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# Deletions in the 7a ORF of feline coronavirus associated with an epidemic of feline infectious peritonitis

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## Abstract

A population of Persian cats experienced an epidemic of feline infectious peritonitis (FIP) over 2 years. Twelve cases of FIP occurred in litters born during this period. Cats contracting FIP were all genetically related through the sire. Feline coronavirus (FCoV) genomic RNA was detected consistently in this study in biologic samples from adult cats, kittens suffering from FIP, and their siblings. Analysis of viral 7a/7b open reading frame (ORFs) were analyzed and revealed two distinct virus variants circulating in the population, one with an intact 7a ORF and one with two major deletions in the 7a ORF. The 7b ORFs were intact and similar among all virus isolates, although point mutations resulting in amino acid changes were present. The sire was determined to be infected with both variants, and was persistently virus-infected. We speculate the deletion variant arose from the non-deletion variant during viral replication in this population, possibly in the sire. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Feline coronavirus; Feline infectious peritonitis; Coronavirus mutation

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## 1. Introduction

Feline infectious peritonitis (FIP) is a serious disease of domestic and wild Felidae. It is an important disease of cats in multi-cat households, catteries, and shelters (Scott et al., 1992; Wolf, 1995). This disease can manifest as an effusive peritonitis and/or pleuritis with a short course ending in death; or it may present as a more insidious disease with

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granulomatous lesions affecting multiple organs that also progresses to death (Pedersen, 1987; Hoskins, 1993; Pedersen, 1995). The etiologic agent of FIP is feline coronavirus (FCoV). In environments where large numbers of cats are housed closely together, 75–100% of the animals may be seropositive to the virus (Pedersen, 1995). Despite these numbers, FIP occurs sporadically — fatal disease is an uncommon manifestation of infection with FCoV. Virus factors are important to disease development, as virus strains vary in virulence (Pedersen, 1987). Virulent FCoV is theorized to arise from mutation of the infecting FCoV during replication in the intestinal tract of infected cats (Poland et al., 1996; Vennema et al., 1998). The 7b open reading frame (ORF), the 3'-most gene, has been speculated to have a role in virulence, as deletions in this region lead to decreased virulence (Vennema et al., 1992; Herrewegh et al., 1995; Vennema et al., 1998). Despite this data, the specific virus and host factors involved in the production of lethal disease are not known.

The occurrence of FIP in feline populations is rarely higher than 5% (Hoskins, 1993). We investigated a colony of 15 adult Persian cats that experienced 12 cases of FIP in their kittens over 2 years. We detected FCoV genetic material in the feces of the adult cats as well as tissue and fluid sample from the FIP victims. The 7a/7b ORF from the FCoV in this population was characterized to determine if mutations in this region were occurring.

## 2. Materials and methods

### 2.1. Samples

A Persian cattery consisting of 15 adult cats was selected for study. All animals in the population were negative for feline leukemia virus and feline lentivirus. They were routinely vaccinated for feline herpesvirus, calicivirus, panleukopenia virus, and rabies. Twelve cases of FIP occurred in six litters over 2 years (1997–1999). Diagnosis was based on clinical signs, CBC and blood chemistry, coronavirus serology, histopathology, and PCR on plasma or effusion. This latter parameter was found to correlate with histopathology for diagnosis of FIP (Kennedy et al., 1998). Fecal samples, whole blood, tissue and ascites from two clinical cases were collected at the time of illness. In addition, fecal and whole blood samples were collected from seven adult cats and unaffected kittens in the household at the same time point.

### 2.2. RNA extraction, reverse transcription, polymerase chain reaction, and sequence analysis

Total RNA was extracted from the specimens using Trizol LS according to the manufacturer's directions for reverse transcription and nested polymerase chain reaction (Gibco BRL, Baltimore, MD). Primers encompassed the 7a/7b ORFs, the 3'-most ORFs of the genome (Kennedy et al., 1998). Reverse transcription was done with Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations with the downstream external primer (Gibco BRL, Baltimore, MD). Nested polymerase chain reaction was done using ExTaq polymerase (Intergen, Purchase, NY) as

described previously (Kennedy et al., 1998). Products were analyzed on a 1% agarose gel. Amplification products were cloned into PCR 2.1 using the TA cloning system (Invitrogen, Carlsbad, CA). Cloned cDNA was sequenced by Molecular Biology Resources Service (University of Tennessee, Knoxville, TN) and analyzed using GCG software (University of Wisconsin, Madison, WI). A minimum of two clones from two separate PCR reactions was used for sequencing and analysis.

### 3. Results

We detected FCoV in a purebred population of cats that had experienced a high rate of FIP over a 2-year period, with 12 cases of FIP in six litters. The FCoV infecting the population was analyzed, focusing on the 7a/7b ORF. This genomic region was chosen for analysis because of its speculated role in virulence (Vennema et al., 1992; Herrewegh et al., 1995; Vennema et al., 1998).

Amplification of FCoV RNA was successful in samples from nine cats. Seven of these were resident adults and included the sire and queens of affected offspring. Two were from kittens that died from FIP. Two distinct virus variants were found, one with an intact 7a ORF and one with two deletions in the 7a ORF (Fig. 1). The deletions encompassed nucleotides 20–120 and nucleotides 164–226 of the 7a gene. Four additional nucleotides (TCTT) were present in all of the deletion mutants at the position corresponding to nucleotide 227 of the undeleted virus. The protein predicted from the nucleotide sequence contains different amino acids in the 3' one-half of the predicted protein due to the altered reading frame caused by the second deletion (Fig. 2). It is not known if the 7a protein predicted from the nucleotide sequence is expressed in the deletion mutant virus.

The remainder of the 7a/7b ORF was highly conserved among all isolates, including the region encoding the 7b ORF (Fig. 3). Interestingly, amino acid residues 202 and 203 of the predicted 7b protein were histidine and lysine. This is contrary to the findings of Vennema et al. (1998) that FIP viruses contained tyrosine and lysine at these positions whereas putative avirulent FCoV contained histidine and arginine.

Both virus variants were identified in one cat, the sire “Dan”, as sequence analysis of clones from a single PCR from this animal revealed the presence of the 7a deletion mutant as well as the intact isolate (Dan 1 and 2 in Fig. 2). This cat was PCR-positive for

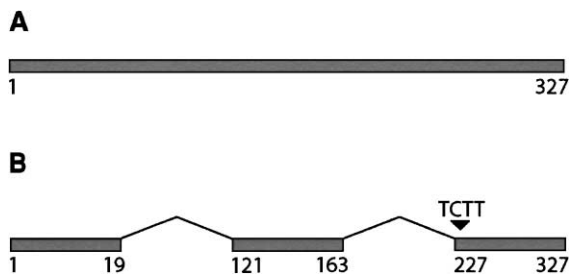


Fig. 1. (A) Intact 7a gene from FCoV of cattery members; (B) 7a gene deletion mutant from FCoV of cattery members. Deletions are indicated by single line, insertion of nucleotides is indicated by inverted triangle.

	1				50
dan1	MRLRTKRMVL	FLHAVLVTA	I LLLI GRIQL	LERLLSHLL	NLTTVSNVLG
por	MRLRTKRMVL	FLHAVLVTA	I LLLI GRIQL	LERLLSHLL	NLTTVSNVLG
scar	MRLRTKRMVL	FLHAVLVTA	I LLLI GRIQL	LERLLSHLL	NLTTVSNVLG
lynx	MRLRTKRMVL	FLHAVLVTA	I LLLI GRIQL	LERLLSHLL	NLTTVSNVLG
brad	MRLRTK----	-----	-----	-----L	NLTTVSNVLD
fp	MRLRTK----	-----	-----	-----L	NLTTVSNVLD
mel	MRLRTK----	-----	-----	-----L	NLTTVSNVLD
rain	MRLRTK----	-----	-----	-----L	NLTTVSNVLD
tort	MRLRTK----	-----	-----	-----L	NLTTVSNVLD
dan2	MRLRTK----	-----	-----	-----L	SLTTVSNVLD
	51				100
dan1	VPDNSLRVNC	LQLLKPDCLD	FNILHKVLA	TRLVLLRV	I FLVLLGFSC
por	VPDNSLRVNC	LQLLKPDCLD	FNILHKVLA	TRLVLLRV	I FLVLLGFSC
scar	VPDSSLRVNC	LQLLKPDCLD	FNILHKVLA	TRLVLLRV	I FLVLLGFSC
lynx	VPDNSLRVNC	LQLLKPDCLD	FNILHKVLA	TRLVLLRV	I FLVLLGFSC
brad	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
fp	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
mel	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
rain	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
tort	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
dan2	VPD-----	-----	---S I LSF	NQI TSSSTAS	DLSSSRVFL
	101				
dan1	YTLLGALF*				
por	YTLLGALF*				
scar	YTLLGALF*				
lynx	YTLLGALF*				
brad	LYTVRCIILT	S*			
fp	LYTVRCIILT	S*			
mel	LYTVRCIILT	S*			
rain	LYTVRCIILT	S*			
dan2	LYTVRCIILT	S*			

Fig. 2. Multiple sequence alignment of the 7a amino acid sequence from FCoV of cattery members. Deleted residues are indicated by tilde signs.

virus in multiple samples and may be persistently infected with FCoV. He was also the sire of all the FIP victims in the first 18 months of the study. Litters resulting from the breeding of this sire to his daughters from previous litters experienced morbidity and mortality of 75–100% within the litter ( $n = 4$ ). During the last 6 months of the FIP outbreak, a new sire was used and bred to the original sire's daughters. Morbidity and mortality has since decreased to 0–25% within litters ( $n = 4$ ), with the last case occurring in spring of 1999. Amplification of FCoV 7a/7b ORF from the most recent cases of FIP ( $n = 4$ ) have not been successful.

Fig. 4 shows the phylogenetic relationship among the virus isolates resulting from nucleotide sequence alignments. The two distinct variants are clearly delineated. The deletion mutants show a very close similarity to one another and form a closely related cluster.

#### 4. Discussion

We have characterized the 7a/7b ORF of FCoV variants in a population experiencing an epidemic of FIP. One variant had an intact 7a/7b ORF, while another had two major

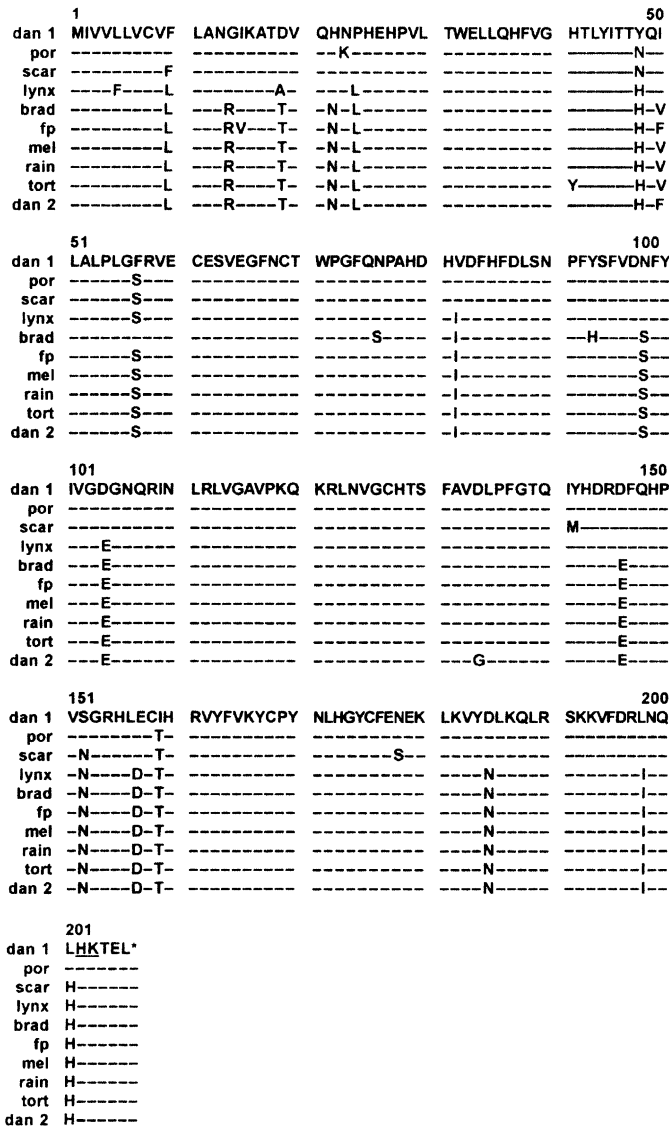


Fig. 3. Multiple sequence alignment of the 7b amino acid sequence from FCoV of cattery members. Amino acid matches to reference strain (Dan 1) are indicated by a dash. Underline indicates residues 202 and 203 (see text).

deletions in the 7a ORF. The latter group appears to be very closely related, and probably arose from a single mutant strain. The first deletion resulted in no change in the reading frame, but the second deletion led to an alteration of the reading frame by one nucleotide leading to an altered amino acid sequence in the predicted protein. We speculate that these deletions arose from “looping out” of RNA regions due to the predicted secondary

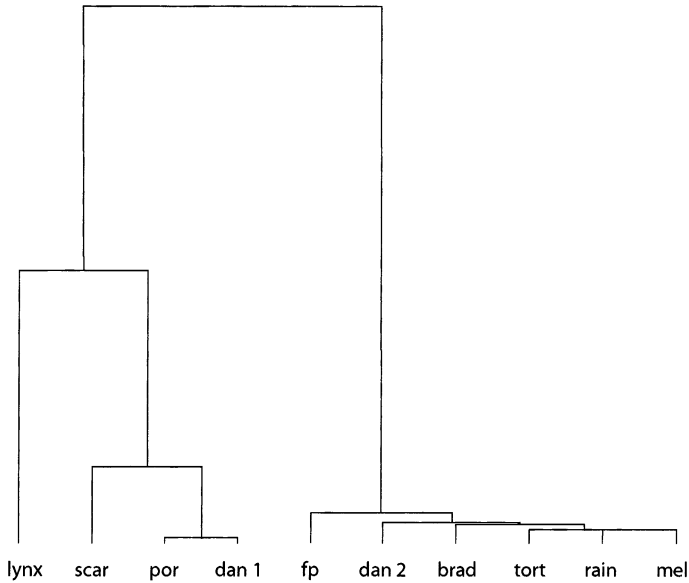


Fig. 4. Phylogenetic tree of the 7a/7b ORFs from cattery member FCOVs.

structure of the single stranded RNA genome in this region (data not shown). This may result in the viral RNA polymerase “skipping” certain regions during transcription.

It is not known if the 7a protein is expressed in the mutant virus. The first 18 nucleotides of the cDNA sequence, which corresponded to nucleotides 1–18 of the 7a gene and included the start codon, corresponded to the upstream internal primer. Some of these nucleotides must be present in the viral template in order for hybridization of primer to template to occur but it is not known how many of nucleotides 1–18 are present in the virus. Thus, it is unclear if this start codon is present. If it is, translation of the 7a ORF in the deletion mutant would result in a predicted protein that is nearly half the size of the native protein (58 amino acids in deletion mutant versus 108 amino acids residues in the native virus).

The sire of the majority of FIP kittens was dually infected with both virus variants as revealed by sequence analysis of cloned 7a/7b genes from this cat. Other cats in this study may have also been dually infected with both virus variants, however, only one virus variant was identified by PCR in each of the remaining cats tested. This may be due to a quantitative difference in the amount of each virus variant in each cat tested. Both variants were circulating as every cat tested was infected with one of the virus variants. Some infected with the intact 7a/7b ORF variant were ill and some infected with the deletion mutant were ill. However, as both variants were circulating and thus, every cat was exposed to both variants, there may be a causal relationship between the mutation that occurred and the increased incidence of disease seen in this population. The variation in disease may be related to host factors, such as generation of an effective immune response, rather than solely to the virus itself. Alternatively, mutations in other genomic regions may be responsible for the variation in virulence observed with these isolates.

Host factors may play an important role in the virulence of FCoV. Increased incidence of FIP in purebred cats, as well as in cheetahs, which are relatively genetically homologous, is known to occur (O'Brien et al., 1985; Foley and Pedersen, 1996). In this investigation, all of the FIP victims were genetically related through the sire. Those related to him through both the queen and sire had the highest morbidity while those related to him through the queen or sire only had lower morbidity. This would support the belief that susceptibility to FIP following infection with FCoV has a host genetic component.

Genetic detection targeting the 7a/7b ORF of FCoV was not successful in all FIP victims. The deletions we have characterized in the 7a ORF occurred near or within the binding site of the internal upstream primer. As the virus has persisted in this population, we postulate that this primer-binding site may have been lost due to the occurrence of additional deletions in the 7a viral gene.

Mutation of FCoV in some cats may lead to changes in virulence ultimately resulting in FIP. Point mutations, recombination, and deletions have been observed (Poland et al., 1996; Herrewegh et al., 1995). Mutations are more likely to occur if virus is not cleared from a host population and viral replication continues at a significant level (Horzinek et al., 1995). It is not known if two virus variants entered this population or if one variant is a mutant of the other. The latter may be more likely, as the cats in this population have remained virus-infected for an extensive period increasing the likelihood of genetic mutation.

Deletions have been noted in the 7b ORF from previous studies (Vennema et al., 1995). This is the first report of a deletion occurring in the 7a ORF in a natural infection of a cat population. Other virus mutations in addition to those which we have characterized may have occurred in the FCoV from this population and may correlate directly with virulence. Further analysis of additional genomic regions of these FCOVs is required to completely explain the increased occurrence of FIP in this population.

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