Veterinary Microbiology 228 (2019) 12–19

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Association of feline morbillivirus infection with defined pathological changes in cat kidney tissues

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ARTICLE INFO

Keywords:
FeMV
Feline morbillivirus
Pathogenicity
Immunohistochemistry
Chronic kidney diseases

ABSTRACT

Feline morbillivirus (FeMV) is an emerging member of morbillivirus discovered in 2012. Although association of FeMV infection with kidney diseases in cats has been suggested, the pathogenicity of the virus has not been clear to date. To study the association between FeMV infection and pathological changes in kidney tissues of infected cats, we performed immunohistochemistry and immunofluorescent assays to detect FeMV antigens and analyzed the effect of FeMV infection on the pathological changes in the kidney tissues. In 38 kidney tissue samples from cats, some tissue injury scores were significantly higher when the FeMV antigens were detected, especially those for the tubular tissues in which the FeMV antigens were mostly localized. Pathological changes associated with the FeMV antigens included the ones typically found in chronic kidney diseases, such as interstitial cell infiltration, glomerulosclerosis, tubular atrophy and fibrosis. We also detected some feline IgG localizations in glomerular tissues, though co-localization or significant association with the FeMV antigens were not found. Our study confirms the association of FeMV infection with some kidney tissue injuries and provides additional information about roles of FeMV infection in chronic kidney diseases.

1. Introduction

Feline morbillivirus (FeMV) is an enveloped negative-sense single-stranded RNA virus. It was originally discovered in Hong Kong in 2012 (Woo et al., 2012) and has been detected across different regions of the world since then (Darold et al., 2017; De Luca et al., 2018; Furuya et al., 2016a, 2014; Koide et al., 2016; Lorusso et al., 2015; McCallum et al., 2018; Park et al., 2014, 2016; Sakaguchi et al., 2014; Sharp et al., 2016; Sieg et al., 2015, 2018; Yilmaz et al., 2017). Although officially classified as *Morbillivirus* (ICTV website), FeMV has a genome sequence relatively distant from the other members of the genus, and has some distinctive biological characteristics, such as infection of kidney tissues and lack of known severe acute symptoms upon infection (Furuya et al., 2014; Park et al., 2016; Sharp et al., 2016; Woo et al., 2012). The biology of FeMV, including its pathogenicity, is still not well understood partly due to this uniqueness. A case-control study in the original report suggested an association of FeMV infection with tubulointerstitial nephritis (TIN) using histopathological samples (Woo et al., 2012) and some studies have been conducted concerning the links between FeMV infection and chronic kidney diseases (CKDs) in cats. FeMV was detected in 40% of the fixed kidney tissues (4 out of 10) from cats with nephritis (Furuya et al., 2014), in urine samples from the cats with lower urinary tract diseases (5 in 120 cats), but not from the ones without the symptoms (86 cats) (Sieg et al., 2015). Inflammatory lesions were detected in the kidney tissues of a higher percentage of cats in the FeMV-positive group [90.0% (26/29)] than in cats in the

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https://doi.org/10.1016/j.vetmic.2018.11.005
Received 21 August 2018; Received in revised form 16 October 2018; Accepted 13 November 2018

-negative group [62.0% (44/71)] (Park et al., 2016). In contrast, the studies in Brazil (FeMV-positive 12 in 52 cats) (Darold et al., 2017) and Turkey (FeMV-positive 3 in 110 cats) (Yilmaz et al., 2017) did not find clear correlation between detection of FeMV and kidney diseases in cats, based on clinical information, and both clinical and histological information, respectively. In the UK, correlation between FeMV infection and azotemic CKDs was not found (FeMV positive in the azotemic and non-azotemic groups, 1/16 and 4/24 cats, respectively) by serum creatinine level and urinary specific gravity (McCallum et al., 2018). Therefore, based on the studies to date, standard clinical records or test results of the cat patients may not be enough and histological examinations may be also required to find the important effects of FeMV infection, including pre-clinical changes of the kidney tissues which lead to CKDs in FeMV-infected cats. For this purpose, we performed immunological assays for detection of FeMV antigens in kidney tissues from cats and analyzed the relationship between FeMV infection and pathological changes in the tissues.

2. Materials and methods

2.1. Reagents and chemicals

All reagents and chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation unless otherwise noted.

2.2. Cat kidney samples

We collected kidney tissues from thirty-eight cats of mixed breeds and genders, which were admitted to clinics for various health conditions (information of some donors are stated in Supplemental Table 1). Urine and other tissue samples from the same patients were not available. Though ages and genders of all the donors were not provided, the average age and male/female ratio were 12.1 years + 1.18 (Mean + SE) and 50% (11/11), respectively, for the ones whose records were provided. All kidney samples were fixed in the 10% neutral buffered formalin, cut in the midsagittal plane, dehydrated in 98% ethanol and embedded in paraffin as formalin fixed paraffin embedded (FFPE) kidney tissues for pathological evaluation and detection of FeMV antigens. This study was reviewed and approved by Ethics Committee for Animal Study and Research at the Faculty of Agriculture, Tokyo University of Agriculture and Technology (Approval Number 0016015).

2.3. Staining and microscopic evaluation of cat kidney tissues

The FFPE kidney samples were sectioned at 3 μm and were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE). For evaluation of the HE sections, we adapted the pathological scoring criteria for kidney section of human lupus nephritis (Hill et al., 2000). The severity of histopathological changes was scored in categories grouped by kidney structures as follows: Glomerular variables; cell proliferation, the thickness of capillaries, glomerulosclerosis, and mesangial area expansion. Renal tubular variables; tubular necrosis, tubular atrophy, luminal expansion, and urinary casts. Interstitial variables; interstitial inflammation, and interstitial fibrosis. All variables were graded from 0 to 3 based on the percentages of observed tissue areas that included lesions: 0, no lesion; 1, less than or equal to 25%; 2, more than 25% and less or equal to 50%; 3, > 50%. To estimate the impact of FeMV on each kidney structure, we calculated the total scores of glomerular variables, tubular scores, and interstitial scores.

2.4. Antibodies

Recombinant FeMV P protein was expressed and purified as reported previously (Arikawa et al., 2017). Briefly, FeMV P gene was inserted into pGEX4T-1 (GE Healthcare) and *E. coli* BL21 (DE3) (Cosmo Bio) was transformed with the resulting plasmid for expression of the gene as a glutathione S-transferase (GST)-fused protein. After initial culture in LB medium the gene expression was induced with Isopropyl-B-d(-)-thiogalactopyranoside at 0.5 mM and the *E. coli* was ultra-sonicated (Handy Sonic UR-20 P, Tomy Seiko Co., LTD) and centrifugated for 10 min at 13,000 rpm at 4°C. The recombinant FeMV P-GST fusion protein was affinity-purified from the soluble fraction with glutathione-conjugated sepharose 4B (GE healthcare). Specific antibody against FeMV protein was produced by a commercial service (Sigma Aldrich). A SPF Japanese white rabbit was immunized with 200 μg of the purified FeMV P-GST protein three times during the 56-day immunization period and whole blood was collected for the serum. A specific antibody against FeMV N protein was produced and provided by Dr. Morikawa. Production of the antibody was described elsewhere (Park et al., 2016).

2.5. Immunohistochemistry and immunofluorescence for detection of FeMV antigens

For immunohistochemistry (IHC), the FFPE kidney tissues were sectioned at 6 μm, deparaffinized, and rehydrated for staining preparation. Then the sections were autoclaved at 121 °C for 20 min in 10 mM citrate buffer (pH 6) for antigen retrieval. The endogenous tissue peroxidase was inactivated by 0.3% H_2O_2 in absolute methanol for 30 min. For blocking non-specific reactions, the sections were incubated with 10% normal goat serum in PBS at room temperature for 30 min. The sections were incubated with 1:200 dilutions of the primary antibodies for overnight at 4 °C in a moist chamber. After washing with PBS, the sections were incubated with HRP-labeled polymer conjugated with the secondary antibody (Envision solution, Dako Envision® + System-HRP Labelled Polymer Anti-Rabbit) at room temperature for 30 min. The signal was developed in 0.05% 3, 3′-diaminobenzidine tetrahydrochloride/H_2O_2 (Wako, Japan). All sections were counterstained with hematoxylin, mounted with a coverslip, and photographed by a photo-micrographic software cell Sens Standard 1.9 (Olympus DP26 model camera).

For immunofluorescence assay (IFA), all FFPE kidney tissues were sectioned at 3 μm. After the same deparaffinization and rehydration process as the immunohistochemistry staining, the section slides were enzymatically treated with 0.05% trypsin for 30 min at 37 °C for antigen retrieval. This antigen retrieval method resulted in better signal/background ratio than the autoclaving method in the case of IFA. The sections were washed with PBS, blocked with 10% normal goat serum in PBS for one hour at room temperature and incubated with rabbit pre-immune serum or the antibody against FeMV P protein diluted to 1:400 with PBS for one hour at room temperature. The tissues were then washed with PBS and incubated with 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Invitrogen) for 1 h at room temperature. For detection of feline IgG, the sections were treated as above until the blocking, and incubated with FITC-conjugated anti-cat IgG (H + L) antibody (Rockland Immunochemicals) diluted to 1:200 with PBS. All stained slides were mounted with coverslips and examined with a fluorescence microscope and digital camera system (FSX 100 Olympus, FSX-BSW Ver 02.02) for observation and image recording.

2.6. Statistical analysis

For the statistical analysis, we decided FeMV test results by taking the most stringent criteria with the obtained results and samples were regarded positive only when all the three immunological examination (two IHC results with antibodies against the FeMV P and N protein, and IFA with antibody against the FeMV P protein) were positive. To evaluate the statistical significance in the differences of the pathology scores between FeMV-positive and -negative kidney tissues, the scores in the two groups were analyzed with two-sided Mann Whitney U test using a publicly available tool (http://astatsa.com/WilcoxonTest/).

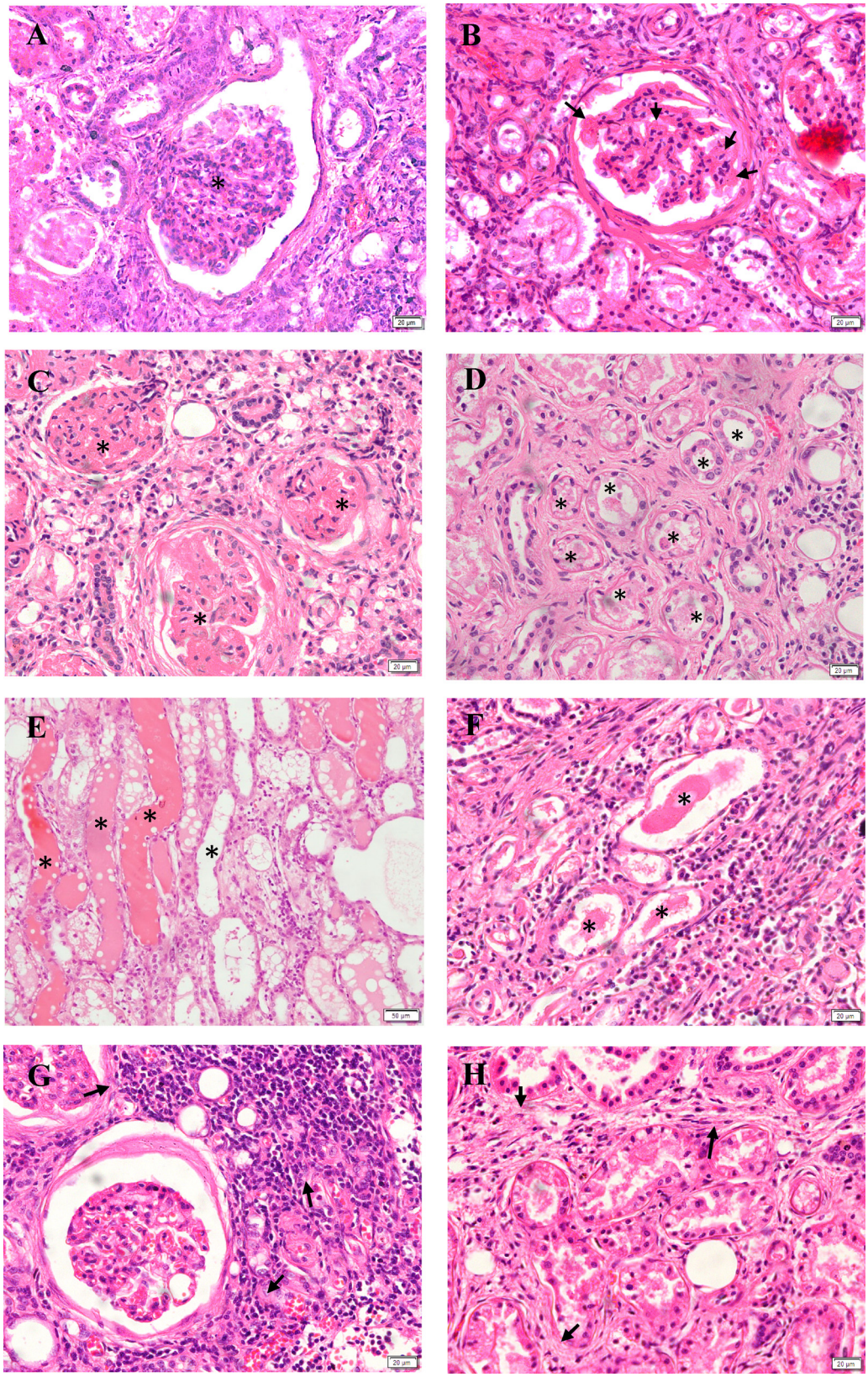

Fig. 1. Representative images for specific pathological changes observed in cat kidney tissues. A, Glomerular cell proliferation; B, Thickness of capillaries; C, Glomerulosclerosis; D, Tubular atrophy; E, Tubular luminal expansion; F, Urinary casts; G, Interstitial cell infiltration; H, Fibrosis. Arrows and asterisks indicate typical affected areas. All images are in 40× magnification and scale bars are 20 µm except E, which is in 20× magnification and has the scale bar of 50 µm.

3. Results

3.1. Microscopic evaluation of pathological changes in cat kidney tissues

To find the morphological abnormalities in the kidney tissues used in this study, we first evaluated HE-stained sections of the cat kidney tissues. Demonstrations of the tissue injuries in the kidney samples were observed microscopically and grouped into the three kidney tissue areas: glomeruli, renal tubules and interstitial areas (categories for each area are shown in Fig. 3). Such morphological changes include; cell proliferation (Fig. 1A), thickness of capillaries (Fig. 1B), glomerulosclerosis (Fig. 1C) and mesangial area expansion (Supplementary Fig. A) for the glomerular tissues; tubular necrosis (Supplementary Fig. B), tubular atrophy (Fig. 1D), luminal expansion (Fig. 1E) and urinary casts (Fig. 1F) for the renal tubules; and inflammatory cell infiltration (Fig. 1G) and fibrosis (Fig. 1H) for the interstitial areas. Also, the severity of the tissue injuries was scored based on the frequencies of the morphological changes above in each tissue (Fig. 1, Supplemental Table 2 for all the scores).

3.2. Immunological detection of FeMV antigens and immune complex in cat kidney tissues

To study association between FeMV infection and cat kidney tissue injuries, we performed immunological detection of the FeMV antigens in the kidney tissues from 38 individual cats using FeMV-specific antibodies. We first performed immunohistochemistry (IHC) with antibodies against FeMV P or N protein (Fig. 2). FeMV P protein was observed in epithelial cells of proximal, distal and collecting duct renal tubules and in transitional epithelial cells (Fig. 2A and B, and Table 1D, negative example). Some positive cells were observed together with inflammatory cells infiltrating into the interstitial areas (Fig. 2B, arrows). IHC was also performed with antibody against the FeMV N protein and the protein was mostly localized on the renal tubular cells, similar to the FeMV P protein localization in the tissues (Fig. 2C, Table 1). With both antibodies, positive signals were observed in the cells of renal tubules and transitional epithelium, but not in the cells of glomeruli or interstitial areas (Table 1). The FeMV antigens were also detected in the renal tubular cells in all the tissue samples in which the FeMV antigens were detected in the transitional epithelial cells (Table 1). Among the 38 total tissues, 20 (52.6%) were positive for the P protein and 16 (42.1%) were positive for the N protein, while 14 (36.8%) tissues were positive for the both proteins (Table 1).

Since eight tissues had signals for only one of the two antigens (21.1%, sample numbers 10, 11, 12, 15, 16, 18, 23, 30), we additionally performed immunofluorescence assay (IFA) with the anti-P protein antibody to confirm the IHC results. In the IFA, signals were detected in 14 samples (36.8%), with the tubular localizations similar to the ones with the IHC (Fig. 1E, and F for a negative sample). We could not use the IFA results for three kidney samples, due to strong backgrounds on the samples (Table 1, indicated as “ND”). Based on the one IFA and the two IHC results, we diagnosed the kidney sample FeMV antigen positive only when all these three tests were positive (Table 1, FeMV antigen diagnosis).

To study association between presence of immune complex formation and FeMV infection in the cat kidney tissues, we performed IFA with anti-cat IgG (H/L) antibody conjugated to FITC. We observed feline IgG-localizations along glomerular tuft of five tissues in total (Table 1 and Fig. 2G), among which two were also positive for FeMV (Table 1, columns for “FeMV antigen diagnosis” and “Feline IgG localization”). In some tissues feline IgG were observed in some interstitial and glomerular spaces (Fig. 2G, Supplemental Fig. 1F). Co-localization of the FeMV antigen and feline IgG was not commonly but only observed in limited occasions in interstitial cells in these tissues (Supplemental Fig. 1F).

3.3. Statistical evaluation of the correlation between the FeMV antigens and pathological changes in the cat kidney tissues

In order to study the association between FeMV infection and kidney tissue injuries, we analyzed the score differences between the FeMV-positive and –negative groups for each pathological category (Fig. 3A–J). The differences between the two groups were especially significant in the three pathological categories of the tubular tissues ($p < 0.0005$, tubular atrophy, luminal expansion, urinary casts, Fig. 3F–H) and the two categories of the interstitial areas ($p = 0.0058$ for inflammatory cell infiltration and 0.0008 for fibrosis). Difference between the FeMV-positive and negative groups was also significant in glomerulosclerosis ($p = 0.0013$) and thickness of capillaries ($p = 0.0390$) of the glomerular tissues, although the latter value was close to the threshold. Based on these results, the presence of the FeMV antigens in cat kidney tissues was significantly associated with some pathological changes, particularly those in the tubular and the interstitial areas in the tissues.

4. Discussion

In this study, detection of the FeMV antigens was significantly associated with some morphological abnormalities in cat kidney tissues, particularly those in renal tubular and interstitial areas. Correlation between FeMV infection and kidney diseases has not yet been clear to date. However, our results are in accordance with lines of evidence which have been suggestive of association between FeMV infection and conditions related to chronic kidney diseases (CKD) (Furuya et al., 2014; Park et al., 2016; Sieg et al., 2015; Woo et al., 2012). On the other hand, in a study in UK, they did not find significant difference in FeMV positive rates between azotemic CKD (6.0%, 1/16) and non-azotemic (17%, 4/24) cat groups when they studied association between detection of FeMV RNA and clinically diagnosed azotemic CKD (McCallum et al., 2018). Although the reason for this discrepancy is not clear at this point, it is possible to point out some potential causes as explained in their report (McCallum et al., 2018). First, TIN and CKD can be developed even after resolution of the viral infection in cats when the viral RNA is no more detected in their urine samples. Also, they could have missed some subclinical azotenic and nonazotemic CKD since they relied on relatively insensitive indirect markers (serum creatinin concentration and urinary specific gravity) to detect azotemic CKD rather than more sensitive means, such as measurement of glomerular filtration rates (McCallum et al., 2018). Finally, they might miss some FeMV RNA due to sub-optimal primers used in their detection method, since the copy numbers of FeMV RNA can be low in the majority of urine samples from FeMV-posibive cats, in which RT-PCR detection of the viral RNA is difficult for some FeMV strains due to sequence variations (Furuya et al., 2016b). Regardless of sensitivity of the diagnostic methods for detection of CKD or FeMV RNA used in the study, they could not detect the histological abnormalities in kidney tissues, since they needed to rely on clinical diagnostic methods for live cat patients, which explains at least some differences observed in their study.

The FeMV antigens were mainly detected in tubular epithelial cells in cat kidney tissues in our study, and such localizations of FeMV were consistent with the analysis results of the severity scores for the pathological abnormalities in which the differences between the FeMV-positive and negative groups were particularly significant in the tubular and interstitial areas (Fig. 3E–J). On the other hand, less morphological changes were affected by presence of the FeMV antigens in the glomerular areas (Fig. 3A–D), agreeing to the fact that no FeMV antigens were localized in glomerular areas (Table). Despite the lack of FeMV antigen localization, severity scores of glomerulosclerosis and thickness of capillaries were significantly affected by detection of the FeMV antigens (Fig. 3B and C). Glomerulosclerosis is known to occur in association with interstitial inflammation and tubular atrophy in cats with

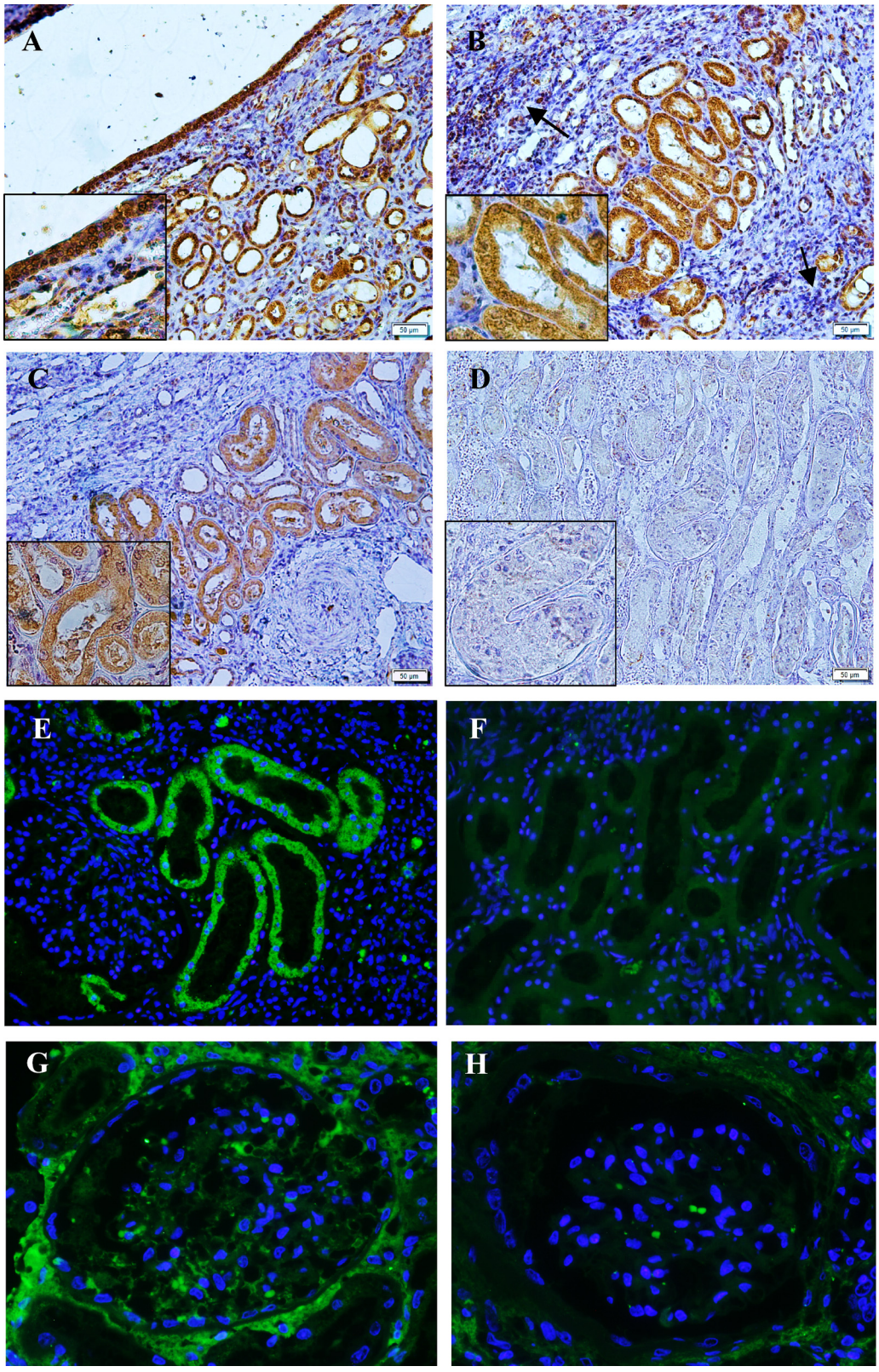

Fig. 2. Representative images for IHC and IFA staining of FFPE feline kidney tissues with antibodies against the FeMV antigens. A–D, IHC images; A, transitional epithelial cells; B and D, renal tubular cells, stained with antibody against the FeMV P protein (D is a sample without the signal for comparison). C, renal tubular cells stained with antibody against the FeMV N protein. Arrows indicate proliferating inflammatory cells. All images in 20× magnification the scale bars are 50 µm. E–H, IFA images; E and F, FeMV P protein-positive and -negative renal tubular cells, respectively (20× magnification). Arrows indicate proliferating inflammatory cells. G and H, feline IgG-positive and -negative glomerular cells (40x original magnification). IFA images (E–H) were taken at the same imaging conditions (exposure time, gain and contrast) for fair comparison.

CKD (Brown et al., 2016), both of which were significantly observed more often in FeMV-positive tissues, which explains the result of our study. It is also possible that thickness of capillaries occurred due to the injuries in the other areas, or secondary injuries such as glomerulosclerosis.

The FeMV antigens were also detected in transitional epithelial cells (Fig. 2A, and Table). However, observation of pathological changes in these cells was difficult, since the tissues were damaged in many samples around that area, and the numbers of the samples were smaller than those with the signals in tubules (Table). Further study will be required to find the effect of FeMV in such cells, including studying more samples with the FeMV localizations in the transitional cells.

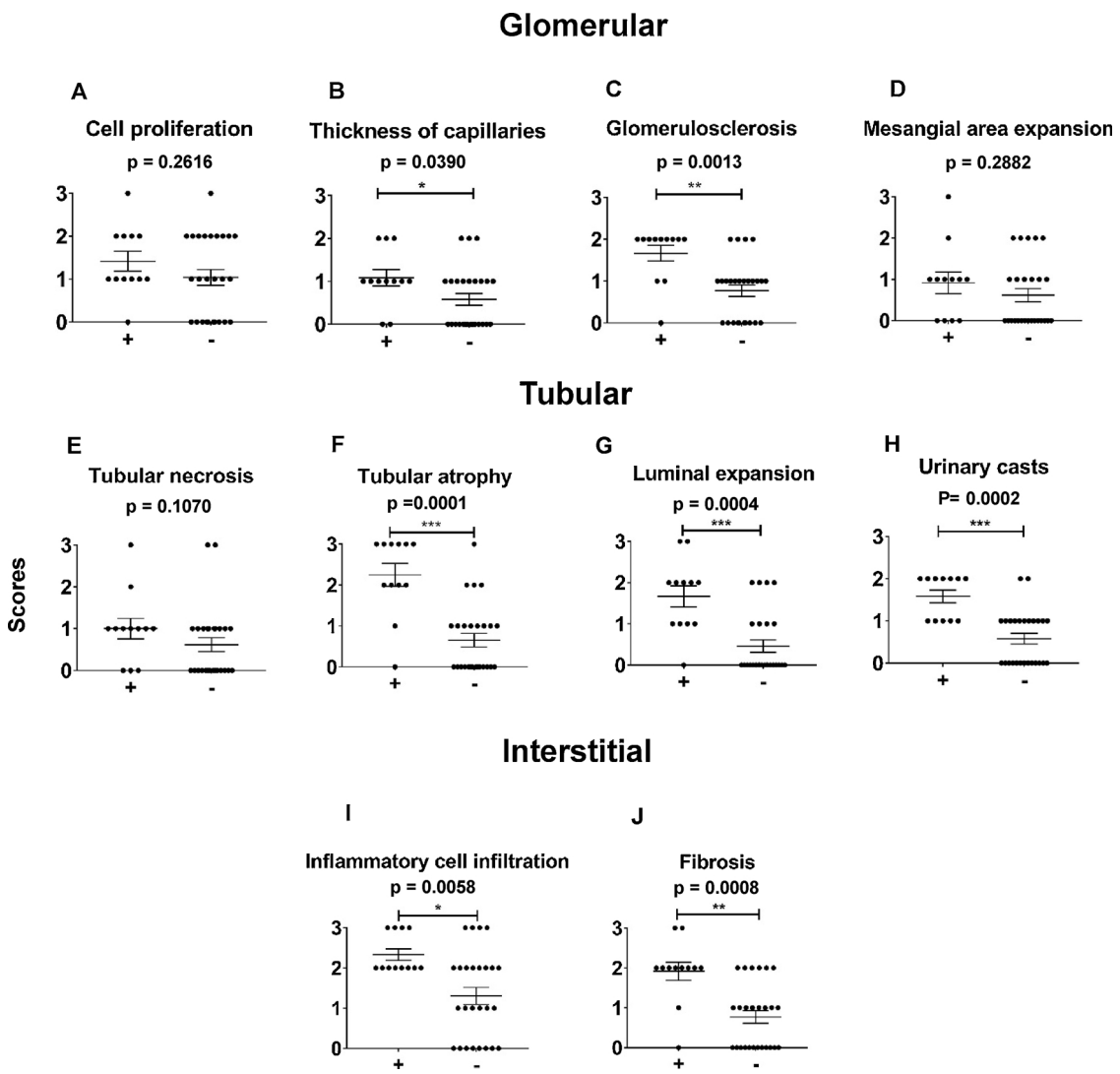

Fig. 3. The correlation between FeMV antigens and pathological changes in the cat kidney tissues. Scores of detailed pathological changes are plotted for groups with (+) or without (-) the FeMV antigens. Significant differences are marked with * ($p < 0.05$), ** ($p < 0.005$), or *** ($p < 0.0005$).

In this study, we detected the feline IgG along capillaries of glomerular tufts and in some interstitial spaces in some kidney tissues (Fig. 2G, Table and Supplementary Fig. 1F). However, we did not find significant association of the FeMV antigens with the feline IgG (Table and Supplementary Fig. 1F), therefore, formation of immune complex in glomerular tissues or interstitial spaces may be secondary to the other injuries associated with FeMV. Further study is needed to find the role of immune complex in FeMV-associated kidney injury.

Although this study suggests correlations between the FeMV infection and the pathological changes in cat kidney tissues, it is still possible that the observed correlations might be results of bystander effects and, thus, FeMV may more frequently infect cats with pre-existing kidney diseases. Any potential cause-effect relationship needs to be examined further with experimental infections of cats with FeMV or studies with continuous clinical records of FeMV-infected cats. However, studies suggest that the irreversible lesions, associated with the FeMV antigens detection in this study, such as interstitial inflammation, fibrosis, and glomerulosclerosis, significantly relate to the later stages of CKDs (McLeland et al., 2015). In humans, infections of viruses such as polyomavirus and herpesviruses are known to induce interstitial and tubular pathological changes in infected kidney tissues (Vanhove et al., 2017). In this regard, it is possible that FeMV-infected cats may have higher chance of developing CKDs later in their lives, particularly at old ages.

In the immunohistochemistry results in this study, some samples became positive for only one of the FeMV antigens (N or P antigen) (Table). Such results might be due to variation of amino acid sequences of the viral proteins among tissues or due to difference in the reactivity between the antibodies. Since it was difficult to additionally perform a genetic test due to RNA degradation in the tissues, we performed IFA as an additional assay since non-specific signal can be lower in IFA in some tissues. Based on the all assays to detect the FeMV antigens, we diagnosed tissue samples positive when all the three results were positive, in order to minimize the chance of false positive results. Although it is difficult to find which assay is the most reliable for the FeMV antigens, we determined the final results stringently as possible in this way, so that the following statistical analysis would be reliable.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgement

Funding for this research was provided in part by JSPS KAKENHI Grant Number 15K07719.

Table 1
Immunological detection of FeMV antigens on the cat kidney tissues.

Sample number	IHC (anti-P)		IHC (anti-N)		IFA (anti-P)	FeMV antigen diagnosis	Feline IgG localization
	Tubules	Transitional epithelium	Tubules	Transitional epithelium			
1	+	−	+	+	+	+	−
2	−	−	−	−	−	−	−
3	+	−	+	−	+	+	−
4	+	−	+	−	+	+	−
5	+	−	+	+	+	+	+
6	−	−	−	−	−	−	−
7	+	+	+	+	+	+	−
8	−	−	−	−	+	−	−
9	+	−	+	−	+	+	−
10	+	−	−	−	−	−	−
11	−	−	+	−	−	−	−
12	+	+	−	−	−	−	+
13	−	−	−	−	−	−	−
14	+	+	+	−	+	+	−
15	+	−	−	−	−	−	−
16	−	−	+	−	−	−	−
17	+	−	+	−	+	+	−
18	+	−	−	−	−	−	−
19	+	−	+	−	−	−	−
20	−	−	−	−	−	−	+
21	−	−	−	−	−	−	−
22	+	−	+	−	+	+	−
23	+	−	−	−	−	−	−
24	+	−	+	−	+	+	−
25	+	−	+	−	+	+	+
26	−	−	−	−	−	−	−
27	+	+	+	+	+	+	−
28	+	−	+	+	−	−	−
29	−	−	−	−	−	−	+
30	+	−	−	−	−	−	−
31	−	−	−	−	+	−	−
32	−	−	−	−	−	−	−
33	−	−	−	−	−	−	−
34	−	−	−	−	ND	−	−
35	−	−	−	−	ND	−	−
36	−	−	−	−	−	−	−
37	−	−	−	−	−	−	−
38	−	−	−	−	ND	−	−

NA: not determined.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.11.005.

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