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Search of the Infections Source of *feline calicivirus* in a Multicat Household

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Abstract: A multicat household experienced an epidemic of *feline calicivirus* (FCV) infection. FCV was isolated from eight of 34 cats. We analyzed molecular evolution of isolated FCVs by a phylogenic tree. All the isolates belonged to the genogroup II, and their nucleotide sequences showed >94% identity. They were subdivided into six distinct clusters by phylogenetic analysis, and Ao198-1, the source of infection, was most closely related to Ao199-1, then Ao212-1, Ao210-1, Ao214-1, Ao213-1, Ao222-1and Ao224-1 in this order. Sequence alignments of the isolates showed that the nonsynonymous substitution/total number of nucleotides ratio was <60% in the regions A, B, D conE and F, and >80% in the regions C and 5' and HVR of E. Our result suggested that the virus, while its transmission to the newborn cats, underwent frequent mutation especially at the regions C and E, suggesting these regions were most often involved in evolution of the FCV genome.

Key words: FCV, phylogenetic analysis, genogroup, molecular evolution, nonsynonymous substitution

Introduction

Feline calicivirus (FCV), a member of *Caliciviridae* [5], is clinically important as it cause respiratory disease in cats [7]. The symptoms of FCV infection include mild rhinitis, conjunctivitis, ulceration of the palate or tongue and occasionally bronchial pneumonia [11]. Experimentally infected cats usually recover after a relatively short period of mild symptoms [20] but may develop fatal interstitial pneumonia [11, 12]. After recovery, the infected cats become carriers shedding the viruses, even though they may not show signs of disease. These latent carriers may remain contagious for more than two years shedding FCV from the oropharynx [19, 33, 34, 35]. Such as viruses are thought to become vaccine-resistant through escape mutations. Also there have been reports that FCV strains different from vaccine strains can be isolated from vaccinated cats that show fever and lameness [17, 18], or that vaccinated cats become infected with FCV after vaccination [6, 21, 28]. These failed vaccinations can occur either within or after 21 days of the last vaccination [6]. The origin of such mutated viruses, whether it is the vaccine strain or naturally infected strains, can be determined by comparative analysis of nucleotide sequences at the 5' hypervariable region (HVR) of the capsid gene. In most of the cases, these mutants are derived from naturally occurring strains that share only 21.3–38.0% homology with the vaccine strain [21].

The 3' end of FCV genome encodes a single major capsid protein [3, 5]. This capsid protein undergoes mutations in response to antibodies and external stimuli,

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and has been thought to play a key role for evolution of FCV and thus extensively studied. The capsid protein is divided into six regions based on sequence conservation between FCV and other *caliciviruses* [16, 29]. Region E is subdivided into 5' and 3' HVRs, which are separated by a central conserved domain [28, 29]. 5' HVR and region C are strongly implicated as a target for virus-neutralizing antibodies [8, 14, 31, 32], as it contains epitopes for neutralizing monoclonal antibody [9]. A synthetic peptide containing this region induces a formation of neutralizing polyclonal antibodies. Based on this, these regions are thought to be a target for immune evasion during virus persistence in the host [22, 23]. In addition, FCV may exist as quasispecies by evolving both in cell culture and experimentally infected cats [22, 23]. It is also reported that FCV related to the vaccine strain [24] or a single strain distinct from the vaccine strain [26] have been isolated from a breeding colony, as well as several strains from a rescue shelter [25]. However, such molecular evolution of FCV has not been reported in domestic cats.

In this report, we examined 34 cats kept in a closed household that had experienced an epidemic of FCV infection, even though all the adult cats were vaccinated. We isolated FCVs from eight cats, and based on the sequence of isolates from the cats that were determined as infectious source, we constructed a phylogenetic tree and examined the molecular evolution of FCV during its transmission.

Materials and Methods

Cell culture and virus isolation [27]

Crandell Rees feline kidney (CRFK) cells were used for viral culture in all experiments [4]. CRFK was grown in Eagle's MEM supplemented with 5% calf serum. Conjunctival, oral and nasal swabs were taken from the 34 cats that developed upper respiratory inflammation. Each sample was prepared in Eagle's buffered saline solution with 1000 μg/ml streptomycin and 1000 U/ml penicillin, and centrifuged at 8000 g for 20 min. 0.1 ml of the supernatant was inoculated to cell culture. The culture media were collected after three days, and all the samples were used for viral RNA extraction at the third passage.

RNA extraction and cDNA synthesis

0.2 ml/l of 2.5MNaCl and 25% (w/v) PG8000 were added to the supernatant obtained by centrifugation of the viral sample, and centrifuged at 8000 g for 10 min. Obtained precipitates were dissolved in TE. Viral RNA was extracted from partially purified viruses using ISOGEN (NIPPON GENE). cDNA was synthesized from extracted RNA mixed with 0.1 mM oligo (dT) primer 12-18 (Rikaken) and MMLV reverse transcriptase (Gibco BRL) after 1 h at 60°C, followed by denaturation at 95°C for 5 min.

Amplification of cDNA

First stage PCR was attempted to amplify residues 1987-1757 of cDNA which corresponds to the capsid regions B - F of FCV strain CFI/68 using a specific primer as described [30], but the target nucleotides were not amplified. We constructed a novel primer (Ao primer) and successfully amplified the target sequence. The amplified sequence also contained region C and HVR of region E [15]. Second stage amplification was performed to prepare cDNA using 100 pmol of each of sense primers (5'-TGCGCTAAC GTGCTTAAAT-3') and antisense primers (5'-WWTTCC ATGTAGGAGGC-3') and 3U Taq. Thermal cycling conditions consisted of 35 rounds of denaturation at 94°C for 30 sec, primer annealing at 40°C for 1 min and primer extension at 72°C for 2 min. The final amplicon of 1938 nucleotides were obtained.

Cloning

The amplified nucleotides were purified using Wizard® SV Gel and PCR Clean-up System (Promega) and cloned into plasmids with TOPO TA Cloning® (Invitrogen) [13]. Plasmids were purified using Q1Aprep® Spin Miniprep Kit (Qiagen). All procedures were performed according to the manufacturer's instruction.

Sequencing

Four clones were randomly selected for each sample and sequenced by an ABI autosequencer. Sequence homology was analyzed by nucleotide and amino acid sequences. Phylogenic analysis was performed by Genetyx-MAC Ver. 10 and Genetyx-MAC/ATSQ Ver. 3.0. The phylogenic tree was reconstructed from amino acid sequences by the neighbor-joining (NJ) method. The bootstrap method was used to determine the confidence interval of each phylogeny from 1000 bootstrap repetitions.

Virus neutralization tests (NT)

CRFK cells were cultured in a 96-well plate. 200 $TCID_{50}$/0.1 ml of virus solution and equivalent amount of diluted rabbit anti-F9 serum were incubated at 37°C for 2 h and inoculated to the cells. CPE was measured after 7 days to determine the viral neutralization titer.

Results

Clinical examination and virus isolation

Symptoms started with nasal discharges and sneezing mainly in cats younger than five months old. These cats were from three different mothers. At sampling, which was done approximately one month after the symptoms started, the symptoms were slightly improved showing conjunctivitis in two cats, eye mucous in two, nasal discharges in one. FCV was isolated from eight cats out of 34 (23.5%), and an ID number was given to each isolate as Ao198-1, Ao199-1, Ao210-1, Ao212-1, Ao213-1, Ao214-1, Ao222-1 and Ao224-1. These isolates were obtained from two of four 3-month-old littermates (50%, Ao222-1 and Ao224-1), all of three 4-month-olds (100%, Ao210-1, Ao212-1 and Ao213-1), one of two 5-month-olds (50%, Ao210-1), and both of two 1-year-old littermates (100%, Ao198-1, Ao199-1). Both of the 1-year-olds were thought to be carriers with persistent infection, as they did not show signs of symptoms (Table 1, Fig. 1)

Neutralization tests (NT)

The results of neutralization tests of the isolates against rabbit anti-F9 serum (1:1024) were shown in Table 2. The isolates from the latent carriers showed significantly low antibody titer at 1:8 (Ao198-1) and 1:16 (Ao199-1). In

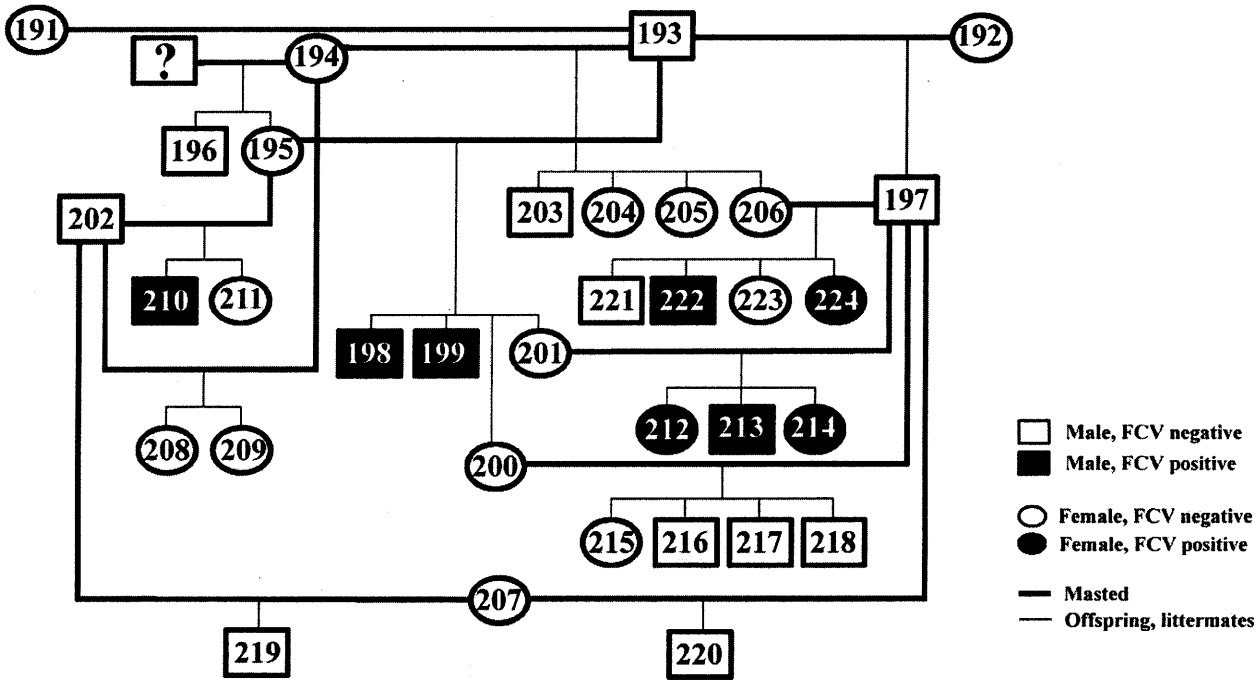

Fig. 1. Family tree of the 34 cats. There is the information of the 34 cats in Table 1.

Table 1 Clinical observations and virus isolation

cats	age	sex	vaccine	virus isolation	Symptoms
Ao191	9years	F	+	-	
Ao192	6years	F	+	-	
Ao193	2years	M	+	-	
Ao194	2years	F	+	-	
Ao195	2years	F	+	-	
Ao196	2years	M	+	-	
Ao197	1years	M	+	-	Conjunctivitis
Ao198	1years	M	+	Ao198-1	
Ao199	1years	M	+	Ao199-1	
Ao200	1years	F	+	-	
Ao201	1years	F	+	-	
Ao202	1years	M	+	-	
Ao203	1years	M	+	-	
Ao204	1years	F	+	-	
Ao205	1years	F	+	-	
Ao206	1years	F	+	-	Eye mucous
Ao207	11months	F	+	-	
Ao208	5months	F	+	-	upper respiratory
Ao209	5months	F	+	-	upper respiratory
Ao210	5months	M	+	Ao210-1	upper respiratory
Ao211	5months	F	+	-	upper respiratory
Ao212	4months	F	+	Ao212-1	upper respiratory
Ao213	4months	M	+	Ao213-1	upper respiratory
Ao214	4months	F	+	Ao214-1	upper respiratory
Ao215	4months	F	+	-	upper respiratory
Ao216	4months	M	+	-	upper respiratory
Ao217	4months	M	+	-	upper respiratory
Ao218	4months	M	+	-	upper respiratory
Ao219	3months	M	-	-	upper respiratory
Ao220	3months	M	-	-	upper respiratory
Ao221	3months	M	-	-	upper respiratory
Ao222	3months	M	-	Ao222-1	upper respiratory
Ao223	3months	F	-	-	upper respiratory
Ao224	3months	F	-	Ao224-1	upper respiratory

Table 2 The result of virus neutralization tests (NT) of the isolates aginst rabbit anti-F9 serum

Viruses	Rabbit anti-F9 polyclonal antibody
Ao198-1	8[a)]
Ao199-1	16
Ao212-1	<5.6
Ao210-1	<5.6
Ao214-1	<2.5
Ao213-1	<2.5
Ao222-1	<2.5
Ao224-1	8
F9	1024

a) reaprocal titer of NT

contrast, antibody titers of other isolates were less than 1:6. These values were significantly lower than the values against F9.

Molecular phylogenetic analysis

The nucleotide sequences of the FCV isolates from the eight cats shared more than 94% identity, while they show less than 75.5% identity with the FCV live vaccine strain, F9. Phylogenic analysis of the nucleotide sequence of the Ao isolates in comparison with the 30 global and 21 Japanese isolates [27] revealed that the Ao isolates formed a distinct cluster that belonged to genogroup II. They were most closely related to strain K10 isolated in Kyushu, Japan, sharing 75.4–77.4% identity. The Ao cluster was subdivided into six distinct groups: Ao198-1 and Ao199-1 in cluster 1 (C1); Ao212-2 in C2; Ao210-1 in C3; Ao214-1 in C4; Ao213-1 in C5 and Ao222-1 and Ao224-1 in C6 (Fig. 2). According to this phylogenetic tree, Ao198-1, the

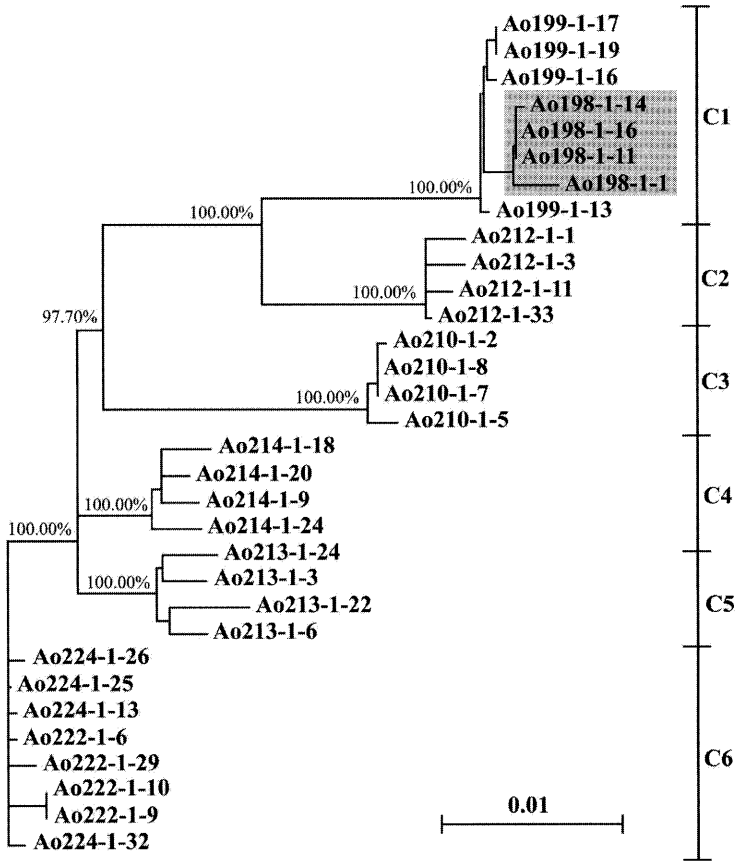

Fig. 2. Phylogenetic tree based on nucleotide sequences obtained by neighbor-joining (NJ) method for capsid fragments of the isolates. Branch lengths are proportional to the distances between the taxa. The values at the branch points indicate the percentage support for a particular node after 1000 bootstrap replicates were performed. Ao198 is the source of infections.

source of infection, was most closely related to Ao199-1, then Ao212-1, Ao210-1, Ao214-1, Ao213-1, Ao222-1 and Ao224-1 in this order. Phylogenetic analysis of amino acid sequences showed a similar result (Fig. 3).

Multialignment analysis of the FCV isolates

Ao198-1 and Ao199-1 in C1 showed 99.5–99.8% nucleotide identity, while Ao198-1 (C1) and Ao224-1 (C6) had slightly lower identity at 96.5–96.8%. Similarly, amino acid sequence identity was 99.1–99.8% between Ao198-1 and Ao199-1 and 94.4–95.6% between Ao198-1 and Ao224-1.

Alignments of nucleotide and deduced amino acid sequences of each Ao subgroup are shown in Table 3. For each of the capsid region, the ratio of nonsynonymous substitution per total number of nucleotides was less than 60% in regions A, B, D, conE and F and more than 80% in regions C, 5' and HVR of E. The ratio of nucleotide substitution per total number of nucleotides was 5/15 (33%), 12/105 (11%) and 15/96 (15.6%) at region C, 5' and 3' HVR of region E, respectively, and less than 10% at the other regions. For amino acid alignments, the ratio was more than 10% at regions C and E: 2/5 (40%), 8/35 (23%), 3/28 (10.7%) and 8/32 (25%), respectively. This suggested the highest variation in region C. The other regions were less than 10%.

Discussion

Neutralization tests showed that Ao198, the isolate from

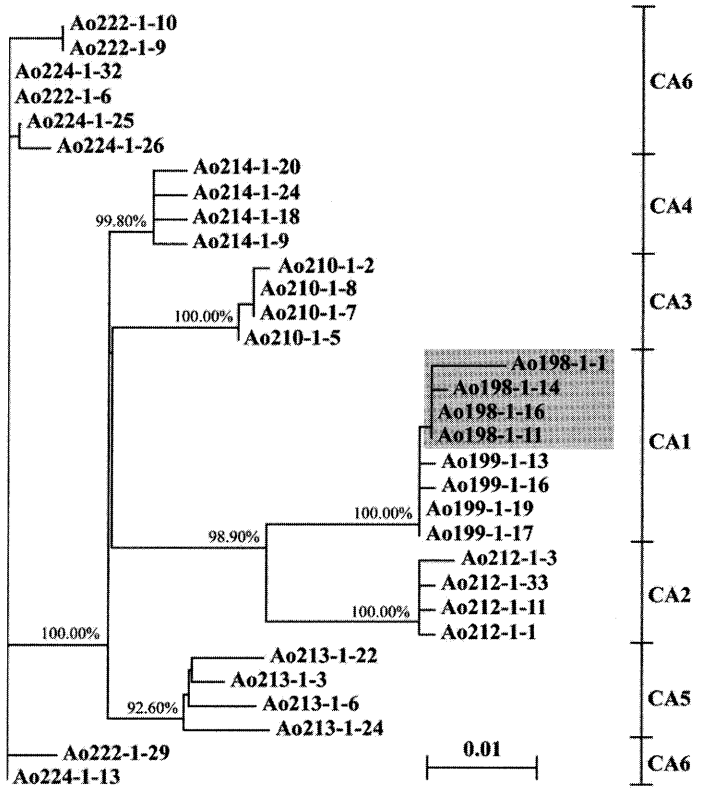

Fig. 3. Phylogenetic tree based on amino acid sequences obtained by neighbor-joining (NJ) method for capsid fragments of the isolates. Branch lengths are proportional to the distances between the taxa. The values at the branch points indicate the percentage support for a particular node after 1000 bootstrap replicates were performed. Ao198 is the source of infections.

Table 3 Variation in nucleotide and amino acid sequences for each capsid region

reagion		Nucleotides		Amino Acid		Nonsynonymous substitution
		Nucleotide substitution / Total number of nucleotides	%	AA substitution / Total AA number	%	Total number of nucleotides
	A	13/341	3.8%	6/113	5.3%	7/13
	B	24/816	2.9%	6/272	2.2%	7/23
	C	5/15	33%	2/5	40%	4/5
	D	6/72	8.3%	1/24	4.2%	1/6
	5'hvr of E	12/105	11%	8/35	22.9%	10/12
E	conE	5/84	6%	3/28	10.7%	3/5
	3'hvr of E	15/96	15.6%	8/32	25%	12/15
	F	24/372	6.5%	7/124	5.6%	10/24

A(nucleotide -372, amino acid -124)
B(nucreotide 373-1188, amino acid 125-396)
C (nucreotide 1189-1203, amino acid 397-401)
D (nucreotide 1204-1275, amino acid 402-425)
E (nucreotide 1276-1560, amino acid 426-520)
5'hvr of E (nucreotide 1276-1380, amino acid 426-460)
3'hvr of E (nucreotide 1456-1560, amino acid 486-520)
F (nucreotide 1561-, amino acid 521-)

the suspected infectious source, was markedly (128-fold) less sensitive to anti-F9 antisera (1:1024). A similar result has been reported by Hodatsu *et al.* [10], and together with our data, it is indicated that if the antibody level against Ao drops, it may result in increased susceptibility to FCV that leads to infection, even though vaccinated.

The isolates in this study formed a distinct subgroup based on the phylogenetic analysis in comparison with other field and global strains. According to the previously established genogroups [27], these isolates belonged to genogroup II, which is distinct from genogroup I vaccine strains (F9 and 255).

In general, when FCV infection occurs in a single multicat household, several field strains can be isolated, because cats are often left unconfined and their origins are variable. In our case, on the other hand, all the cats were kept indoors, and the adult cats were all vaccinated. Our clinical and phylogenetic study showed that a single FCV strain was transmitted to the newborns from the latent carriers and to the other cats. Through transmission, antibody titers against F9 became low in the all samples, and the especially high sequence variability in regions C and E indicates an escape from immunity mediated by antibody against the vaccine strain. We suggest that when cats susceptible to FCV are infected with the virus, immunity to the infected FCV may result in reduced antibody production against F9.

Our case was suitable to investigate transmission and mutation of an FCV field strain. The constructed phylogenetic tree suggested that the virus underwent mutations from C1 → C2 → C3 → C4 → C5 → C6, as transmission occurred. It is possible that the infection started from C6, but phylogenetic distance was closely related to C1, C2, C3, C4, C5 in this order, and the two newborns in C6 (3 months old) were not vaccinated and thus infected due to reduced neutralization ability of antibodies transmitted from their mother. The two 1-year-olds in C1 had been vaccinated but shedding FCVs without any sign of symptoms. Thus, we concluded that the FCV was transmitted from C1 and mutated as the virus was passed on to C2, C3, C4, C5 and finally to C6.

In general, male cats do not show an interest in newborn cats. We therefore suspected viral transmission from mothers to the kittens. However, FCV was not isolated from the mothers. The two male 1-year-olds in C1 are not fathers of the infected kittens, but the virus was somehow transmitted between them. This resembles the transmission of feline *Coronavirus*, which is more likely transmitted by cats other than mothers [1, 2].

Nucleotide and amino acid substitution ratios of Ao isolates were low at regions A, B, D and F and highest in regions C and E. These mutations suggested immune escape of the infected FCV, as suggested by the nonsynonymous substitution ratio higher than the synonymous ratio, indicating positive selection. On the contrary, there were more synonymous than nonsynonymous substitutions at regions A, B, D and F, indicative of a less positive selection force on these regions. 5' HVR of region E and region C are possible targets of neutralization antibodies [8, 14, 31, 32]. Together with our data, FCV may evolve by mutations of region C and E to escape the host's antibody-mediated immunity. Radford and his colleagues [22, 23] have shown that FCV evolves both in cell culture and persistently infected cats, and that the mutation in the latter case is the result of immune selection. In the naturally infected cats in our study, it was also shown that the capsid regions that contain epitopes for neutralization antibodies underwent frequent mutation upon transmission and thereby evaded host's immunity. The relationship between such molecular evolution of FCV and antigenicity is of strong interest, and further studies to elucidate other variable and non-variable regions are encouraged.

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References

1) Addie, D.D., Jarrett, O., Control of feline coronavirus infection in kittens. *Vet Rec.* **126**, 164 (1990).

2) Addie, D.D., Jarrett, O., A study of naturally occurring feline coronavirus infections in kittens. *Vet Rec.* **130**, 133-137 (1992).

3) Carter, M.J., Milton, I.D., Meanger, J., Bennett, M., Gaskell, R.M., Turner, P.C. The complete nucleotide sequence of feline calicivirus. *Virology* **190**: 443-448 (1992).

4) Crandell, R.A., Fabvicant, C.G., Nelson-Rees, W.A. Development and characterization and viral susceptibility of a feline (Felis catus) renal cell line (CRFK). In Vitro Cellular and Developmental Biology. *Animal* **9**: 76-85 (1973).

5) Cubitt, D., Bradley, D.W., Carter, M.J., Chiba, S., Estes, MK., Salf, L.J., Shaffer, F.L., Smith, A.W., Studdert, M.J., Thied, H.J. Classification and nomenclature of viruses. In: Sixth Report of the International Committee on Taxonomy of Viruses. Virus taxonomy. Springer-Verlag Wien New York, Austria, pp359-363 (1995).

6) Dawson, S., Gaskell, R.M. Investigation of vaccine reactions and breakdowns after *feline calicivirus* vaccination. *Vet Rec* **132**: 342-350 (1993).

7) Gaskell, R.M., Dawson, S. Feline respiratory disease. In: Greene CE (ed) Infections Diseases of the Dog and Cat. 2nd edn. W. B. Saunders Co., Philadelphia, pp 97-106 (1998).

8) Geisler, K., Sheneider, K., Truyen, U. Mapping neutralizing and non-neutralizing epitopes on the capsid protein of *feline calicivirus*. *J Vet Med B Infect Dis Vet Public Health* **49**: 55-60 (2002).

9) Guiver, M., Littler, E., Caul, E.O., Fox, A.J. The cloning, sequencing and expression of a major antigenic region from the *feline calicivirus* capsid protein. *J Gen Virol* **73**: 2429-2433 (1992).

10) Hodatsu, T., Sato, K., Tajima, T., Koyama, H. Neutralizing feature of commercially available *feline calicivirus* (FCV) vaccine immune sera against FCV field isolates. *J Vet Med Sci* **61**: 299-301 (1999).

11) Holizinger, E.A., Kahn, D.E. Pathologic Features of *Picornavirus infection* in cats. *Am J Vet Res* **31**: 1623-1630 (1970).

12) Love, D.N. Pathogenicity of strain of *feline calicivirus* for domestic Kittens. *Aust Vet J* **51**: 541-546 (1975).

13) Mead, D.A., Pey, N.K., Herinstadt, C., Marcil, R.A., Smith, L.M. A universal method for the direct cloning of PCR amplified nucleic acid. *Bio/Technology* **9**: 657-663 (1991).

14) Milton, L.D., Turner, K., Teelan, A., Gaskell, R., Turner, P.C., Carter, M.J. Location of monoclonal antibody binding sites in the capsid protein of feline calicivirus. *J Gen Virol* **73**: 2435-2439 (1992).

15) Neill, J.D., Reardon, I.M., Heinrikson, R.L. Nucleotide sequence and expression of the capsid protein gene of feline calicivirus. *J Virol.* **65**: 5440-5447 (1991).

16) Neill, J.D. Nucleotide sequence of the capsid protein gene of two serotypes of *San Miguel sea line virus*: identification of conserved and non-conserved amino acid sequences among calicivirus sequences. *Virus Res* **24**: 211-222 (1992).

17) Pedersen, N.C., Laliberte, L., Ekman, S. A transient febrile limping syndrome of kittens caused by two different strains of *feline calicivirus*. *Feline Practice* **13**: 26-35 (1983).

18) Pedersen, N.C. Feline infectious diseases, American Veterinary Publications, Inc., U.S.A., pp 58-64 (1993).

19) Povey, R.C., Wardley, R.C., Jessen, H. *Feline picornavirus* infection: the in vivo carrier state. *Vet Rec* **92**: 224-229 (1973).

20) Povey, R.C., Hale, C.J. Experimental infections with feline calicivirus (picornaviruses) in specific-pathogen-free kittens. *J Comp Pathol.* **84**: 245-56 (1974).

21) Radford, A.D., Bennett, M., McArdle, F., Dawson, S., Turner, P.C., Glenn, M., Gaskell, R.M. The use of sequence analysis of a *feline calicivirus* (FCV) hypervariable region in the epidemiological investigation of FCV related disease and vaccine failures. *Vaccine* **15**: 1451-1458 (1997).

22) Radford, A.D., Turner, P.C., Bennett, M., McArdle, F., Dawson, S., Glenn, M.A., Williams, R.A., Gaskell, R.M. Quasispecies evolution of a hyper variable region of feline calicivirus capsid gene in cell culture and in persistently infected cats. *J of Gen Virol* **79**: 1-10 (1998).

23) Radford, A.D., Bennett, M., McArdle, F., Dawson, S., Turner, P.C., Williams, R.A., Glenn, M.A., Gaskell, R.M. Quasispecies evolution of a hyper variable region of feline calicivirus capsid gene in cell culture and in persistently infected cats. *Vet Microbiol* **69**: 67-68 (1999).

24) Radford, A.D., Sommerville, L., Ryvar, R., Cox, M.B., Johnson, D.R., Dawson, S., Gaskell, R.M. Endemic infection of a cat colony with a feline calicivirus closely related to an isolate used in live attenuated vaccines. *Vaccine*. **19**: 4358-62 (2001a).

25) Radford, A.D., Sommerville, L.M., Dawson, S., Kerins, A.M., Ryvar, R., Gaskell, R.M. Molecular analysis of isolates of feline calicivirus from a population of cats in a rescue shelter. *Vet Rec.* **149**: 477-81 (2001b).

26) Radford, A.D., Dawson, S., Ryvar, R., Coyne, K., Johnson, D.R., Cox, M.B., Acke, E.F., Addie, D.D., Gaskell, R.M. High genetic diversity of the immunodominant region of the feline calicivirus capsid gene in endemically infected cat colonies. *Virus Genes.*

27: 145-55 (2003).

27) Sato, Y., Ohe, K., Murakami, M., Fukuyama, M., Furuhata, K., Kishikawa, S., Suzuki. Y., Kiuchi, A., Hara, M., Ishikawa, Y., Taneno. A. Phylogenetic analysis of field isolates of *feline calicivirus* (FCV) in Japan by sequencing part of its capsid gene. *Vet Res Commun* **26**: 205-219 (2002).

28) Sato, Y., Ohe, K., Fukuyama, M., Furuhata, K., Kishikawa, S., Sakai, S., Kiuchi, A., Hara, M., Watanabe, T., Ishikawa, Y., Taneno, A. Properties of a calicivirus isolated from cats dying in an agitated state. *Vet Rec.* **155**: 800-805(2004).

29) Seal, B.S., Ridpath, J.F., Mengeling, W.L. Analysis of *feline calicivirus* capsid protein genes; identification of variable antigenic determinant region of the protein. *J Gen Virol* **74**: 2519-2524 (1993).

30) Seal, B.S. Analysis of capsid protein gene variation among divergent isolates of feline calicivirus. *Virus Res.* **33**: 39-53 (1994).

31) Shin. Y.S., Tohya, Y., Oshikamo, R., Kawaguchi, Y., Tomonaga, K., Miyazawa, T., Kai, C., Mikami. T. Antigenic analysis of *feline calicivirus* capsid precursor protein and its polypeptides produced in a mammalian cDNA expression system. *Virus Res* **30**: 17-26 (1993).

32) Tohya, Y., Yokoyama, N., Maeda, K., Kawaguchi, Y., Mikami, T. Mapping of antigenic sites involved in neutralization on the capsid protein of *feline calicivirus. J of Gen Virol* **78**: 303-305 (1997).

33) Wardley, R.C. *Feline calicivirus* carrier state: a study of the host / virus relationship. *Arch Virol* **52**: 243-249 (1976).

34) Wardley, R.C., Povey, R.C. The clinical disease and patterns of excretion associated with three different strains of *feline caliciviruses. Res Vet Sci* **23**: 7-14 (1977).

35) Wardley, R.C., Povey, R.C. The pathology and sites of persistence associated with three different strains of *feline calicivirus. Res Vet Sci* **23**: 15-19 (1977).