**Short Communication**

Genetic diversity of feline morbilliviruses isolated in Japan

Shoichi Sakaguchi,1 So Nakagawa,2 Rokusuke Yoshikawa,1 Chieko Kuwahara,3 Hiroko Hagiwara,3 Ken-ichi Asai,3 Kazuo Kawakami,3 Yu Yamamoto,4 Makoto Ogawa5 and Takayuki Miyazawa1

Correspondence

Takayuki Miyazawa
takavet@gmail.com

Received 1 March 2014
Accepted 9 April 2014

1Laboratory of Signal Transduction, Department of Cell Biology, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan
2Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
3Advanced Technology Development Center, Kyoritsu Seiyaku Corporation, 2-9-22 Takamihara, Tsukuba, Ibaraki 300-1252, Japan
4National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan
5Ogawa Pet Clinic, 6–12 Shino-cho, Kameoka, Kyoto 621-0822, Japan

Feline morbillivirus (FmoPV) is an emerging virus in domestic cats and considered to be associated with tubulointerstitial nephritis. Although FmoPV was first described in China in 2012, there has been no report of the isolation of this virus in other countries. In this report, we describe the isolation and characterization of FmoPV from domestic cats in Japan. By using reverse transcription (RT)-PCR, we found that three of 13 urine samples from cats brought to veterinary hospitals were positive for FmoPV. FmoPV strains SS1 to SS3 were isolated from the RT-PCR-positive urine samples. Crandell-Rees feline kidney (CRFK) cells exposed to FmoPV showed cytopathic effects with syncytia formation, and FmoPV N protein was detected by indirect immunofluorescence assays. In addition, pleomorphic virus particles with apparent glycoprotein envelope spikes were observed by electron microscopy. By sequence analysis of FmoPV H and L genes, we found that FmoPVs showed genetic diversity; however, signatures of positive selection were not identified.

Morbilliviruses belong to the family Paramyxoviridae and are negative-sense, single-stranded RNA viruses that include pathogens of humans and animals, including measles, rinderpest and canine distemper (CDV) viruses (Lamb & Parks, 2013). Although CDV infections are sporadically observed among large felids (Deem et al., 2000; Meli et al., 2010; Quigley et al., 2010), the presence of morbilliviruses in domestic cats (Felis catus) was unknown until Woo et al. (2012) reported the isolation of feline morbillivirus (FmoPV) in domestic cats in Hong Kong. Recently, Furuya et al. (2014) demonstrated evidence for the presence of FmoPV in Japanese domestic cats by reverse transcription (RT)-PCR; however, FmoPV has not been isolated in countries other than China (Hong Kong). Genetic analysis revealed that although FmoPV isolates in Hong Kong were members of the morbillivirus lineage, they were substantially different from any reported previously (Woo et al., 2012). Importantly, a histological study suggested that there might be a correlation between FmoPV infection and tubulointerstitial nephritis, which is commonly seen in cats (Woo et al., 2012). In this report, we describe the isolation and genetic diversity of FmoPV, for the first time to our knowledge, from domestic cats in Japan.

Urine samples were randomly collected from cats brought to veterinary clinics in Kyoto and Ibaraki prefectures, Japan. These cats were admitted to the clinics for a variety of reasons. RNA was extracted from the samples using Viral RNA kits (Qiagen) following the manufacturer’s instructions. From the extracted RNA, part of the L gene of FmoPV was amplified using SuperScript III (Invitrogen) with random primers according to the manufacturer’s instructions, followed by PCR with a set of specific FmoPV primers (5’-GGAACATGGCCTCCTGTAGA-3’/5’-CTCC-ATGGGAATCCAGGTTTT-3’) used previously (Furuya et al., 2014), yielding a final product of 487 bp in size. For sequencing, amplicons were purified from agarose gels

The GenBank/EMBL/DDBJ accession number for the N, P/V/C, M, F, H and L gene sequences of feline morbillivirus SS1 is AB910309. The accession numbers for the H gene sequences of feline morbillivirus strains SS2 and SS3 are AB910310 and AB910311, respectively.

One supplementary table is available with the online version of this paper.

The Authors

Printed in Great Britain
with a Gel Extraction kit (Qiagen) and nucleotide sequences were determined by a DNA sequencing service (FASMAC, Kanagawa, Japan). We found that three out of 13 cats were positive for FmoPV genomic RNA and the cDNA showed 90–99% nucleotide similarity to the Hong Kong isolates (Woo et al., 2012) (data not shown).

Next, we attempted to isolate FmoPV from urine samples. Five hundred microlitres of the urine samples were centrifuged at 3000 r.p.m. (2320 g) for 5 min to remove debris, and filtered through 450 nm disc filters (PALL). 1-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma-Aldrich) was then added to the samples at the final concentration of 0.1 μg ml⁻¹ and they were incubated at 37 °C for 15 min. The mixture was inoculated into Crandell-Rees feline kidney (CRFK) cells grown in 25 cm² flasks in serum-free Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with penicillin (100 units ml⁻¹) and streptomycin (100 μg ml⁻¹) (Invitrogen). After 16 h, inocula were replaced by DMEM supplemented with 2% heat-inactivated FCS and antibiotics (see above). Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂ and observed daily for cytopathic effect (CPE) by light microscopy. Subsequently, CRFK cells inoculated with three urine samples (cats 138, 140 and 143) that were positive for FmoPV RNA showed syncytia formation (Fig. 1a). Using RT-PCR, the culture supernatants and cell lysates of these three cultures were found to be positive for FmoPV RNA (data not shown).

To detect viral antigens, we conducted indirect immunofluorescence assay (IFA) using a specific antibody against FmoPV N produced as described by Fischer et al. (2003) and Krach et al. (2000). Briefly, rabbits were immunized with a mixture of three synthetic peptides (VTAPDTAAEENRR, DNINDRGEDQNSNI and SGVTNVHNNDTDLK) conjugated with keyhole limpet haemocyanin. CRFK cells showing CPE were fixed in 4% paraformaldehyde at room temperature for 15 min. The fixed cells were incubated with 1 : 200-diluted anti-FmoPV N rabbit antibody, followed by 1 : 1000-diluted anti-rabbit IgG conjugated with Alexa Fluor 488 (Life Technologies). Cells were embedded in VECTASHIELD mounting medium (Vector Laboratories) and subjected to confocal fluorescence microscopy (Digital Eclipse Spectral Imaging Confocal Laser Microscope C1si, Nikon). Uninfected cells were used as a negative control. We observed specific green granular and diffuse cytoplasmic fluorescence in CRFK cells showing CPE (Fig. 1b). We designated the three FmoPV isolates from cats 138, 140 and 143 strains as SS1, SS2 and SS3, respectively.

The morphology of SS1 was examined by electron microscopy (EM). CRFK cells collected 72 h after viral inoculation were fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined under a Hitachi H-7500 transmission electron microscope. By EM, pleomorphic virus particles that appeared to have glycoprotein envelope spikes were observed (Fig. 1c). The virions were highly variable in size, ranging from 100 to 500 nm in diameter. There were also rare aggregates of filamentous virus nucleocapsids in the cytoplasm of the infected cells. These ultrastructural findings are consistent with those reported in other morbilliviruses such as measles virus (Nakai & Imagawa, 1969).

To detect antibodies against FmoPV, plasma samples from the 13 cats we investigated were subjected to immunoblot analysis using FmoPV strain SS1 propagated in CRFK cells as antigens. Immunoblot analysis was performed as described previously (Nakaya et al., 2013) using 1 : 1000 dilutions of cat plasma samples and a 1 : 30 000 dilution of horseradish peroxidase-conjugated goat anti-cat IgG antibody (Bethyl). As a result, one of the three (33%) RT-PCR-positive and two of the 10 (20%) RT-PCR-negative samples were positive for anti-FmoPV N IgG antibodies (Fig. 1d). Moreover, patterns of FmoPV proteins recognized by cat plasma were different in the positive cats.

Nucleotide sequences of six genes (N, P/V/C, M, F, H, L) of one strain (SS1) and one gene (H) of two strains (SS2 and SS3) were determined. Pairwise comparisons of nucleotide and amino acid sequences of the FmoPV H and L genes are shown in Tables 1 and 2. Note that no gaps were observed in the alignments. As a result, nucleotide and amino acid sequence similarities of the six FmoPV H genes obtained from three Japanese and three Hong Kong strains were 98.9–99.8% and 93.3–99.0%, respectively (Table 1). Interestingly, we found that the SS3 (Lpn) and M252A (HK) H genes showed the second highest nucleotide and amino acid sequence similarities (98.8% and 99.0%, respectively) among the six strains (15 pairwise combinations). By contrast, the SS1 H gene sequence was more distant from those of the other strains (Table 1). Similarly, nucleotide and amino acid sequence similarities of the SS1 L gene were found to be relatively distant from those of the three Hong Kong strains (Table 2). These observations were also supported by phylogenetic analyses of FmoPV H (Fig. 2a) and L genes (data not shown). We also calculated the ratio of non-synonymous to synonymous substitution rates (dN/dS) for each branch of the FmoPV H and L maximum-likelihood trees using the codeml program in PAML 4.7 (Yang, 2007). We could not detect any evidence that positive selection may have operated during evolution of the FmoPV strains (mean dN/dS=0.0752 and 0.0392 for H and L gene trees, respectively). In addition, variable selective pressures along branches were not supported statistically for the FmoPV H and L trees (data not shown).

To infer the phylogeny of FmoPVs among other viruses, we conducted phylogenetic analyses using amino acid sequences of H/G (Fig. 2b) (G proteins of hendraviruses and some of the unclassified paramyxoviruses are equivalent to H proteins of morbilliviruses and FmoPVs) and L genes (data not shown) of FmoPVs, morbilliviruses, henipaviruses and unclassified paramyxoviruses (Table S1, available in the online Supplementary Material). This
procedure was the same as reported previously (Shimode et al., 2014). In both trees, FmoPV strains formed a distinct clade that was distantly related to the cluster consisting of the other morbilliviruses. In addition, the FmoPV clade was also distant from henipaviruses and other unclassified paramyxoviruses. These results indicate that FmoPVs do not belong to morbilliviruses but are unclassified paramyxoviruses.

In this study, we report the isolation of FmoPV from domestic cats in Japan. Although FmoPV Hong Kong

### Table 1. Pairwise comparison of nucleotide (bottom left) and amino acid (upper right) sequences of FmoPV H genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Similarity with (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS1 Jpn</td>
</tr>
<tr>
<td>SS1 Jpn</td>
<td>93.6</td>
</tr>
<tr>
<td>SS2 Jpn</td>
<td>90.6</td>
</tr>
<tr>
<td>SS3 Jpn</td>
<td>90.7</td>
</tr>
<tr>
<td>761U HK</td>
<td>90.3</td>
</tr>
<tr>
<td>776U HK</td>
<td>89.8</td>
</tr>
<tr>
<td>M252A HK</td>
<td>90.7</td>
</tr>
</tbody>
</table>

Jpn, Japan; HK, Hong Kong.

### Table 2. Pairwise comparison of nucleotide (bottom left) and amino acid (upper right) sequences of FmoPV L genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Similarity with (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS1 Jpn</td>
</tr>
<tr>
<td>SS1 Jpn</td>
<td>95.9</td>
</tr>
<tr>
<td>761U HK</td>
<td>89.7</td>
</tr>
<tr>
<td>776U HK</td>
<td>89.7</td>
</tr>
<tr>
<td>M252A HK</td>
<td>90.3</td>
</tr>
</tbody>
</table>
isolates have been shown to be a single species (Woo et al., 2012), our present study revealed that FmoPV is genetically diverse. Phylogenetic analyses using nucleotide sequences of the H and L genes showed that Japanese strain SS1 was related relatively distantly to Hong Kong isolates (Fig. 2a). Low dN/dS values between isolates may indicate that FmoPV had not been subjected to positive selection to escape from the feline immune system, possibly because infections are limited and result in low levels of virions. These results may reflect the findings that we could not detect any anti-F and -H antibodies in RT-PCR-positive cats (Fig. 1d).

Intriguingly, one of our isolates, strain SS3, had a high similarity to strain M252A from Hong Kong. Similarly, Furuya et al. (2014) reported that a partial sequence of FmoPV from a cat living in Tokyo was quite similar to that of strain M252A. These data may suggest that FmoPV had been transmitted by unidentified vector(s) that traversed between China and Japan. According to phylogenetic analyses, bats and rodents have morbilli-related viruses (Drexler et al., 2012). As several species of bats migrate very long distances (Calisher et al., 2006), it is possible that FmoPV-infected bats flew to Japan from China, or vice versa. Alternatively, there could be additional explanations, i.e. transportation of virus-carrying cats between the two regions and/or unusually high genetic constraints of this virus.

The isolation rate of FmoPV was relatively high in cats living in Kyoto. Furthermore, antibody positive rates were also high in both RT-PCR-positive and -negative cats. From our data, we suspect that FmoPV may be prevalent in cats from the Kyoto area, and some cats may be persistently infected with FmoPV irrespective of detectable antibodies; however, the sample size was too small to draw any significant conclusions.
The humoral immune responses against FmoPV in cats were varied in immunoblot analysis (Fig. 1d). In support of this observation, it was reported that persistently CDV-infected dogs also showed different reactions to envelope and core antigens (Krakowka et al., 1975). These data indicate that individual cats elicit antibodies against FmoPV proteins in different ways. However, we cannot exclude the possibility that FmoPV strains have different antigenicity. Furthermore, Appel et al. (1974) reported that about 10% of serum samples originating from cats in the USA had neutralizing antibodies against CDV. We also reported that certain cat populations in Japan and Taiwan had neutralizing anti-CDV antibodies (Ikeda et al., 2001). Because anti-CDV dog sera responded to FmoPV antigens and anti-FmoPV N antibody detected CDV N protein (Fig. 1e), cross-reactivity between FmoPV and CDV should be considered in interpretation of immunoblot analyses. Further studies are needed to discriminate anti-FmoPV from anti-CDV antibodies in cats.

A previous report by Woo et al. (2012) suggested that FmoPV infection is related to tubulointerstitial nephritis. However, in this study, we could not find any clear relationship between FmoPV infection (as determined by the presence of FmoPV RNA in urine and/or anti-FmoPV antibodies) and nephritis. Further epidemiological studies with larger numbers of cats and experimental infection are needed to determine the pathogenesis of FmoPV in cats.

Acknowledgements

We would like to express our gratitude to Dr Masato Fujimura for his help and support in collecting specimens. We thank Professor George Rohrmann (Oregon State University) for his generous help in the preparation of this manuscript. Shoichi Sakaguchi was supported by a fellowship of the Japan Society for the Promotion of Science.

References


