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Mutation of the *S* and *3c* genes in genomes of feline coronaviruses

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ABSTRACT. Feline coronavirus (FCoV) is classified into two biotypes based on its pathogenicity in cats: a feline enteric coronavirus of low pathogenicity and a highly virulent feline infectious peritonitis virus. It has been suspected that FCoV alters its biotype via mutations in the viral genome. The *S* and *3c* genes of FCoV have been considered the candidates for viral pathogenicity conversion. In the present study, FCoVs were analyzed for the frequency and location of mutations in the *S* and *3c* genes from faecal samples of cats in an animal shelter and the faeces, effusions, and tissues of cats that were referred to veterinary hospitals. Our results indicated that approximately 95% FCoVs in faeces did not carry mutations in the two genes. However, 80% FCoVs in effusion samples exhibited mutations in the *S* and *3c* genes with remainder displaying a mutation in the *S* or *3c* gene. It was also suggested that mutational analysis of the *3c* gene could be useful for studying the horizontal transmission of FCoVs in multi-cat environments.

KEY WORDS: *3c* gene, feline coronavirus, multi-cat environment, mutation, *S* gene

The genome of feline coronavirus (FCoV), a member of the *Alphacoronavirus 1* species of the genus *Alphacoronavirus*, comprises single-stranded positive-sense RNA [9]. FCoV infection is prevalent in cats worldwide and is divided into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). The former has low pathogenicity, causing mild enteritis or unapparent infection, and the latter is highly virulent and lethal. FIP is characterised by the accumulation of body cavity effusions (effusive or wet form) and the formation of granulomatous lesions affecting multiple organs (non-effusive or dry form) [10]. FIPVs are considered mutants of FECVs [11, 12, 14].

Although the viral genes responsible for biotype conversion have not been completely elucidated, the candidate genes have been identified. The *S* gene encodes spike protein on the viral membrane. It was reported that 95.8% of 118 serotype I FIPVs displayed missense mutations in codon 1,058 or 1,060 of the *S* gene, whereas none of the sample of 183 FECVs exhibited these mutations [6]. The non-synonymous mutations in codons 1,058 and 1,060 substituted methionine to leucine (M1,058L) and serine to alanine (S1,060A), respectively. The *3c* gene encoding an accessory viral protein was also reported to be mutated in 60–100% of FIPVs, resulting in the loss or truncation of the 3c protein, whereas most FECVs carried an intact *3c* gene [3–5, 8, 11, 13, 14]. Accordingly, it was considered that mutation of the *S* gene, *3c* gene or both was involved in the acquisition or augmentation of lethal pathogenicity in the majorityof FIPV field strains. In the present study, we analyzed the *S* and *3c* genes of FCoVs detected in faecal materials, effusion samples, and tissues that were obtained from cats in Japan to determine the frequency and location of the mutations. An analysis of the *3c* gene suggested the horizontal infection of FCoVs, which were detected in effusions and tissues, among several housemate cats in a multi-cat environment.

MATERIALS AND METHODS

Collection of clinical samples

Clinical specimens were obtained from 93 cats referred to private veterinary hospitals in Japan for suspected FIP based on clinical symptoms, including pyrexia, vomiting, diarrhoea, jaundice, emaciation, anaemia, ascites, pleural effusion, ophthalmologic abnormalities, neurological signs, and death. Some animals displayed an enlargement of abdominal organs that was noticed on palpation, radiography, or ultrasound. The samples of abdominal and pleural effusions, whole blood, serum, rectal swabs, faeces and tissues were sent to our laboratory under refrigeration. Tissues were obtained via autopsy of four cats that had been kept by the

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same owner and referred to a veterinary hospital. The analyzed tissues included kidneys, mesenteric lymph nodes, a spleen, and an eye and its vitreous humor. Whole blood samples were treated with ethylenediaminetetraacetic acid as an anticoagulant.

Faecal samples were collected from an animal shelter wherein each cat was housed alone or with a few other cats per cage. To prevent the redundant analysis of a cat when ≥2 cats were kept in a single cage, only one faecal sample was taken.

Nucleic acid extraction and complementary DNA synthesis

Total RNA samples were extracted from effusions, supernatants of phosphate-buffered saline-homogenised faecal and rectal swab samples, serum, plasma, and a vitreous humor sample from an eye using a QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany) or ISOGEN-LS reagent (NIPPON GENE, Tokyo, Japan). RNA samples of whole blood were extracted using ISOGEN-LS reagent. In some cases, erythrocytes were lysed using 0.2% sodium chloride to isolate leukocytes, and their RNA was extracted using an RNeasy® Mini Kit (QIAGEN) in combination with a QIA shredder (QIAGEN). Tissues were homogenised in ISOGEN reagent (NIPPON GENE) using a TissueRuptor with TissueRuptor disposable probes (QIAGEN). cDNAs were synthesised using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan). All reagents and kits were used according to the manufacturers' instructions.

Amplification of the S and 3c genes by reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the *S* and *3c* genes using GoTaq® Green Master Mix (Promega, Madison, WI, U.S.A.), previously reported primers [1, 5, 6] and our designed primers (Supplementary Table 1). The primers were used at a final concentration of 0.5 μM. The *S* gene fragments were amplified to determine the FCoV serotype (I or II) in each animal. Amplification of the *3c* and *S* genes, including codons 1,058 and 1,060, via first-round PCR was performed as follows: initial denaturation at 94°C for 2 min; 50 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec; and final extension at 72°C for 7 min. In some cases, the *3c* and *S* genes were amplified via nested RT-PCR, in which a second-round reaction was performed using the same PCR cycle parameters. The *S* gene-based serotyping was carried out together with the *3c* gene amplification under the same reaction protocol or separately via single or nested RT-PCR, wherein the reaction protocol was the same except for a shortened extension time of 20 sec. The PCR products were electrophoresed on a 2% agarose gel and amplified DNA fragments were retrieved using the Wizard® SV Gel and PCR Clean-Up System (Promega). The extracted product was directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit on a genetic analyzer (Applied Biosystems 3130, Thermo Fisher Scientific, Waltham, MA, U.S.A.). Some amplicons were cloned into a pCR2.1-TOPO vector using a TOPO® TA Cloning® Kit (Thermo Fisher Scientific) and sequenced using M13 primers or the primers used for RT-PCR. The obtained *3c* gene sequences were analyzed to determine the types and locations of mutations via comparisons with type I FECV strains RM (FJ938051) and UU19 (HQ392470) and type II FIPV strain KUK-H/L (AB781789), none of which carry mutations resulting in the production of truncated proteins. GENETYX 13 (Genetyx Corp., Tokyo, Japan) and BioEdit 7.1.3.0 [7] software were used for sequence analysis. All determined *3c* gene sequences were submitted to the DNA Data Bank of Japan. Accession numbers are shown in Supplementary Tables 2 and 3.

RESULTS

Detection of FCoVs from clinical samples

Of the 53 samples obtained from 40 out of 93 cats that had been referred to animal hospitals, 55 *3c* gene sequences were obtained (Supplementary Table 2). FCoV serotypes I and II were detected in 38 and 2 cats, respectively. The ages of 39 animals with FCoV positivity in any sample ranged from 2 months–17 years (median, 9.5 months), and 30 animals were younger than two years old. Cat 19 was of unknown age. Twenty cats were male, 18 were female and the sex was not recorded for two animals. In stools collected from the animal shelter, *3c* genes were detected in 19 samples (Supplementary Table 3).

Analysis of the S gene

Partial *S* gene fragments of FCoV including codons 1,058 and 1,060 were amplified from the faeces of 19 cats from the animal shelter. Codon mutations were not present in all samples. The FCoV *S* gene in faecal samples from 14 cats that had been referred to animal hospitals was also examined. Four of these cats were fed by a single owner, and the M1,058L or S1,060A mutation was detected in their faeces. Half of the 14 hospital cases presented with ascites or pleural effusion in which FCoV genomes were detected. Of the 30 ascites and pleural effusion samples that contained type I FCoVs, M1,058L and S1,060A mutations were discovered in 24 and 4 samples, respectively, and the remaining 2 samples did not carry the mutations.

Six tissue samples were obtained from four deceased cats that were 4–6 months of age. All tissues contained FCoVs that carried the M1,058L mutation.

An FCoV that was detected in the blood sample from cat 55 had the M1,058L mutation, but because other samples were not taken, this cat was not analyzed further.

Analysis of the 3c gene

Previously reported information regarding the open reading frame (ORF) lengths of the *3c* gene was obtained from the National Center for Biotechnology Information online database. The majority of non-truncated ORFs consisted of 714 nucleotides coding 237 amino acids (aa). Some ORFs were longer because of one or more insertions of several nucleotides. Accordingly, in the present

study, an intact *3c* ORF was defined as a sequence of at least 714 bases that did not contain a premature stop codon due to any mutation type.

The *3c* ORF was 714 bases long in 18 out of 19 FCoV-positive stool samples from the animal shelter. The ORF of the virus detected in the stool sample from cat S10 [shelter cat] was 711 bases due to a 3-base deletion spanning codons 23–24, resulting in the deletion of 1 aa. This mutation did not generate a premature stop codon (Fig. 1A). The *3c* genes were also analyzed from the 14 FCoV-containing faecal samples from cats that had been referred to animal hospitals. One faecal FCoV from cat 37 had a longer intact *3c* gene of 720 bases. This sequence was genetically closest (96.3%) in a BLAST search to two intact *3c* gene sequences of FIPV strains DSKUU48 (GU053649) [5] and UU9 [6].

Ascites and pleural effusion samples containing FCoVs were taken from 32 cats in animal hospitals. Two ascites samples contained type II FCoVs with truncating mutations in the ORF of *3c*. The other 30 samples were type I FCoVs. Of these, 26 samples carried truncating mutations in the *3c* genes (Fig. 1B). Some FCoVs were not expected to express the 3c protein because of a mutation involving the start codon.

All FCoVs identified in the six tissue samples of four cats contained a truncated ORF in each *3c* gene (Fig. 1C). In a kidney and mesenteric lymph node from cat 80, two FCoV variants were detected in each tissue, in which the *3c* ORFs were 712 and 684 bases, respectively. Both variants shared an identical two-base deletion at codon 153, and one variant had an additional 28-base deletion located 46 bases downstream of the two-base deletion site. An FCoV in blood of cat 55 had an intact *3c* gene.

The lengths of truncated 3c proteins expressed by FCoVs were predicted to range from 3 to 235 aa, corresponding to 1.3–99.2% of the length of the wild-type protein.

Mutation types leading to truncation or deletion of the 3c protein

Mutations that resulted in the production of truncated 3c proteins less than 237 aa or complete protein loss were detected in 39 samples collected from 33 cats. This included one faecal sample from a cat housed in an animal shelter and clinical samples from 32 hospital-referred cats. Two deletions (faeces from cat S10 and ascites from cat 75) and one insertion (faeces from cat 37) did not create premature stop codons. The other *3c* genes amplified from 37 samples of 31 cats had mutations resulting in premature stop codons or no protein expression because of a mutation that involved the start codon of each sequence. The most common mutation type that generated premature stop codons was a frameshift resulting from a deletion or insertion (18 samples [48.6%] from 16 cats). Deletions accounted for the majority (17 of 18 samples) of the frameshifts. The second most common cause of premature termination was a nonsense mutation (15 samples [40.5%] from 11 cats). A missense mutation at the start codon was found in three samples (8.1%) from three cats, and an ATG codon next to the original start codon in each sequence was out of frame in all three samples. Deletion of a region including the start codon was found in one sample (2.7%).

Relationship of the mutation of S and 3c genes

The relationship of *S* and *3c* gene mutations in each sample type is indicated in Table 1. For FCoVs in 19 faecal samples that were obtained from the animal shelter, no viruses carried missense mutations at codons 1,058 and 1,060 of the *S* gene. Only one sample showed a deletion of three consecutive nucleotides in the *3c* gene, causing the lack of one aa.

In the four cats belonging to a single owner, the faecal samples contained FCoVs where the M1,058L mutation was found together with truncating mutations of the *3c* genes. FCoVs in the other ten hospital samples did not carry mutations in the *S* and 3c genes.

In the effusion samples, type I FCoVs had mutations in both the *S* and *3c* genes in 24 of 30 samples. A mutation at either codon 1,058 or 1,060 was present in 4 out of 30 samples. The remaining two effusion samples carried only *3c* gene truncating mutations. The present study detected two type II FCoVs in ascites samples, both viruses carrying truncating mutations in the *3c* gene. In the tissue samples of the four cats that belonged to one owner, all FCoVs in tissues had both the M1,058L mutation and *3c* gene truncating mutation.

Sequence relationship among co-habitants

Six cats included in this study were co-habitants (80, 81, 82, 85, 87 and 88) that were fed by a single owner. Cats 80–82 were 4-month-old littermates that died within a month of disease onset and were autopsied. Cat 87 died approximately 2 months later and was also autopsied. Consequently, 14 samples including faecal samples and rectal swabs from the six cats were analyzed, and some identical and closely related mutations were identified (Fig. 2). The ORF homology among the samples ranged from 95.66–99.86%.

A 714-base consensus sequence generated from these samples was identical to the *3c* gene of an FCoV in a rectal swab from cat 80. Deletion of the second and third nucleotides at codon 153 was found in FCoVs detected in the lymph node and kidney tissues of cat 80. An identical deletion was shared in a virus detected in ascites from cat 85. A frameshift caused by this deletion resulted in the generation of a premature stop codon. The kidney and lymph node samples of cat 80 demonstrated another virus variant featuring a 28-base deletion located 45 bases downstream of the two-nucleotide deletion site. An FCoV in a rectal swab from cat 85 had a closely related 29-base deletion at the same position in the *3c* ORF.

A nonsense mutation at codon 210 was identified in the rectal swab and vitreous humor sample from cat 82. The same mutation was detected in viruses in a rectal swab, ascites, and kidney samples from cat 87. The homology of the sequences between the rectal swab from cat 80 and samples from cats 82 and 87 ranged from 99.44%–99.86%. Another nonsense mutation at codon 205 was found in the spleen and lymph node samples of cat 81. A two-nucleotide deletion at codon 123 was detected in an FCoV

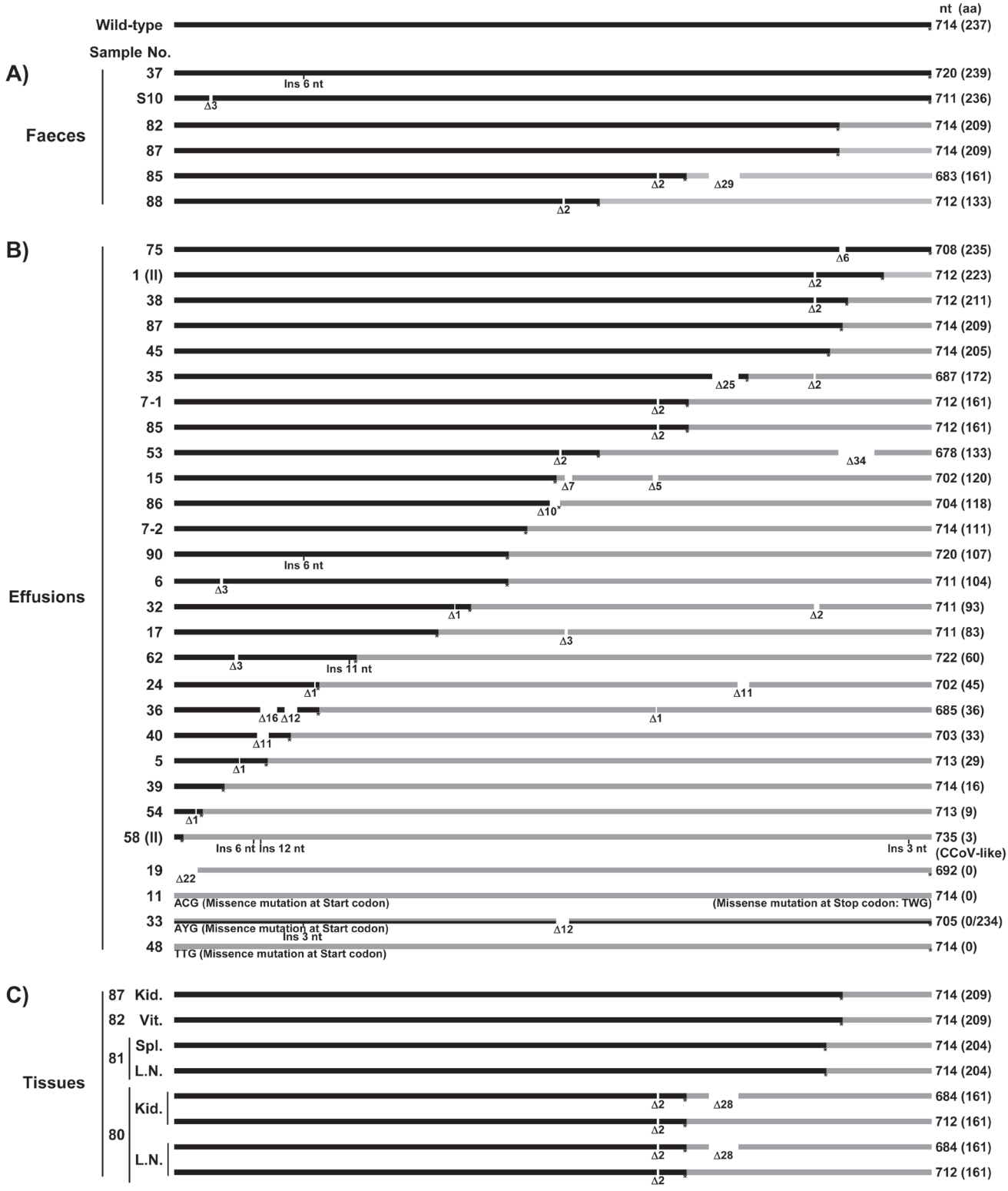

Fig. 1. Schematic representation of feline coronavirus *3c* gene sequences determined in this study. 'Wild-type' indicates a sequence encoding 237 amino acids (aa), which is present in most feline enteric coronaviruses. Every sequence shown below contains a mutation resulting in loss, truncation or elongation of the 3c protein in comparison with the wild-type sequence. Arabic numerals on the left indicate the number assigned to the cat. S10 was a cat from an animal shelter. The virus serotype in cat 1 and cat 58 was II, as indicated in parentheses. Each sequence is shown as a horizontal line with the number of nucleotides (nt) and predicted number of amino acids (aa). A deletion is indicated by a white break with 'Δ' and the number of deleted nt. An insertion (Ins) is shown at the insertion position with the number of inserted nucleotides. An asterisk (*) denotes a stop codon. Black colour indicates the portion of the sequence expected to be translated. Grey colour denotes the portion of the sequence that will not be translated due to a premature stop codon. The sequences are arranged in order of descending protein length for each sample type. An effusion sample from cat 33 contained two variant viruses, one of which harbored an nt substitution in the start codon. Abbreviations: Kid., kidney; Vit., vitreous humor; Spl., spleen; L.N., lymph node.

Table 1. Relation of mutatons between *S* and *3c* genes

Mutated gene in type I FCoVs	No mutation	*S* gene[a)]	*3c* gene[b)]	*S* and *3c* genes	(Total)
Faeces of shelter cats	18	0	1	0	19
(n=19)	94.7%	0.0%	5.3%	0.0%	100.0%
Faeces of hospital cats	10	0	0	4	14
(n=14)	71.4%	0.0%	0.0%	28.6%	100.0%
Effusions of hospital cats	0	4	2	24	30
(n=30)	0.0%	13.3%	6.7%	80.0%	100.0%
Tissue of hospital cats	0	0	0	6	6
(n=6, 4 cats)	0.0%	0.0%	0.0%	100.0%	100.0%
Blood of a hospital cat	0	1	0	0	1
(n=1)	0.0%	100.0%	0.0%	0.0%	100.0%
Mutated gene in type II FCoVs	No mutation		*3c* gene		(Total)
Effusions of hospital cats	0	-	2	-	2
(n=2)	0.0%	-	100.0%	-	100.0%

a) Mutation at codon 1,058 or 1,060. b) Truncating mutation.

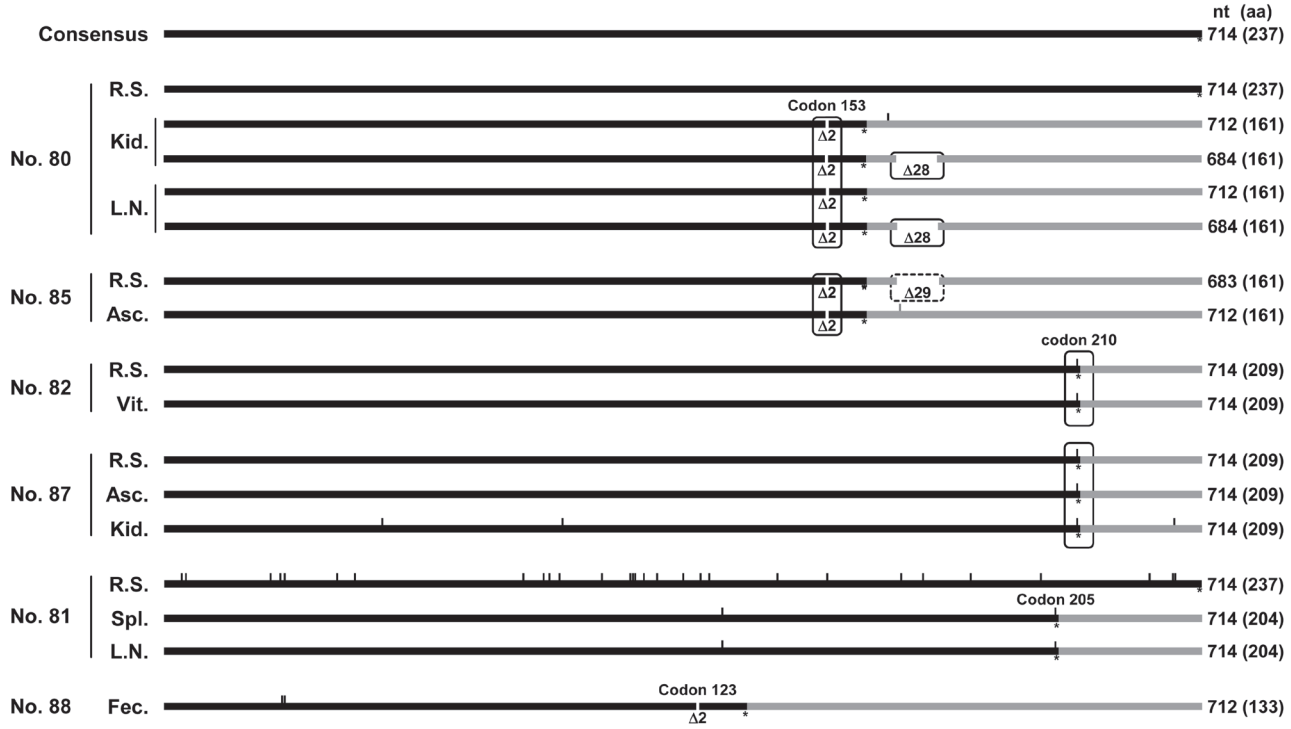

Fig. 2. Sequence similarity of *3c* genes in viruses of cats living in a multi-cat environment. A consensus sequence (top) was determined from all *3c* gene ORFs via software analysis. Each short vertical line on the sequence diagram represents the replacement of a nucleotide from the consensus sequence. Deletions and nonsense mutations, which are identical in length and position, are enclosed by solid lines. A 29-base deletion, closely related to the 28-base deletions indicated, is enclosed by a dashed-line box. Abbreviations: R.S., rectal swab; Kid., kidney; Asc., ascites; Vit., vitreous humor; Spl., spleen, L.N., lymph node; Fec., faeces.

isolated from the faeces of cat 88.

Cats 7-1 and 7-2 were 3-month-old kittens that were housed together. Both cats displayed the accumulation of ascites and pleural effusion over the same period, and the effusion samples were obtained from the hospital on the same day. The sequence homology of the samples was 99.44%, but premature stop codons were caused by a deletion and frameshift in cat 7-1 and a nonsense mutation in cat 7-2.

DISCUSSION

A previous investigation had determined that 96.2% FIPVs causing wet form FIP had either an M1,058L (89.9%) or S1,060A

(6.3%) mutation in the *S* gene [6]. Our present study revealed that type I FCoVs in ascites and pleural effusion samples had the M1,058L and S1,060A missense mutations at a rate of 80.0 and 13.3%, respectively. Because histopathological examinations of the cats were not performed, the biotypes of FCoVs analyzed in this study could not be determined. Therefore, the relationship between the biotypes and gene mutations was not analyzed. However, it is considered that approximately ≥90% FCoVs in effusion samples have one of the *S* gene mutations. On the contrary, neither M1,058L nor S1,060A mutations were found in FCoVs in any of the 19 faecal samples from shelter cats, some of which had soft stools, indicating enteritis. A previous report indicated that none of the FECVs in rectal swabs carried a mutation at codon 1,058 or 1,060 [6]. Accordingly, it is suggested that majority FCoVs in faeces of clinically healthy cats and cats with only mild enteritis carry the *S* genes without any of these mutations. The M1,058L mutation was also detected in faeces and tissues of four young diseased cats that had died. The biotype of the viruses was unknown, but faecal FCoVs with the M1,058L or S1,060A mutation would require experimental infection for pathogenicity determination, even when FIP was confirmed via histopathological examination.

Previous studies have identified truncating mutations of the *3c* gene in the genomes of >60% of FIPVs, whereas most FECVs carried intact *3c* genes. Although the precise molecular function of the 3c protein is unknown, it has been reported to play an essential role in FECV replication in the intestines [2, 13]. Our present study determined that 87.5% FCoVs in body cavity effusions, which included type I and II viruses, and all type I FCoVs, in six tissues collected from four cats, carried *3c* gene mutations resulting in the truncation or loss of 3c protein. However, such mutations were not detected in the majority of faecal FCoVs of shelter cats that did not exhibit any clinical symptoms except for soft stools. This finding is similar to previously published data [3, 6]. Therefore, it is considered that in addition to the M1,058L and S1,060A mutations in the *S* gene, a truncating mutation of the *3c* gene is another genetic feature that is relatively characteristic of FCoVs in effusions and tissues. Molecular functional analysis of the 3c protein is required to elucidate the influence of the *3c* gene mutation on the FCoVs.

The M1,058L or S1,060A mutations were found along with a *3c* gene truncating mutation in 80.0% type I FCoVs from 30 effusion samples, whereas the mutation of either the *S* or *3c* gene was detected in 13.3 and 6.7% of the FCoVs in effusions, respectively. Because the *S* genes were not mutated and *3c* genes were intact in the majority of faecal FCoVs, it is suggested that a mutation of either or both the genes are involved in the alteration of tissue tropism of FCoVs.

A frameshift due to a deletion and a nonsense mutation is the most common truncating mutation of the *3c* gene. This result correlated with previous studies [3, 5]. Interestingly, 22.5% *3c* gene ORFs contained two or three deletions at different positions in each sequence. For example, the *3c* sequence of an FCoV in ascites from cat 35 had a 25-base deletion and another downstream two-base deletion. Therefore, it is considered that mutations often accumulate in the *3c* gene of an FCoV in effusions.

FIPV is not usually considered to transmit horizontally [10]. However, in an outbreak of FIP in an animal shelter in Taiwan, an identical nonsense mutation at codon 210 in the 714-base *3c* ORF was shared by serotype II FCoVs in the effusions of two cats that died from FIP within a 5-month interval [15]. Our present research analyzed six housemate cats that were referred to a veterinary hospital. All cats died within 3 months with or without ascites and granulomatous lesions in organs upon gross examination. Some identical mutations were shared in the *3c* gene sequences with >99% homology. Accordingly, it is considered that an analysis of the *3c* genes is useful to determine whether a specific viral strain horizontally transmits among cats.

In the present study, it was shown that approximately 95% faecal FCoVs in an animal shelter had an intact *3c* gene and that the *S* gene that was not mutated at codons 1,058 and 1,060. In contrast, all FCoVs in effusion samples carried a mutation in either or both of the *S* and *3c* genes. Determination of FCoV biotypes is required to elucidate the correlation of pathogenicity of the virus to gene mutations. Further investigations also need the analysis of protein function alterations caused by the mutations.

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