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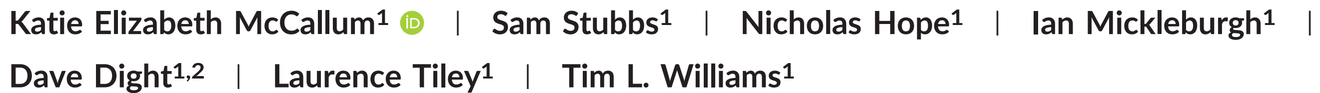
Detection and seroprevalence of morbillivirus and other paramyxoviruses in geriatric cats with and without evidence of azotemic chronic kidney disease



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Background: Feline morbillivirus (FeMV) is associated with the presence of tubulo-interstitial nephritis (TIN) in cats, however the seroprevalence of FeMV in the UK and the association between the presence of FeMV and renal azotemia is unknown

Hypothesis/Objectives: To identify whether paramyxoviruses are present in urine samples of geriatric cats and to develop an assay to assess FeMV seroprevalence. To investigate the relationship between both urinary paramyxovirus (including FeMV) excretion and FeMV seroprevalence and azotemic chronic kidney disease (CKD).

Animals: Seventy-nine cats (40 for FeMV detection; 72 for seroprevalence).

Methods: Retrospective cross-sectional, case control study. Viral RNA was extracted from urine for RT-PCR. PCR products were sequenced for virus identification and comparison. The FeMV N protein gene was cloned and partially purified for use as an antigen to screen cat sera for anti-FeMV antibodies by Western Blot.

Results: Feline morbillivirus RNA from five distinct morbilliviruses were identified. Detection was not significantly different between azotemic CKD (1/16) and nonazotemic groups (4/24; $P = .36$). Three distinct, non-FeMV paramyxoviruses were present in the nonazotemic group but their absence from the azotemic group was not statistically significant ($P = .15$). 6/14 (43%) azotemic cats and 40/55 (73%) nonazotemic cats were seropositive ($P = .06$).

Conclusions and Clinical Importance: Feline morbillivirus was detected in cats in the UK for the First time. However, there was no association between virus prevalence or seropositivity and azotemic CKD. These data do not support the hypothesis that FeMV infection is associated with the development of azotemic CKD in cats in the UK.

KEYWORDS

azotemia, feline, morbillivirus, paramyxovirus

Abbreviations: CDV, canine distemper virus; CKD, chronic kidney disease; CRFK, Crandell-Rees feline kidney; FLUTD, feline lower urinary tract disease; FeMV, feline morbillivirus; GFR, glomerular filtration rate; IRIS, International Renal Interest Society; PCR, Polymerase Chain Reaction; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SDMA, symmetric dimethylarginine; SDS-PAGE, Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis; TT4, total thyroxine; TIN, tubulointerstitial nephritis; UAC, urine albumin: creatinine ratio; UCC, urine cystatin C: creatinine ratio; UPC, urine protein: creatinine ratio; USG, urine specific gravity; WB, Western blot.

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1 | INTRODUCTION

Feline morbillivirus (FeMV), family Paramyxoviridae, was originally discovered in Hong Kong in 2012. Sequencing and phylogenetic analyses confirmed that FeMV is a distinct virus species.[1] Paramyxoviruses are enveloped, single-stranded, negative RNA viruses, which have been previously identified in a wide variety of vertebrate hosts.[2] Upon discovery, the presence of FeMV was associated with histopathologically confirmed tubulointerstitial nephritis (TIN) in stray cats[1] and the virus has since been detected in the urine of cats in Japan, Germany, Italy, USA, South America, and Turkey.[3–10] Furthermore, there is an association between paramyxoviruses and renal pathology in a range of species, including humans,[11] rats,[12] bats,[13] and squirrels.[14] Tubulointerstitial nephritis is the most common lesion in cats with chronic kidney disease (CKD)[15] and FeMV RNA is more prevalent in renal tissues of cats with TIN compared with blood and urine samples from the same animals.[5] Furthermore, in-vitro studies of Crandell-Rees Feline Kidney (CRFK) cells inoculated with known FeMV positive samples have demonstrated virus replication and cytopathic effects, including syncytium formation,[1,4] and immunohistochemistry has detected the presence of FeMV N protein in renal tubular cells.[1,10,16] However, at time of writing, no studies have evaluated if FeMV is more prevalent in cats with biochemically confirmed azotemic CKD compared with nonazotemic cats.

CKD is a common condition of older cats, with a 4% prevalence in UK 1st opinion practice.[17] Current treatment protocols have limited efficacy and therefore, CKD is an important cause of morbidity and mortality in geriatric cats.[18] The etiopathogenesis of TIN in cats with CKD is unclear at present, and determination of risk factors for CKD could help to further clarify the inciting factors that lead to TIN and CKD in cats, which could in turn lead to better preventative strategies, such as vaccination.

The primary aims of our study were to (1) identify if paramyxoviruses (including FeMV) are present in the urine of cats in the UK, (2) to develop a serological assay to establish if there is evidence of exposure of UK cats to FeMV, and (3) to evaluate if a relationship exists between the presence of azotemic CKD and the detection of serum anti-FeMV antibodies, the urinary excretion of paramyxoviruses (including FeMV) or both.

2 | MATERIALS AND METHODS

2.1 | Sample selection

A free-of-charge screening programme at three UK 1st-opinion practices between March 1, 2013 and April 30, 2015 was designed to collect urine samples from cats with and without azotemic CKD. This protocol was approved by the Ethics and Welfare Committee at the Department of Veterinary Medicine, University of Cambridge (CR56). Blood samples were obtained by jugular venipuncture and urine samples were taken by cystocentesis. A commercial laboratory analyzed complete blood count, serum biochemistry, including total thyroxine concentration (TT4) and urinalysis, including measurement of urine specific gravity (USG) by refractometry, urine dipstick, sediment analysis and UPC. Cats with CKD were staged according to the IRIS guidelines for cats with CKD.[19]

To be included in the study, cats were aged ≥ 8 years old with no significant systemic disease (eg, diabetes mellitus or hyperthyroidism). Exclusion criteria included recent or ongoing treatment with corticosteroids, diuretics, or angiotensin-converting enzyme inhibitors, and recent or concurrent IV fluid treatment at the time of sampling. Samples were excluded from the study if TT4 >40 nmol/L, if urine samples were older than 3 days at the time of analysis, if there was evidence of significant systemic illness on complete blood count or biochemistry or if there was evidence of lower urinary tract inflammation on urinalysis (pyuria, bacteriuria, and hematuria).

Excess urine was stored at -80°C until batch RNA isolation. Renal azotemia was diagnosed by documentation of an increased concentration of serum creatinine in conjunction with reduced urine concentrating ability.[20] Cats were assigned to either the azotemic CKD group (defined as serum creatinine concentration >1.7 mg/dL or 153 μmol/L (the upper limit of our laboratory reference interval) and concurrent USG < 1.035) or the nonazotemic group. Table 1 summarizes the results for the cat population studied (79 cats in total). Urine RT-PCR was performed in 16 azotemic cats (designated A1-A16) and 24 nonazotemic cats (designated S1-S11; and H1-H13). Serology was performed in 72 cats in total (designated A1-A16; S1-S8; H1-H9; H14-H52). 33/40 (82%) cats had concurrent serology and urine RT-PCR performed.

A power calculation performed based on the prevalence of FeMV in cats with and without TIN[1] indicated that the study needed a minimum of 14 cats per group to be 80% powered to find significant differences between the groups.

2.2 | RT-PCR and nested RT-PCR amplification

RNA isolation was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Manchester) following the manufacturer's instructions.

We used a broadly reactive PCR assay reported to detect all known paramyxoviruses.[2] The primer targets correspond to highly conserved regions of the genome (L protein) and can detect 25 reference viruses from the paramyxovirus family. The published method was slightly modified by using random hexamers to generate the cDNA as we found this resulted in greater and more consistent yields of PCR product. cDNA synthesis was performed using Superscript III (Invitrogen, Carlsbad, California) following the manufacturer's instructions. 10 μl of the cDNA reaction was used as input for the 1st round of seminested PCR using 1 μM forward (PAR-F1) and reverse primers (PAR-R) subjected to 40 cycles of amplification (94°C for 15 seconds, 48°C ramping to 50°C for 30 seconds, 72°C for 30 seconds) and a final extension at 72°C for 7 minutes. The 2nd amplification in the seminested PCR used 2 μL of template from the 1st amplification under the same conditions as the 1st except the forward primer PAR-F2 replaced PAR-F1.

The final PCR products were visualized by ultraviolet light after electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

TABLE 1 Summary of clinical status, PCR, and WB results

CKD status (A versus NA)	FPV PCR	FeMV PCR	WB	Number of cats	Sample numbers
A	NEG	POS	SP	1	A1
A	NEG	NEG	SP	1	A2
A	NEG	NEG	P	2	A3, A4
A	NEG	NEG	WP	2	A5, A6
A	NEG	NEG	HB	2	A7, A8
A	NEG	NEG	NEG	8	A9-A16
NA	NEG	POS	SP	2	S1, H1
NA	NEG	POS	P	2	S2, H3
NA	NEG	NEG	P	1	S3
NA	NEG	NEG	WP	5	S4, S5, H4, H5, H6
NA	NEG	NEG	NEG	5	S7, S8, H7, H8, H9
NA	POS	NEG	NEG	1	S6
NA	POS	NEG	NT	2	S9, H10
NA	NEG	NEG	NT	5	S10, S11, H11, H12, H13
NA	NT	NT	POS	9	H14, H17, H21, H24, H27, H28, H35, H37, H45
NA	NT	NT	SP	3	H16, H31, H43
NA	NT	NT	WP	17	H15, H19, H20, H22, H23, H25, H26, H36, H38, H39, H40, H41, H46, H48, H49, H50, H51
NA	NEG	NEG	SP	1	H2
NA	NT	NT	HB	1	H18
NA	NT	NT	NEG	9	H29, H30, H32, H33, H34, H42, H44, H47, H52

RT-PCR was performed on 16 azotemic cats (A1-A16) and 24 non-azotemic cats (S1-S11, H1-H13). serology was performed on 72 cats (A1-A16, S1-S8, H1-H9, H14-H52). FPV, feline paramyxovirus; FeMV, feline morbillivirus; A, azotemic; NA, nonazotemic; SP, strong positive; POS, positive; WP, weak positive; NEG, negative; NT, not tested; HB, high background (therefore, could not be scored definitively).

2.3 | DNA sequencing

Bands of the appropriate size (~500 bp) were excised from the gel and DNA was extracted using the Purelink Quick Gel Extraction Kit (Invitrogen, Carlsbad, California) before being sent for Sanger sequencing using the primers PAR-F2 and PAR-R (Source BioScience, Cambridge, UK). The sequences were compared with the complete nonredundant nucleotide database using BLASTn (http://blast.ncbi.nlm.nih.gov).

2.4 | Sequence alignment

Multiple sequence alignment and phylogeny representation of the amplified region of the L gene were performed using the MUSCLE, GBlocks, PhyML, and TreeDyn tools as implemented in the "One-click" mode of Phyogeny.fr.[21] The alignment was displayed using MView.[22]

2.5 | N protein PCR amplification

The N protein is highly conserved with >90% nucleotide sequence identity amongst the FeMVs but distinct from other morbilliviruses.[4] The N protein has previously been assessed by indirect immunofluorescence assay for seroprevalence studies of FeMV where a seroprevalence of 21% was identified.[16] Amplification of the N gene of FeMV from urine sample RNA was performed using nested PCR. cDNA was produced as described above (except for using primer FMV_N_For (5′GAGGRGRAGGAATCAGGTATTTCASAATG 3′) instead of random hexamers). The N gene was amplified for 30 cycles (95°C for 30 seconds, 59°C for 30 seconds, 72°C for 60 seconds) followed by 72°C for 10 minutes using 0.5 μM of each primer FMV_N_For and FMV_N_Rev (5′CRGTYGTGAACYTTGAGGTCCTAAGT 3′), 2 μL template cDNA and 2.5U DreamTaq DNA Polymerase (Thermo Scientific). The secondary nested PCR procedure was carried out as above except for using pASG-IBA144 insertion primers (N_pASG_N_termF (5′AGCGCGTCTCCAATGTCTAGTCTATTGAGGTCACTTGCTG3′) and N_pASG_Rev (5′AGCGCGTCTCCTCCCTTTTAGAAGGTCAGTATCATTATAATG 3′; annealing temperature 61°C). These are designed to fuse the full-length N gene open reading frame in frame with the N-terminal ompA-twin Strep tag and the C-terminal 6xHis tag intended to be used for subsequent protein expression and purification. PCR products were subsequently purified using a QIAQuick PCR Purification kit, digested with Esp31, ligated into pASG-IBA144, cloned in *E. coli* DH5-alpha

(Invitrogen) and subsequently transferred into *E. coli* BL21 DE3. The complete sequence of the N-gene and associated affinity tags for the chosen clone was subsequently verified.

2.6 | Expression and partial purification of FeMV N protein

One milliliter of overnight starter culture was added to 10 mL LB Amp_{100} medium and incubated at 37°C shaking at 225 rpm until the OD_{600} reached 0.4-0.8 at which point 200 μg/mL of anhydrotetracycline was added to induce protein expression from the vector. At the optimum time-point for maximum soluble protein (2 hours post-induction), the *E. coli* BL21 DE3 cells were pelleted and resuspended in 3 mL/g wet weight of pellet of lysis buffer (0.15 M NaCl, 10% sucrose, 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100 and Lysozyme at 0.2 ng/mL). and incubated for 30 minutes on ice. Subsequently, 0.5 μL of Benzonase (Sigma 250 units μL) was added to the sample for a further 10 minutes to digest DNA to reduce sample viscosity. The supernatant was harvested by centrifugation at 10 000*g* for 25 minutes at 4°C. The pellet fraction (which contained ~50% of the yield) was solubilized using 8 M urea in 10 mM Tris-HCl pH 8 (5 mL/g wet weight of pellet) incubated for 60 minutes at RT. After incubation, the mixture was centrifuged at 10 000*g* for 25 minutes and the solubilized protein in the supernatant collected. The soluble and solubilized fractions of N protein were purified via the 6xHis tag, using Ni-NTA Magnetic Agarose Beads following the manufacturer's standard protocol.

2.7 | SDS PAGE and Western blotting (WB)

SDS PAGE was performed using a 8% resolving gel and 4.5% stacking gel under reducing conditions. After semidry transfer to a nitrocellulose membrane and blocking [5% nonfat-dried milk powder dissolved in 19 mM Tris pH 7.4. 137 mM NaCl, 2.7 mM KCl, 0.1% Tween20 (TBST)], the blot was probed for His-tagged N protein expression and enrichment using 1:500 mouse anti-6xHis antibody (Merck Millipore/cat #05–949) followed by a 1:2000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich, cat #A3673).

To screen multiple cat sera simultaneously for reactivity to the N-protein, 50 μL of Ni-NTA bead-enriched N protein was loaded onto a full gel-width well, electrophoresed, transferred, and blocked as before. The nitrocellulose filter was loaded into a 20-channel Biorad Multiscreen unit. The cat sera (diluted 1:100 with TBST + 0.5% Roche Blocking Reagent) were loaded separately into the individual channels and incubated for 2 hours at room temperature. A mixture of mouse anti-His tag 1° antibody and anti-mouse HRP 2° antibody was added to the end channel to provide a marker for the migration of the His-tagged N protein band (s). The filter was processed as before except for using 1:1000 dilution of BioRad horseradish peroxidase conjugated goat anti-cat as the secondary antibody (Bio-RAD/Cat #AA126P). Blots were developed using the BM Chemiluminescence Blotting kit following the manufacturer's

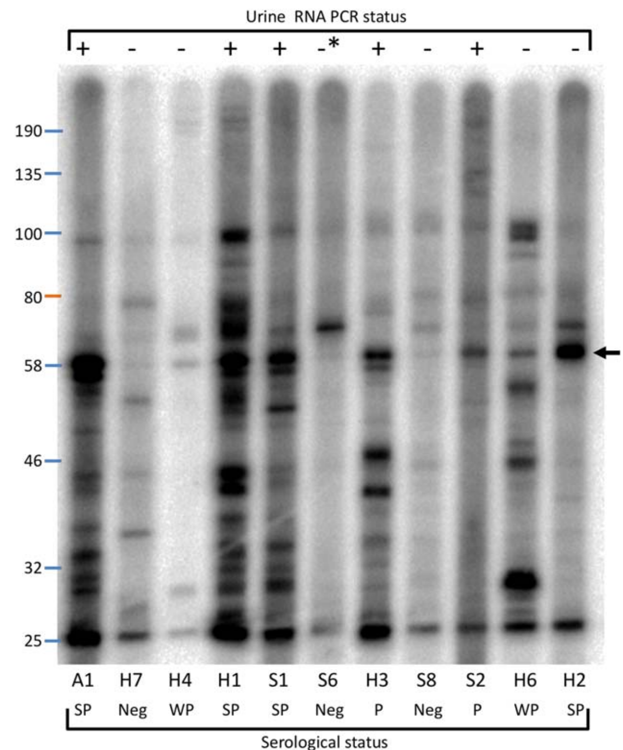

FIGURE 1 WB using Biorad Multiscreen apparatus. Individual cat sera (designated A1, H7, H4, etc) were tested for binding to *E. coli* expressed, NTA-bead purified His-tagged FeMV N protein. Urine RT-PCR status for each sample is indicated by + or –. * sample S6 was positive for non-FeMV viral RNA. The ~60 kDa band of interest is indicated by the black arrow

instructions. Luminescence imaging was recorded using a Biorad Chemidoc MP imager (exposure time of 2 seconds).

A primary assay validation of the WB assay was performed using sera from all 5 cats that tested positive for FeMV in urine via PCR, 1 cat that was positive for non-FeMV and 5 FeMV RNA negative cats to test for reactivity to the 58 kDa doublet (Figure 1).

2.8 | Statistical analysis

Comparisons between groups were made using Fisher's exact test and Mann Whitney *U* test were used as appropriate. Data are presented as median [25th, 75th percentiles] and statistical significance defined as <.05.

3 | RESULTS

3.1 | Clinical description of the cohort

A total of 40 cats were included in the 1st part of the study (Table 1). Sixteen cats were in the azotemic CKD group and 24 cats in the nonazotemic group. The azotemic CKD group (A1-A16) comprised 6 female neutered and 10 male neutered cats. Breeds represented in this group included 11 domestic short-haired cats, 2 domestic long-haired cats plus 1 British Short Hair, Burmese, and Tonkinese cat, respectively. The age of the cats ranged from 8.7 to 18.5 years old (median: 12.5 years

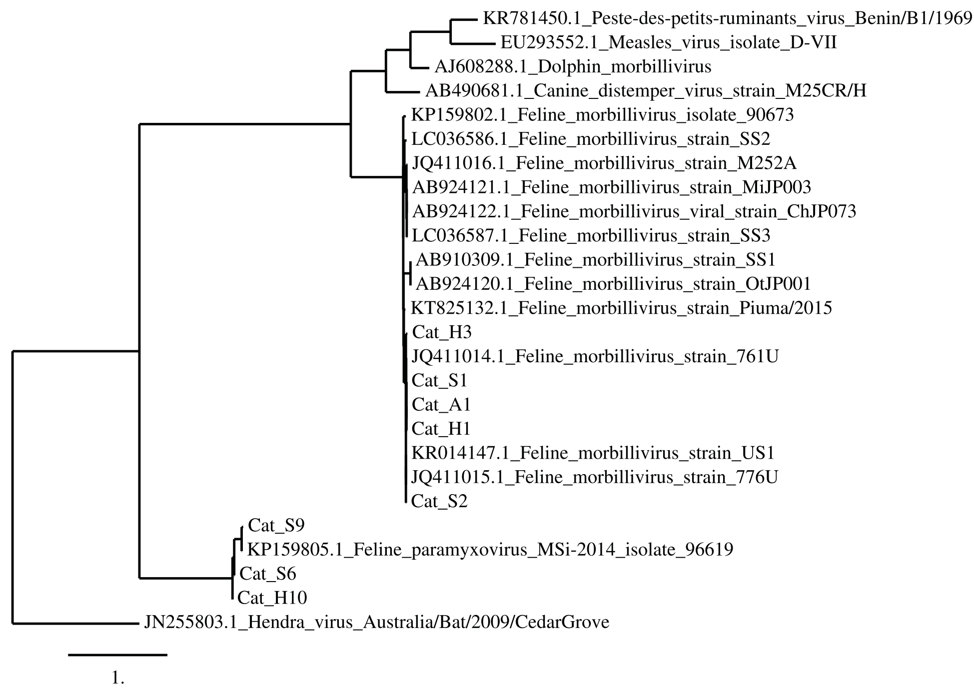

FIGURE 2 Phylogenetic tree showing comparison of FeMV and non-FeMV sequences to previously reported sequences. GenBank accession numbers A1 (MG640027), S9 (MG640028), H10 (MG640029), H1 (MG640030), S6 (MG640031), S1 (MG640032), H3 (MG640033), and S2 (MG640034)

[11.3, 16.4]). The median serum urea concentration was 42.3 mg/dL [29.9, 50.1] or 15.1 mmol/L [10.7, 17.9] and median serum creatinine concentration was 2.08 mg/dL [1.82-2.57] or 184 μmol/L [161, 227]. Thirteen cats had a serum creatinine value between 1.58 and 2.82 mg/dL (140 and 250 μmol/L) (corresponding to IRIS stage II) and 3 cats had serum creatinine value between 2.82 and 4.98 mg/dL (250–440 μmol/L; corresponding to IRIS stage III).

The nonazotemic group (S1-S11 and H1-H13) comprised 11 female neutered and 13 male neutered cats. Breeds represented in this group included 3 domestic long-haired cats and 21 domestic short-haired cats. The age of the cats ranged from 8 to 19 years old (median: 11 years [9.8, 14.0]). Median serum urea concentration was 29.4 mg/dL [26.6, 35] or 10.5 mmol/L [9.5, 12.5] and median serum creatinine concentration was 1.49 mg/dL [1.23, 1.59] or 132 μmol/L [109, 141].

3.2 | Detection and characterization of paramyxovirus RNA in urine samples

Morbillivirus L gene sequences were detected by RT-PCR in 5 out of 40 of the urine samples (Table 1). Analysis of the aligned sequences (Figure 2) showed that they were all highly similar (Table 2) and all 5 were phylogenetically most closely related to the USA strain FeMV-US1 (accession number KR014147) with the Hong Kong strains 761U and 776U (JQ411014 and JQ411015, respectively) being the next most closely related. Samples A1, S2, and H1 clustered as a single group with FeMV-US1. S1 and H3 mapped as two further distinct isolates. The N gene coding region cloned from sample S1 also most closely matched these three isolates (see below). Three distinct non-FeMV paramyxoviruses were also detected (samples S6, S9 and H10) that were most closely related to strain FPV-MSi-2014 from Germany (accession number KP 159805). Of these, H10 and S6 formed a distinct subgroup. S9 was the most closely related to FPV-MSi-2014.

3.3 | Correlation of paramyxovirus RNA in urine and health status

FeMV RNA detection was not significantly different between azotemic CKD and non-azotemic groups (1/16 versus 4/24 respectively; $P = .36$. Table 1). Although the non-FeMV paramyxoviruses were only detected in the nonazotemic group, this difference from the azotemic group was not significant (3/24 versus 0/16, respectively; $P = .15$; Table 3).

To assess the effect of urine concentration on the likelihood of detecting FeMV and non FeMV paramyxovirus, the proportion of cats with USG >1.035 that were positive for FeMV and non-FeMV paramyxovirus in their urine was compared with that of cats with USG <1.035. FeMV detection was not significantly different between cats in the USG <1.035 and USG >1.035 group (2/12 versus 2/12; $P = 1.0$). Non-FeMV paramyxovirus detection was also not significantly different between USG <1.035 and USG >1.035 group (1/12 versus 2/12; $P = .55$).

3.4 | N Protein expression

The N sequence from cat S1 (a nonazotemic cat testing positive for FeMV RNA) was cloned and sequenced. It was most closely related to the 761U and US1 strains (1538 and 1537 out of 1557 nucleotide identity; 514 and 515 out of 518 amino acid identity, respectively). WB analysis using antibody to the C-terminal 6xHis-tag introduced during cloning showed a doublet band straddling the 58 kDa prestained

TABLE 2 Comparison of percentage nucleotide sequence identity between the aligned sequences identified in our study and their nearest paramyxovirus relatives, FmoPV-US1 (KR014147) and FPV-MSi-2014 (KP159805)

Comparison of sequences	MSi-2014	S9	H10	S6	US1	S2	AI	S1	H1	H3
Msi-2014	100	98	92	92	73	73	73	72	72	72
S9	98	100	91	92	73	73	73	72	72	72
H10	92	91	100	98	73	67	74	73	73	67
S6	92	92	98	100	72	72	73	72	72	71
US1	73	73	73	72	100	99	99	97	99	98
S2	73	73	67	72	99	100	99	97	99	99
A1	73	73	74	73	99	99	100	97	99	98
S1	72	72	73	72	97	97	97	100	97	96
H1	72	72	73	72	99	99	99	97	100	98
H3	72	72	67	71	98	99	98	96	98	100

Cells are shaded to indicate the isolates belonging to the FeMV (light shading) and non-FeMV (dark shading) groups.

marker band (the predicted size of the 57 kDa N protein plus the tags is 63.6 kDa) plus several smaller presumed N-terminal truncation products ~29–32 kDa in size (Figure 3A). NTA-bead purification of the protein led to a substantial enrichment of the ~60 kDa doublet in the primary eluate and the detection of numerous lower molecular weight products (Figure 3B). A 2nd elution from the column released only a small additional amount the protein. A substantial proportion remained associated with the beads that was released upon boiling in sample buffer.

The partially purified protein was used as antigen to analyze single dilutions of individual cat sera by WB (Figure 4). The cat sera reacted with a variety of different sized proteins in addition to the ~60 kDa doublet on the blot. Some of these may be N protein degradation products but others are likely to be *E. coli* proteins present in the partially purified antigen. All the sera from the cats that were PCR-positive for viral RNA in their urine were also highly reactive to the ~60 kDa doublet (cats A1, H1, S1, H3, and S2). Cat H2 (which was FeMV negative) was also strongly positive (as was H6 to a lesser extent). The remaining sera from RNA negative cats in this figure had low level (weak positive or negative) reactivity to the ~60 kDa doublet. Cat S6, which was positive for non-FeMV paramyxovirus was negative on WB. The presence of FeMV RNA in urine was therefore predictive of N protein reactivity by WB, but this correlation did not extend to all the WB positive samples.

TABLE 3 Paramyxoviral status (FeMV and non-FeMV paramyxoviral positive cats) of azotemic (A1-A16) and nonazotemic CKD groups.

Paramyxoviral status	Azotemic CKD (A1-A16)	Non-azotemic CKD (S1-S11; H1-H13)
Non-FeMV paramyxovirus positive	0/16 (0%)	3/24 (13%)
FeMV positive	1/16 (6%)	4/24 (17%)

Further screening of sera from both azotemic and normal cats was performed (Figure 4) and in total 22/72 sera tested positive or strongly positive by WB and a further 24 had detectable but weak positive reactivity. However, the level of reactivity was quite variable, with the majority of cats showing weak positive reactivity compared to cat A1 which was routinely included as a benchmark for strong positivity. Three cats (two azotemic and one nonazotemic) had high background on the WB and so were excluded from further analysis. 6/14 (43%) azotemic cats and 40/55 (73%) nonazotemic cats were seropositive on WB ($P = .06$).

4 | DISCUSSION

Our study identified RNA from FeMV and non-FeMV paramyxoviruses in the urine of cats, thus confirming the presence of these viruses in cats in the United Kingdom. In addition, a high percentage of UK cats had detectable serum antibodies to FeMV (31% or 64% depending on whether the weak positives were included), indicating that a substantial proportion of UK cats have evidence of exposure to this/or a related virus. However, we did not detect an association between urinary excretion of these viruses, or the presence of serum anti-FeMV antibodies, and the presence of azotemic CKD. This is in contrast with the previously documented association between FeMV and the presence of histopathologically confirmed TIN (the most common lesion in cats with CKD),[1] but is in agreement with a subsequent study from Japan which found no association between TIN and FeMV infection.[16] In both of these previous studies, FeMV status was determined by a combination of RT-PCR, serology and immunohistochemistry.

Recently, Sieg et al[6] reported an association between urinary excretion of FeMV and CKD in cats, which is in contrast to the findings of our study. However, it should be noted that the Sieg study did not use a homogenous group of biochemically confirmed azotemic cats, as the CKD group also included cats diagnosed with feline lower urinary tract disease (FLUTD), nephritis, hematuria, urolithiasis, cystitis,

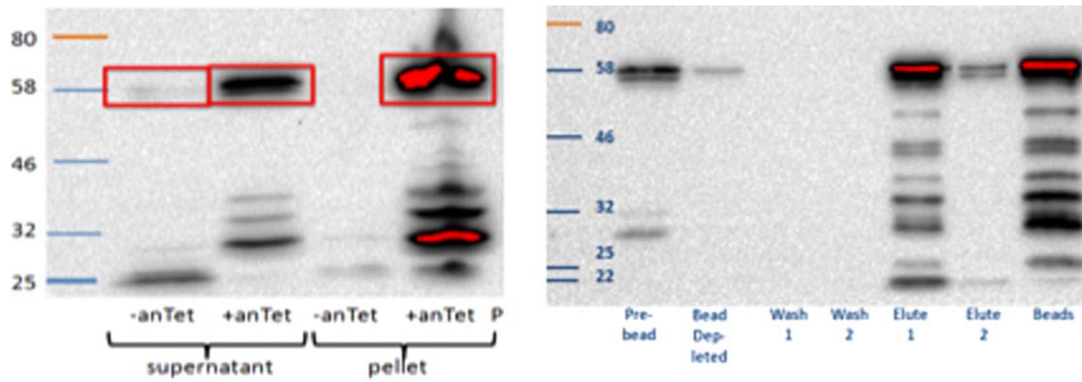

FIGURE 3 A, WB analysis of His-tagged N protein expression in *E. coli* before and after induction with anhydrotetracycline. Protein reactive with mouse-anti 6xHis IgG is present in the soluble and insoluble fractions after induction. B, WB analysis after NTA-bead purification showing N protein concentrations in the initial lysate pre and post-binding to the beads. Equal total protein levels were loaded on lanes 1, 2, and 5 to permit assessment of the depletion because of binding to the beads (compare lanes 1 and 2) and subsequent enrichment post elution (lane 5). A substantial amount of protein remained bound to the bead after two rounds of elution (lane 7)

urostase, chronic kidney failure, chronic nephropathy, proteinuria, and bacteriuria. In contrast, our study excluded cats with evidence of other etiologies such as lower urinary tract inflammation and only included cats with biochemical evidence of renal azotemia.

Whilst our results suggest that exposure to, or urinary excretion of, FeMV is not associated with the presence of azotemic CKD in cats, this does not entirely rule out the possibility of a causal association between renal FeMV infection and CKD. It is possible that the infection could result in intermittent shedding or could trigger a self-sustained immunopathological process leading to the development of TIN and CKD even after the viral infection had been completely resolved (both possibilities manifesting as an RNA PCR negative, WB positive, azotemic CKD result). FeMV infected nonazotemic cats (initially scored as RNA PCR positive, WB positive, CKD negative) might subsequently develop azotemic CKD. Longitudinal observational studies would be required to investigate the association between exposure to, or infection with, FeMV and the eventual development of azotemic CKD.

The seropositive rate of UK cats was higher (31%-64%) than has been reported in previous studies (21%-28%).[1,4,16,23] All of the previous studies evaluated the seroprevalence in Japanese cat populations, therefore it appears that a greater number of UK cats have been exposed to the virus. This could reflect increased proportion of outdoor cats given that a greater proportion of cats with street access had detectable FeMV in urine in a recent study.[10] It has also been reported

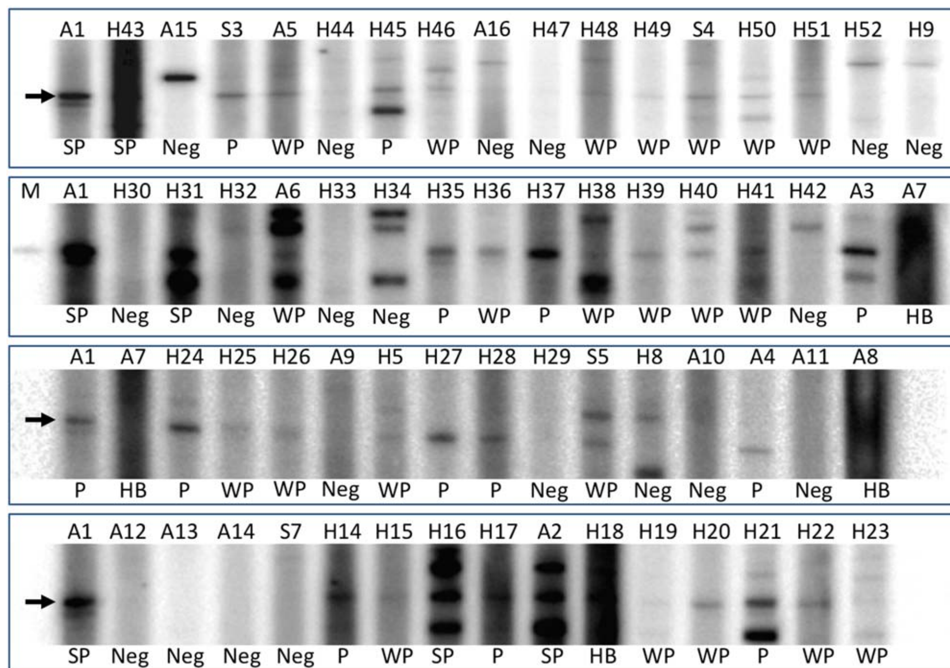

FIGURE 4 Further WB screening of 1/100 dilutions of sera from azotemic and nonazotemic cats showing strong positive, positive, weak positive and negative reactivity to NTA-bead purified his-tagged FeMV N protein. Data are summarized in Table 1. Lane M was developed using a mixture of mouse anti-6xHis 1° Ab and goat anti-mouse HRP 2° antibody

that there can be antigenic cross-reactivity between FeMV and canine distemper virus,[4] and ~10% of serum samples from cats had neutralizing antibodies against CDV,[24] therefore it is also possible that the higher seropositive rate in our cats could partly be explained by the presence of anti-CDV antibodies rather than anti-FeMV in some cats. If the weak positive samples were excluded, the 31% prevalence is more consistent with the results from Japan. The overall prevalence of FeMV in the urine of UK cats was 13%, which was similar to the prevalence documented in Hong Kong (12%)[7] and Japan (6%-23%)[3–5,16,25] but higher than that documented in Turkey (3%).[10]

Many cats with positive or strong positive levels of anti-FeMV antibodies did not have detectable FeMV in the urine. This could reflect previous infection with the virus that has subsequently been cleared or could reflect active infection without urinary FeMV excretion at the time of sampling.[16] WB analysis in our study documented a seroprevalence of 31%-64% and also revealed a high correlation between positive FeMV urinary PCR results and seropositivity. Similarly, in Hong Kong, a significantly higher proportion (76.7%) of FeMV RT-PCR positive cats were seropositive against FeMV N protein compared to FeMV RT-PCR negative cats (19.4%).[1]

Phylogenetic analysis of the L protein sequences identified in our study demonstrated that the most closely related FeMV sequences corresponded to those in Japan and the USA. The sequence variation in this region of the virus was so limited that it was not possible to determine the likely recent ancestral relationship.

The diagnosis of azotemic CKD in our study was based on the measurement of serum creatinine concentration, which is a relatively insensitive indirect marker of GFR, because serum creatinine concentrations only increase above reference intervals when ~75% of functional renal mass lost. Direct measurement of GFR was not possible in the 1st opinion clinics where these samples were collected. One study has indicated that increased concentrations of symmetric dimethylarginine (SDMA) occur earlier in the time course of progressive feline CKD in comparison to increased concentrations of serum creatinine.[26] Future studies investigating the seroprevalence of FeMV in cats with and without CKD when using this novel biomarker could be warranted.

There are a several limitations of our study. We have not confirmed the presence of live virus by viral isolation studies, however previous studies have identified live virus from all RNA positive cats.[1,4] Furthermore qRT-PCR has been reported to be more sensitive than conventional PCR for detecting FeMV RNA in urine samples.[25] However, we repeated our assays using the qRT-PCR method but did not identify any additional cats with urinary FeMV that were not detected by our semi-nested PCR assay (data not shown). It would also have been interesting to document if FeMV infection was persistent in FeMV positive cats in the present study. Persistence of viral shedding over a 2 week and 15 month period has been reported in two previous studies,[7,8] however regrettably we were not able to obtain follow up samples from any RT-PCR positive cats in the present study. Bias in virus detection and serological screening may have been introduced by the assessment of solely geriatric cats, however, this represents the population in which CKD occurs in general. Although there were only small number of cats included, power calculations suggested that the group sizes were adequate to detect significant differences in the prevalence of FeMV between azotemic and non-azotemic cats, if the proportion of cats with CKD that had FeMV infection was similar in the UK and Hong Kong. Histopathology would have been required to further investigate if there is an association between TIN and FeMV detection, however this would have required renal biopsy, which is not currently recommended for the diagnostic workup of most cases of feline CKD. It also is possible that some nonazotemic cats had subclinical nonazotemic CKD, however direct measurement of GFR was not possible in the 1st opinion clinics where these samples were obtained. It is also possible that in some cases FeMV RNA degradation may have occurred during transport of urine samples to our laboratory or during freezing. However, a previous study documented that freeze-thawing samples did not affect virus titers and FeMV was stable at environmental temperatures.[27] Furthermore, it would have been useful to perform complete viral genome sequencing to allow for further studies evaluating the genetic diversity of morbillivirus compared to other strains detected worldwide. However, detecting multiple differences in the highly conserved L gene region suggests that these viruses were genetically distinct.

In conclusion, our study reports the detection of RNA from FeMV and non-FeMV paramyxoviruses in the urine of cats in the UK. No association was found between the presence of clinically appreciable (azotemic) CKD and urinary excretion or past exposure to these viruses (seroprevalence). Despite a lack of association with CKD, the high seroprevalence of paramyxovirus in this cohort suggests that further studies are required to further evaluate the seroprevalence of both FeMV and feline paramyxovirus in the UK cat population and establish if these viruses are associated with other diseases.

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CONFLICT OF INTEREST DECLARATION

The authors declare that they have no conflict of interest with the contents of this article.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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