of passively acquired maternal antibodies. However, it is also likely that some of the samples were false negatives on the FIV provirus PCR. This is because, unfortunately, owing to an issue with the courier, the samples sent from the UK arrived in Germany thawed following a very long transit time, and this is likely to have caused degradation of sample nucleic acid, thus resulting in false negatives among the FIV PCR results. Furthermore, levels of proviral DNA and viral RNA may have been below the limit of detection,^{23,24,53} or a cat may have been infected with a clade of FIV not detected by the PCR assay.^{24,53} It is worth mentioning here that vaccination against FIV is not performed in Serbia and none of these cats were vaccinated against FIV.

Potential explanations for the discordant results for FeLV (three ELISA-positive but PCR-negative) are that

these were falsely positive by ELISA, as has been previously reported with this and other similar tests that detect the FeLV p27 antigen tests,^{54,55} or that these were false negative PCR results.²²

In conclusion, regarding the discrepancies in retroviral testing, we acknowledge that not all results are available for all the cats in our study as PCR and ELISA testing were performed on different but overlapping subpopulations of these, which limits interpretation of the available results. These tests detect different aspects of infection and thus these discrepancies may be genuine; however, the tests may also have inherent flaws, resulting in false positives and false negatives, which may confound the interpretation of the results.

Furthermore, as the presence of false negative FIV and FeLV ELISA results among the samples tested cannot be excluded, and given the lack of molecular data on the seronegative samples, the prevalence of these retroviral infections may be underestimated.

As a further consideration, we appreciate that we did not differentiate between chronic and acute haemoplasma infections and the significance of the seasonal data reported may not always be relevant, in particular in the chronically infected cats, as the time of sampling is unlikely to reflect the time of infection in these. However, as this has been previously investigated in similar studies,^{13,29} this is still considered to be an interesting point and may contribute to further understanding of this disease.

We acknowledge that the present study utilised a convenience sample of cats. The limitations of such samples have been previously described,⁵⁶ and it is likely that these results may not be truly representative of the general prevalence of feline haemoplasma infection in Serbia. Furthermore, it is unfortunate that owing to insufficient funds and/or sample volume we were unable to perform PCR and serology on all samples for the retroviruses. The described problems during shipping of the samples for FIV PCR analysis may also have affected our results regarding the prevalence of FIV and its association with haemoplasma infection.

Conclusions

Feline haemoplasmas were present in a population of Serbian cats and all three known species were detected, with CMhm being the most prevalent; the potential risk factors identified were mostly in accordance with other similar studies. Furthermore, we reported a high prevalence of FIV infection in cats from Serbia, and, interestingly, identified clade D to be the most prevalent clade, whereas FeLV infection was found to be much less prevalent.

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