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Phylogenetic analysis of feline calicivirus *capsid gene* in infected cats

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SUMMARY

To investigate the genetic diversity of feline calicivirus (FCV) in cases of field infection, we isolated FCV-S and FCV-B strains from 2 domestic cats (of 5 pet cats in the same house) that had an acute course and died, and an FCV-SAKURA strain from 1 of the remaining asymptomatic cats, and then RT-PCR-amplified part of the capsid region. The amplified cDNA products were cloned into TA cloning vectors, and the clones obtained were genetically analyzed. FCV-S and FCV-B yielded clones with same nucleotide sequences as FCV-SAKURA or containing somewhat different molecular species, with homologies ranging from 99.4% to 100%. On the other hand, FCV-SAKURA included clones with sequences common to FCV-S and FCV-B and, in addition, clones with different nucleotide sequences, with approximately 71.6% nucleotide homology. Phylogenetic analysis of these clones using 13 other Japanese isolates and 11 isolates from around the world showed that they formed 2 clearly different groups with a bootstrap value of 99.8%, suggesting that the asymptomatic cat was infected at least with a mixture of 2 FCV species with markedly different nucleotide sequences, and became super-infected with FCV from another cat which died.

Key words: capsid region, cat feline calicivirus, Japanese field isolates, phylogenetic analysis.

INTRODUCTION

Feline calicivirus was initially isolated as the etiologic virus of feline respiratory disease by Fastier in 1957.¹⁾ In addition to respiratory disease,²⁾ it has been noted that febrile lameness syndrome,^{3,4)} chronic stomatitis and gingivitis,^{5,6)} abortion,^{7,8)} urinary tract infection⁹⁾ and haemorrhagic fever¹⁰⁾ are associated with this virus. FCV serotypes have been investigated using the virus neutralization test, and the FCV serotypes were highly crossreactive.¹¹⁾ Therefore, FCV is regarded as consisting of variants of 1 serotype,¹²⁻¹⁴⁾ which makes epidemiological studies difficult.

Since the relationship between antigenicity and the 33 amino acids of the E region of FCV *capsid gene* was reported,¹⁵⁾ epidemiological studies of FCV infectious diseases based on phylogenetic analysis of the FCV gene

have been attempted.¹⁶⁻¹⁹⁾ These findings from the genetic analysis led us to consider that FCV is a virus with quasispecies diversity and has genetically diverse molecular species. It has been reported that viruses infecting the host occur as a wide variety of molecular species, and that FCV molecular species diversity evolves experimentally in cats latently infected with FCV.²⁰⁾ However, the nature of the molecular diversity in field cases of FCV infection remains largely obscure. It is difficult to evaluate and compare the phylogenetic relationships of field isolates of FCV based on the nucleotide and amino acid homology of these quasispecies alone, and therefore phylogenetic analyses have been attempted for this purpose, with the goal of inferring the route of infection. In this study, to investigate FCV molecular species diversity and the route of infection, we isolated FCV that caused rare cases of

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fatal field infection in cats (2 of 5 domestic cats housed together) and performed phylogenetic analysis of the FCV isolates.

MATERIALS AND METHODS

Cells and viruses

For the virus cultivation, CRFK cells (feline kidney cells) were used in all the experiments.²¹⁾ The CRFK cells were cultured in Eagles's MEM supplemented with 5% fetal serum. The three strains designated as FCV-S, -B and -SAKURA were selected from isolates from an outbreak manifested by sudden death after excitement (Table 1). The nucleotide sequences of 11 isolates from around the world, including isolates from Germany, Australia and USA, and of 13 Japanese isolates were obtained from GenBank and used for gene analysis. The origins of these isolates are summarized in Table 2.

RNA extraction

NaCl was added to the FCV culture supernatant to a concentration of 0.4 M and polyethylene glycol 8000 was added to 8%, and then the supernatant was centrifuged at 8,000 g for 10 minutes. The precipitate was dissolved in 150 µl of TE buffer.²²⁾ The RNA was extracted from this partially purified virus using ISOGEN (NIPPON GENE, Tokyo).²³⁾

cDNA of capsid gene (2,021 bps)

The RNA extracted from the partially purified virus was supplemented with 0.1 mM oligo (dT) 12-18, and reverse transcribed by incubation with Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Gibco BRL, Tokyo) at 60°C for 1 hour, followed by heat-denaturation at 95°C for 5 minutes, to obtain the cDNA.²⁴⁾

Polymerase chain reaction (PCR)

The cDNA prepared as described above was amplified

Table 1. Three FCV isolates from a field outbreak.

No. of cat	Clinical sign	Breed	Sex	Age	Vaccination	Virus isolation
1	Death two days post exciting	Siamese	Male	7 yr.	Unknown	Cervical spinal cord (FCV-S) Lungs, tonsil
2	Death two days post exciting	Mongrel	Male	4 yr.	Yes	Medullar oblongata (FCV-B) Cervical spinal cord Ileum
3	No sign	Chinchilla	Male	6 yr.	Yes	Oral (FCV-SAKURA)
4	No sign	Chinchilla	Male	2 yr.	No	Negative
5	No sign	Unknown	Unknown	Unknown		Negative

Table 2. Origins of FCV isolates analysed in the phylogenetic tree.

Isolate	Year	Country	Location	Disease complex	GenBank
KS 8	1994	Germany		Acute stomatitis	X 99449
KS 109	1995	Germany		Chronic stomatitis	X 99446
V 274	1989	Australia		Upper respiratory disease and ocular disease	AF 031877
FPL (Bolin)	1957	USA		Panleukopenia	U 06652
FS	1958	USA		Stomatitis	U 06650
FRI-NCI	1960	USA		Upper respiratory disease, conjunctivitis	U 06647
Urbana	1960	USA		Upper respiratory disease and ocular disease	L 40021
F 9	1960	USA		Upper respiratory disease and ocular disease	M 86379
CFI/68	1960	USA		Upper respiratory disease and stiffness	U 13992
FPL-NCI	1960	USA		Enteritis	U 06653
FCV 255	1970	USA		Pneumonia and Ocular disease	U 07130
ML 1	1996	Japan	Kanagawa	Unknown	AB 055454
FCV-2	1989	Japan	Kanagawa	Acute respiratory disease	AB 055446
K 2	1994	Japan	Fukuoka	Upper respiratory disease	AB 058650
K 4	1994	Japan	Osaka	Upper respiratory disease	AB 058652
K 5	1994	Japan	Fukuoka	Upper respiratory disease	AB 058653
K 7	1994	Japan	Osaka	Upper respiratory disease	AB 058654
K 10	1995	Japan	Fukuoka	Upper respiratory disease	AB 058657
K 11	1995	Japan	Fukuoka	Upper respiratory disease	AB 058658
IJ-F-3	1998	Japan	Fukuoka	Upper respiratory disease	AB 058660
IJ-F-4	1998	Japan	Fukuoka	Upper respiratory disease	AB 508661
IJ-F-5	1998	Japan	Fukuoka	Upper respiratory disease	AB 058662
IJ-F-7	1999	Japan	Fukuoka	Upper respiratory disease	AB 058663
IJ-F-9	1999	Japan	Fukuoka	Upper respiratory disease	AB 058664

by PCR as described by Seal,¹⁵⁾ using the following primers to obtain a fragment consisting of the nucleotide sequence from positions 1087 to 1757 in the capsid region (B-F) of the CFI/68 strain and including the hypervariable E region (Fig. 1). For this PCR amplification, 100 pmol each of the sense primer 5'-TTCGGCCTTTTGTGTTCC-3' and the antisense primer 5'-TTGAGAATTGAACACAT-3', and 3 units of Taq polymerase were added to the cDNA preparation. After heat-denaturation at 94°C for 30 seconds, the DNA was annealed with primers at 50°C for 30 seconds and elongated at 72°C for 10 minutes. This reaction was repeated for 25 cycles to amplify the DNA. A 671-nucleotide DNA fragment was the main product.¹⁵⁾

Fig. 1. PCR-amplified FCV capsid region, including E hypervariable region (B-F), and TA cloning vector.

Cloning

The PCR-amplified DNA products of the Japanese isolates were cloned using TA cloning kits (Invitrogen, USA) following the manufacturer's manual.²⁵⁾ The sequences of these cloned DNA fragments were determined and recombinant plasmids containing 2 identical sequences were selected and used for the genetic analysis.

Sequence determination

The plasmid nucleotide sequences of the FCV capsid gene were determined using an ABI autosequencer (Applied Biosystems, Weiterstadt) with Taq polymerase and fluorescence-labeled dideoxynucleotides.^{22,27)}

Sequence analysis

In the phylogenetic analysis, cDNA sequences consisting of 658 bases encoding for amino acids from positions 377 to 576 excluding the primer regions at both ends of the PCR products were used. The phylogenetic analysis of the nucleotide and amino acid sequences was performed by the neighbor-joining (NJ) method.²⁸⁾ The Deoxy Nucleic Acid sequencing, amino acid sequencing, homology comparison, and phylogenetic analysis were

Fig. 2. Electrophoretic patterns of RT-PCR products of FCV-S, -B and -SAKURA.

M: molecular weight marker.

Fig. 3. Electrophoretic patterns of *EcoRI* restriction fragments of cloned RT-PCR product-containing TA cloning vector.

M: molecular weight marker.

Clone (No. of nucleotide)

(1087) sense primer	
FCV-S 1	1:TTGCGCCTTTTGTGTTCAAGCCAATA--GACACTTTGACTTCAAGCAGGAGACTGCAGGATGGAGCACCCCGAGATTAGACCTATGAGGGTA--A-C-C-ATCAGTCA-AA-AA-GG-TG 111
FCV-S 2	1:..... 111
FCV-S 5	1:..... 111
FCV-S 6	1:..... 111
FCV-B 3.4.7.11	1:..... 111
SAKURA 10.25	1:..... 111
SAKURA 19	1:..... 111
SAKURA 20	1:..... 111
SAKURA 29	1:..... 111
SAKURA 3	1:..... 111
SAKURA 8	1:.....CC.T..T..T..C..A..G..G..T..T..A..G..G..C..CT.T.A.G..T..G..G..C..C.. 114
SAKURA 14	1:.....CC.T..T..T..C..A..G..G..T..T..A..G..G..C..CT.T.A.G..T..G..G..C..C.. 114
SAKURA 15	1:.....CC.T..T..T..C..A..G..G..T..T..A..G..G..C..CT.T.A.G..T..G..G..C..C.. 114
*****	
FCV-S 1	112:GGGCAATG-TTAGGAATTGG-AGTGGCTACCGACT-CTATAGTCCCTGGCATACCCGATGGGTGGCTGACACACAATACCCAGAGAACTCACCCAGCTGGTGATTATTCAATTGTTA-- 228
FCV-S 2	112:..... 228
FCV-S 5	112:..... 228
FCV-S 6	112:..... 228
FCV-B 3.4.7.11	112:..... 228
SAKURA 10.25	112:..... 228
SAKURA 19	112:..... 228
SAKURA 20	112:..... 228
SAKURA 29	112:..... 228
SAKURA 3	115:.....A..G.....C..T..A..T..T..A..G..T..G.....T..C.....T..C..TG.....G.....G.....C.....CG.....C..CC 227
SAKURA 8	115:.....A..G.....C..T..A..T..T..A..G..T..G.....T..C.....T..C..TG.....G.....G.....C.....CG.....C..CC 227
SAKURA 14	115:.....A..G.....C..T..A..T..T..A..G..T..G.....T..C.....T..C..TG.....G.....G.....C.....CG.....C..CC 227
SAKURA 15	115:.....A..G.....C..T..A..T..T..A..G..T..G.....T..C.....T..C..TG.....G.....G.....C.....CG.....C..CC 227
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FCV-S 1	229:ATCA--GACTAAG-AATGACATCACCAGGCTGC--TCAGTACGACAGC-GCC-ACTGAAATCACAA-ACAACACAACTTT-AAGAGTATGTACATATGTGG-TGCTCTT-CAGCGTGGCT 339
FCV-S 2	229:..... 339
FCV-S 5	229:..... 339
FCV-S 6	229:..... 339
FCV-B 3.4.7.11	229:..... 339
SAKURA 10.25	229:..... 339
SAKURA 19	229:..... 339
SAKURA 20	229:.....C..... 339
SAKURA 29	229:..... 339
SAKURA 3	228:.....GG..A..G..T.....T..T..T..AAC..T.....A..A.....A..G.....CCG..G..G.....T..C.....A.....A..A..A..C.. 339
SAKURA 8	228:.....GG..A..G..T.....T..T..T..AAC..T.....A..A.....A..G.....CCG..G..G.....T..C.....A.....A..A..A..C.. 339
SAKURA 14	228:.....GG..A..G..T.....T..T..T..AAC..T.....A..A.....A..G.....CCG..G..G.....T..C.....A.....A..A..A..C.. 339
SAKURA 15	228:.....GG..A..G..T.....T..T..T..AAC..T.....A..A.....A..G.....CCG..G..G.....T..C.....A.....A..A..A..C.. 339
*****	
EcoR I	
FCV-S 1	340:GGGGTGATAAGAAAATATCAAACTACTGCTTTGTGACGACCGGGGACT-CT-T---GATGGGA-ATTC-CATTC-TTCCTTCCAACACAATTGACCCA--ACAAAATT-ATAGTTTCCAGG 450
FCV-S 2	340:.....A..... 450
FCV-S 5	340:.....A..... 450
FCV-S 6	340:.....A..... 450
FCV-B 3.4.7.11	340:.....A..... 450
SAKURA 10.25	340:.....A..... 450
SAKURA 19	340:.....A..... 450
SAKURA 20	340:.....A..... 450
SAKURA 29	340:.....A..... 450
SAKURA 3	340:.....G..T..G..C.....C..TT.....G..G..AAA..A..T..A..A..GC..AAG..A.....TG.....GC.....G..... 450
SAKURA 8	340:.....G..T..G..C.....C..TT.....G..G..AAA..A..T..A..A..GC..AAG..A.....TG.....GC.....G..... 450
SAKURA 14	340:.....G..T..G..C.....C..TT.....G..G..AAA..A..T..A..A..GC..AAG..A.....TG.....GC.....G..... 450
SAKURA 15	340:.....G..T..G..C.....C..TT.....G..G..AAA..A..T..A..A..GC..AAG..A.....TG.....GC.....G..... 450
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FCV-S 1	451:ACAATCAGC-T---CAAAGCTGAAGTTCAAACATCTGACGTACACATTGGCAATGT--TAGGATACACTGGAATTGGTGAAGAAGTC-ATTGGATCTGATAGAAACAAAGTTGTCGATCAAC 566
FCV-S 2	451:..... 566
FCV-S 5	451:.....G.....C..... 566
FCV-S 6	451:.....G.....C..... 566
FCV-B 3.4.7.11	451:..... 566
SAKURA 10.25	451:..... 566
SAKURA 19	451:.....A..... 566
SAKURA 20	451:..... 566
SAKURA 29	451:.....A..... 566
SAKURA 3	451:..T..C.....T..AGG.....GC.....A..GC.....C..CC..T..G.....C..C..C.....A.....C..G.....G.....GG..G..G..A.. 564
SAKURA 8	451:..T..C.....T..AGG.....G.....A..GC.....C..CC..T..G.....C..C..C.....A.....C..G.....G.....GG..G..G..A.. 564
SAKURA 14	451:..T..C.....T..AGG.....G.....A..GC.....C..CC..T..G.....C..C..C.....A.....C..G.....G.....GG..G..G..A.. 564
SAKURA 15	451:..T..C.....T..AGG.....G.....A..GC.....C..CC..T..G.....C..C..C.....A.....C..G.....G.....GG..G..G..A.. 564
*****	
Antisense primer (1757)	
FCV-S 1	567:GTGC-TACCTG--AGACCAGC---A-CGCGTGGTGGGAATCA-CCCAGTGAT-TATTTGAATAAGTC---AAA-TTAGGATATGTAATTAGAG-AAATTGATGTGTTCAATTCTCAA 671
FCV-S 2	567:..... 671
FCV-S 5	567:..... 671
FCV-S 6	567:.....C..... 671
FCV-B 3.4.7.11	567:..... 671
SAKURA 10.25	567:..... 671
SAKURA 19	567:..... 671
SAKURA 20	567:..... 671
SAKURA 29	567:..... 671
SAKURA 3	565:.....A.....CC.....TGG..C..C.....C..C..T..C..C.....C..G.....TAT..G..G.....TCT.. 671
SAKURA 8	565:.....A.....CC.....TGG..C..C.....C..C..T..C..C.....C..G.....TAT..G..G.....TCT.. 671
SAKURA 14	565:.....A.....CC.....TGG..C..C.....C..C..T..C..C.....C..G.....TAT..G..G.....TCT.. 671
SAKURA 15	565:.....A.....CC.....TGG..C..C.....C..C..T..C..C.....C..G.....TAT..G..G.....TCT.. 671
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Fig. 4. Sequence variabilities of the partial capsid gene (fragments of 671 bp) of three FCV-S, B and SAKURA clones obtained from two cats with sudden death and 1 latent infection.

* : identical nucleotide.

Clone (No. of nucleotide)

(1087)sense primer			
FCV-B 8	1:	TTCCGGCCTTTTGTGTTCC	100
FCV-S 9	1:	.....G.....	100
FCV-B 6	1:	.....	100
FCV-S 7	1:	.....	100
.....			
FCV-B 8	101:	TCAAAAAGGTGGGGCAATGTTAGGAATTGGAGTGGCTACCGACTCTATAGTCCCTGGCATAACCGATGGGTGGCCTGACACAACAATACCAGAGAACTC	200
FCV-S 9	101:	.....	200
FCV-B 6	101:	.....	200
FCV-S 7	101:	.....	200
.....			
FCV-B 8	201:	ACCCAGCTGGTGATTATTCAATTGTTAATCAGACTAAGAATGACATCACCACGGCTGCTCAGTACGACAGCGCCACTGAAATCACAAACAACACAACT	300
FCV-S 9	201:	.....	300
FCV-B 6	201:	.....	300
FCV-S 7	201:	.....	300
.....			
FCV-B 8	301:	TTAAGAGTATGTACATATGTGGTGCTCTTCAGCGTGCCTGGGGTGATAAGAAAAATATCAAATACTGCATTGTGACGACCGGGACTCTTGATGG	400
FCV-S 9	301:	.....	400
FCV-B 6	301:	.....	400
FCV-S 7	301:	.....	400
.....			
FCV-B 8	401:	CATTCTTCTTCCAACACAATTGACCCAACAAAAATTATAGTTTTCCAGGACAATCAGTCAAAGCTGAAGTTCAAACATCTGACGTCACATTGGCAATG	500
FCV-S 9	401:	.....	500
FCV-B 6	401:	.....	500
FCV-S 7	401:	.....	500
.....			
FCV-B 8	501:	TTAGGATACACTGGAATTGGTGAAGAAGTCATTGGATCTGATAGAAACAAAGTTGTCGATCAACGTGCTACCTGAGACCAGCACGCGTGGTGGGAATC	600
FCV-S 9	501:	.....G.....	600
FCV-B 6	501:	.....C.....	600
FCV-S 7	501:	.....	600
.....			
Antisense primer (1757)			
FCV-B 8	601:	ACCCAGTGATTATTTGAATAAGCTCAAATTAGGATATGTAATTAGAGAAAT	700
FCV-S 9	601:	.....G.....T..T.....	700
FCV-B 6	601:	.....	700
FCV-S 7	601:	.....G.....T..T.....	700
.....			
sense primer (1874)			
FCV-B 8	701:	TAATAATTACTTACTTGACCCTGACTGTTTGTCTGTTTATAGGATCACTGACTCTAATGGCTCTTGTTT	788
FCV-S 9	701:	.....GGAACACAAAAGGCCGAA	788
FCV-B 6	701:	.....AC.TTGGT.TA.ATT.TTCTGG.TTTTCT	800
FCV-S 7	701:	.....AC.TTGGT.TA.ATT.TTCTGG.TTTTCT	800
.....			
EcoRI			
FCV-B 6	801:	TATGTTGGTGTCTTACTATTGGAAAATTAGAGTTTCCCTTACTGCCTCTACATGG	900
FCV-S 7	801:	TATGTTGGTGTCTTACTATTGGAAAATTAGAGTTTCCCTTACTGCCTCTACATGG	900
.....			
sense primer (2001)			
FCV-B 6	901:	ACACAAAAGGCCGAA	915
FCV-S 7	901:	ACACAAAAGGCCGAA	915

Fig. 5. Sequence variabilities of the partial *capsid gene* (long DNA fragments of 788 bp and 915 bp) of FCV-S and B clones obtained from two fatal cats with sudden death.

* : identical nucleotide.

performed using Genetyx-MAC Ver. 10 and Genetyx-MAC/ATSQ 3 software (Software development, Tokyo).

RESULTS

Outbreak of FCV infection in cats

In June 1993 in Yokohama, cat No.2 of 5 domestic cats developed symptoms. Subsequently, cat No.1 and 2 became severely agitated and died 2 days later. Of the viruses isolated from these 5 cats, the following 3 strains were subjected to PCR amplification followed by nucleotide sequencing for genetic analysis: FCV-S isolated from cat No.1's spinal cord, FCV-B from cat No.2's medulla oblongata (these nerve tissues were probably involved in the disease process), and FCV-SAKURA from the oral cavity of 1 of the 3 asymptomatic cats (Table 1).

PCR amplification

Electrophoresis of PCR products from the 3 strains gave a band of near a marker of 726 bp (Fig. 2). The PCR products of each isolate were cloned into the TA cloning vector. The resulting plasmid DNA was cleaved by *EcoRI* and the fragments were electrophoresed. As shown in Fig. 3, a band near a marker of 427 bp was detected for clones of FCV-S (1, 2, 5, 6, 7 and 9), FCV-B (3, 4, 7, 11, 6 and 8) and FCV-SAKURA (10, 19, 20, 25 and 29). FCV-S 7 and FCV-B 6, and FCV-S 9 and FCV-B 8 showed similar electrophoretic patterns. This long DNA fragment was observed in 2/6 (33%) clones of FCV-S and FCV-B respectively, but not in the products amplified from FCV-SAKURA. The 4 clones of FCV-SAKURA (3, 8, 14 and 15) gave a band of near a marker of 726 bp.

Fig. 6. Unrooted phylogenetic tree of FCV clones produced using nucleotide sequences of 658 bases.

The tree was produced using the NJ method. The clones were divided into 2 major groups of A and B. Bootstrap values (%) are included at the relevant nodes, Evolutionary distances are to scale. The origins of each isolate are also indicated by a country letter and year, as in Table 2 (A: Australia, G: Germany, J: Japan, USA: America).

Genetic analysis

As shown in Fig. 4, the nucleotide sequencing showed that the clones of 671 bp from FCV-S 2, FCV-B 3, 4, 7 and 11, and FCV-SAKURA 10 and 25 were identical, and other clones (FCV-S 1, 5 and 6, and FCV-SAKURA 19, 20 and 29) also showed high nucleotide homology of over 99.4%. In addition, in FCV-SAKURA (3, 8, 14 and 15), the sequence of these clones were different from that of clones noted above, and the nucleotide and amino acid sequence homologies of these clones were about 71.6% and 72.7%, respectively. In Fig. 5, FCV-S 7 and FCV-B 6, and FCV-S 9 and FCV-B 8 showed 788 and 915 bp, respectively, which were longer than the clones of 671 bp. Although the antisense primer site of FCV-S clones was not changing, the 2 clones of FCV-B 6 and 8 contained the nucleotide sequence GATGT (A) TTCAA (C) TC (C) CAA with 3 substitutions (A, C and C) in the antisense primer-annealing region; thus, the antisense primer failed to anneal to this region, and the sense primer annealed to the region upstream from the

3' end of the antisense primer.

Phylogenetic tree analysis

A phylogenetic tree was constructed the clones described above, and strains obtained from the Genbank (Table 2). These clones were found to consist of 2 groups (A and B in Fig. 6), and had a bootstrap value of 99.8%.

DISCUSSION

On electrophoresis, PCR products of the viruses from the 3 cats showed a band with the same size, revealing no significant difference. PCR using these primers amplified a region extending from inside the capsid B region to inside the F region and containing the hyper-variable E region.¹⁵⁾ Studies by seal using slight modifications of these primers in field infection cases and in experimental cases produced a single band of the same size as that observed in this study.²⁹⁾ Using those modified primers, we obtained the same results as those reported by Seal (data not shown).¹⁵⁾ Thus, the PCR amplification in this study was performed according to his method. The PCR products were cloned into TA vectors, and the resulting FCV clones were cleaved with *EcoRI* for electrophoresis. As a result, FCV clones showing electrophoretic patterns common to the 3 strains and fragments showing bands of different lengths for FCV-S and FCV-B were detected for 33% of the clones of each strain. Such FCV-B clones contained 3 different nucleotide substitutions in the antisense primer-annealing region, which prevented their annealing to the antisense primer, and resulted in annealing of the sense primer to the region upstream from the 3' end of the antisense primer. Thus, the clones represented the product of amplification of the region flanked by 2 sense primers. Although the long clones of FCV-S had antisense primer of the same sequence as the clones of 671 bp, it formed the clones of the same length as FCV-B. It seems that the sense primer as FCV-B exists in 3' upstream of antisense primer of FCV-S. Such long clones were not detected from FCV-SAKURA clones. Since it was not thought that FCV infected in the 2 dead cats produced mutant for such a short period of time, it seemed that cat No. 3 was infected with FCV from a dead cat, and became a latent infection.

On the other hand, 44% of FCV-SAKURA clones showed a different electrophoretic pattern. These clones had markedly different sequences due to base deletions and substitutions, which were not detectable in clones from the 2 dead cats. These clones formed another group with phylogenetic analysis. However, an attempt to isolate viruses corresponding to these different clones was unsuccessful, making it impossible to examine their antigenicity. The finding that FCVs with markedly different clones were isolated from the same cat suggested the quasispecies diversity of FCV

field isolates.

We hypothesize that this cat with a latent infection with FCV-SAKURA became superinfected with FCVs with 2 different clones. It has been reported that FCV undergoes mutations in 0.10-1.07% of its bases during 1 year in cell culture, and in up to 7.3% of nucleotides through selection and elimination due to immunity in the feline body.²⁰⁾ Kreutz et al.³⁰⁾ observed antigenic variation and variation in the hypervariable E region of the *capsid gene*, but it was not so marked result in different clones and groups, as shown by clones in the primer-annealing region and by phylogenetic analysis of FCV-SAKURA.

Although the outbreak of this fatal infection was terminated, the findings in this study suggest that similar highly pathogenic viruses may emerge in the future because this virus undergoes severe mutations.¹⁰⁾ We are considering further investigation of molecular species diversity in a variety of cases of field infection.

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