Feline infectious peritonitis; insights into feline coronavirus pathobiogenesis and epidemiology based on genetic analysis of the viral 3c gene

Running title: 3c gene mutations in the feline coronavirus pathotype switch

Authors: Hui-Wen Chang, Raoul J. de Groot, Herman F. Egberink, and Peter J.M. Rottier*

Affiliations: Virology Division, Department of Infectious Diseases & Immunology, Veterinary Faculty, Utrecht University, Utrecht, The Netherlands

Correspondent footnote: P.J.M. Rottier, Virology Division, Department of Infectious Diseases & Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands. Phone: +31 30 253 2462, Fax: + 31 30 253 6723, E-mail: p.rottier@uu.nl

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Abstract

Feline infectious peritonitis (FIP) is a lethal systemic disease caused by FIP virus (FIPV), a virulent mutant of apathogenic feline enteric coronavirus (FECV). We analyzed the 3c gene - a proposed virulence marker - in 27 FECV- and 28 FIPV-infected cats. Our findings strongly suggest that functional 3c protein expression is crucial for FECV replication in the gut but dispensable for systemic FIPV replication. While intact in all FECVs, the 3c gene was mutated in the majority (71.4%) but not in all FIPVs, implying that mutation in 3c is not the (single) cause of FIP. Most FIP cats had no detectable intestinal FCoV and had apparently cleared the primary FECV infection. In those that had, the fecal virus always had an intact 3c and seemed acquired by FECV superinfection. Apparently, 3c-inactivated viruses do not - or only poorly - replicate in the gut, explaining the rare incidence of FIP outbreaks.

TEXT

Feline coronaviruses (FCoVs; family *Coronaviridae*, order Nidovirales), important pathogens of cats, occur in two distinctly different pathotypes. Feline enteric coronavirus (FECV), the pathotype most common in the field, seems mainly confined to the intestinal tract and causes mild, often unapparent enteritis. The virus efficiently spreads via the fecal-oral route and, as infections may persist subclinically for up to a year and perhaps even longer (Herrewegh *et al*., 1997; Pedersen *et al*., 2008), FECV prevalence is high, reaching up to 90% seropositivity in multi-cat environments. The other pathotype, designated feline infectious peritonitis virus (FIPV), occurs only sporadically. In sharp contrast to FECVs, FIPVs do not seem to be well transmitted and they are highly virulent. By efficiently infecting macrophages and monocytes, FIPVs can escape from the gut and cause a lethal systemic disease with multi-organ involvement, in classical cases accompanied by accumulation of abdominal exudate (ascites; for reviews, see de Groot & Horzinek,1995; Haijema *et al*., 2007; Pedersen, 2009).

There is genetic and animal experimental evidence to indicate that the virulent pathotype time and again evolves from the avirulent one by mutation in individual infected cats. Comparative sequence analysis of feline coronavirus laboratory strains and field variants revealed that FECVs and FIPVs come in genetically closely related pairs, more identical to each other than to other feline coronaviruses (Herrewegh *et al*., 1995; Pedersen *et al*., 1981; Poland *et al*., 1996; Vennema *et al*., 1998). Direct support for the 'internal mutation' hypothesis comes from an experiment in which cats with an immunosuppressive feline immunodeficiency virus (FIV) infection were superinfected with FECV. A number of these animals developed FIP in response. The (systemic) virus

variants isolated from the diseased cats were isogenic to the original FECV strain yet, unlike the parental virus, readily induced FIP when inoculated into specific pathogen-free cats (Poland *et al*., 1996; Vennema *et al*., 1998); virulence thus appears to be an acquired genetic trait.

So far, the critical mutations that would convert apathogenic FECV into FIPV have not been identified in the huge 29 kb FCoV RNA genome. It has been noted, however, that FIPV strains frequently carry mutations that inactivate the gene for 3c (Pedersen, 2009; Vennema *et al*., 1998), an accessory triple-spanning membrane protein with a predicted topology similar to that of SARS coronavirus 3a (Oostra *et al*., 2006). Loss of 3c function thus seemingly correlates with acquisition of virulence (Haijema *et al*., 2004; Pedersen, 2009; Vennema *et al*., 1998).

Recently, the internal mutation theory as the basis for the pathotype switch was fundamentally challenged by Siddell and coworkers (Dye & Siddell, 2007). Departing from the assumption that cats with FIP should harbor distinct enteric and non-enteric FCoV populations, these authors determined and compared the sequences of the 3'-most third of viral genomic RNAs isolated from the jejunum and liver of a case of FIP. The ~10 kb sequence of the two viruses, which includes gene 3c, appeared to be completely identical, an observation the authors considered to be in flat violation with the mutation hypothesis.

To try to resolve this controversy and to gain more insight into feline coronavirus epidemiology, we compared naturally occurring FECV and FIPV variants with respect to the 3c gene. We screened 198 cats to identify animals positive for feline coronavirus using a RT-nested PCR targeting the highly conserved 3'-untranslated region of the viral

84 genome (Herrewegh *et al*., 1995) and then amplified gene 3c by RT-PCR using specific
85 primers (sense 5′-CAAGTACTATAAAACGTAGAAGMAG-3′, antisense 5′-
86 CAGGAGCCAGAAGAAGACACTAA-3'), applying 30 cycles of 94 °C for 60 s, 50 °C
87 for 30 s, and 72 °C for 1 min and additional extension at 72 °C for 7 min at the end of
88 amplification. Thus, gene 3c sequences were obtained from the feces of 27 apparently
89 healthy FECV-infected cats as well as from organs, ascites or typical pyogranulomatous
90 lesions of 28 pathologically confirmed FIP cats (Table 1) collected during 2007-2008 in
91 the Netherlands. In addition, from 17 of the 28 FIP-confirmed cats also fecal material was
92 obtained and used for 3c gene analysis.
93 Without exception, the FECVs possessed an intact 3c gene, specifying a 238-
94 residues polypeptide. In contrast, 3c gene sequences amplified from FIPV-affected
95 tissues fell into two categories. Of the 28 sequences, only eight had an intact open reading
96 frame (ORF) the size of that in FECV; the vast majority (71.4%) exhibited various
97 aberrations, as depicted in Fig. 1. Some had small in-frame deletions (cats # 12 and 97)
98 and insertions (cat # 48); as these mutations preserve the reading frame and result in the
99 loss or gain of just 1-3 amino acid residues, it is difficult to assess to what extent 3c
100 protein function is affected. Importantly, however, such changes, minor as they might
101 seem, were never observed in any of the FECV-derived ORF3c sequences. The
102 remaining 3c sequences amplified from FIP lesions displayed more serious aberrations,
103 including various out-of-frame deletions (n=12) or insertions (n=2), nonsense mutations
104 (n=5), and combinations of deletions and point mutations (n=1), as depicted in Fig. 1.
105 Most of these mutations will result in premature termination of translation and severe
106 truncation of the 3c polypeptide (Fig.1) and will almost certainly be incompatible with 3c

function. In one FIP case (cat #46), 3c translation was even blocked completely by a point mutation in the initiation codon (AUG→ACG). The combined findings lead us to conclude that the 3c protein is strictly required for efficient feline coronavirus replication in the gut, but dispensable for systemic propagation.

From 17 of the 28 FIP cats fecal material was also available. This gave us the opportunity to characterize the viruses present in the intestinal tract and to establish their relationship to the systemic FCoVs. We reasoned that any information about the presence of fecal viruses, their 3c sequence and the pathotypic signature thereof would provide crucial insight both into FIPV etiology and epidemiology. Remarkably, in most samples (n=11) FCoV RNA could not be detected, not even by using a well-established highly sensitive nested RT-PCR (Herrewegh *et al.*, 1995). Apparently, most cats with FIP had cleared the primary enteric infection or, at least had suppressed the infection to levels below detection limits. In five out of six animals, where we did succeed in detecting FCoV and amplifying 3c sequences from the feces, the 3c open reading frame was fully intact and identical in size (714 nt) to that observed in FECVs. In the one remaining animal (cat # 12) the 3c gene sequence was identical to the one amplified from FIP lesion-derived RNA and carried an in-frame 3-nt deletion resulting in the loss of a single 3c residue (Thr^{187}).

Of the animals with FIP that harbored FCoV both in the gut and systemically, the 3c nucleotide sequences amplified from these compartments were always more similar to each other than to those found in the other FIP cats (Figs. 2A, B). Still, tempting as it would seem to assume an immediate ancestral relationship between the enteric and systemic viruses, we noted that in most cases the extent of nucleotide sequence variation

(up to 3.4%, Fig. 2A) was higher than to be expected for such close relatives; the data rather suggested the FIP cats to be doubly-infected with genetically closely related FECVs that they might have acquired in their multi-cat environments. To study this possibility, we sampled feces from apparently healthy contacts of the FIP cats and obtained FECV sequences from companions of cat # 23 (cat # 25; household A), cat # 150 (cat # 152; household B) and cat # 107 (cats # 113, 176 and 179; household C). Consistent with our previous observations (Herrewegh *et al*., 1995, 1997), the FCoVs in the three multicat households form separate clades each with a distinctive genetic signature (Fig. 2C). Our findings are best illustrated by the results for FIP cat #107. This animal harbored a systemic virus (FIPV 107) that, with respect to its 3c sequence, was most closely related to FECV isolated from cat #179, while the virus from its bowels ("feces 107") was virtually identical to FECVs from companion animals #113 and #176. Similarly, for cats #23 and #150, the viruses in the feces were more closely related to FECVs from healthy contacts (FECV #25 and FECV #152, respectively) than to their systemic viruses (FIPV #23 and #150, respectively; Fig. 2C). Our findings, while still fully consistent with the internal mutation hypothesis, indicate that by the time FIP signs become overt, most cats will have resolved the primary FECV infection. Even in those FIP cats where we did detect FCoV in the feces, the virus represented the systemic FIPV nor its FECV predecessor, but rather a super-infecting FECV. These findings are remarkable, given the fact that FECV may persist for very long periods of time in apparently healthy carriers (Herrewegh *et al*., 1997; Pedersen *et al.,* 2008). Possibly, in early stages of FIP, the mutation-induced systemic FCoV infection (i.e. FIPV) (re)activates immune mechanisms that lead to viral clearance from the gut. The severe

153 immune dysregulation and collapse of key effectors of the immune system in cats with
154 end-stage FIP (de Groot-Mijnes *et al*., 2005; Haagmans *et al*., 1996) might then create an
155 opportunity for FECVs circulating in surrounding healthy carriers to cause the
156 superinfections that we apparently observe in a fraction of the animals.

157 In view of its supposed role in the pathogenesis of FIP the aim of the present
158 study was to sequence and compare the 3c gene of FCoVs in a large number (55) of
159 symptomatic and asymptomatic infected cats. The results show that FECVs invariably
160 carry an intact 3c gene whereas in the majority (71.4%) of FIPVs the gene has mutations,
161 unique for each virus, consistent with earlier studies (Pedersen *et al*., 2009; Vennema *et*
162 *al*., 1998) and with the internal mutation theory. Our key observation, however, is that
163 the viruses replicating in the gut invariably had an intact 3c gene while those replicating
164 outside the gut mostly had not. Importantly, intact 3c genes were also found in the
165 intestines of all those FIP cats that carried a mutated 3c in their lesion-derived FCoV
166 genome, except in one case, cat #12. It is, however, conceivable that the single-residue
167 deletion found in ascitic and intestinal virus of this cat does not affect the functionality of
168 the 3c protein, hence leaving the gene actually intact; alternatively, the fecal appearance
169 of the virus might just have resulted from passive leakage of systemic FIPV into the gut.

170 The key question regarding FIP remains whether mutations in the 3c gene are the
171 cause of this disease, as has been suggested (Pedersen *et al*., 2009; Vennema *et al*., 1998).
172 Our results clearly indicate that, if the gene is involved at all, it is certainly not the only
173 one. Though FIPVs carrying 3c gene mutations were present in the large majority of the
174 FIP cats studied, the absence of mutations in a considerable proportion of cases implies
175 that either additional or alternative mutations can generate the virulent pathotype. We

favor the idea that the 3c gene product is critical for the replication of the avirulent pathotype in its specific biotope, the enteric tract, but that 3c function becomes nonessential once virulence mutation(s) elsewhere in the genome enable the virus to infect monocytes/macrophages and spread systemically. Loss of 3c might not only be tolerated but may possibly even enhance the mutant virus' fitness in its new biotope.

Sequence comparisons of our collection of 3c genes initially seemed to confirm the expected relatedness in each FIP cat between the fecal and lesion-derived virus, consistent with the former being the immediate ancestor of the latter. However, more careful inspection of the data obtained from some multi-cat households revealed that in each of these cases the FIPV 3c sequence was more similar to the fecal 3c sequences found in surrounding healthy FECV carriers than to that in the respective FIP cat. Combined with the remarkable lack of detectable fecal FCoV in a large fraction of FIP cats it seems like the original FECV infection is generally cleared from the gut following the pathotype switch, the cats sometimes becoming FECV infected again later by contact animals.

FECV replicates in the gastrointestinal tract (Herrewegh *et al*., 1997; Pedersen *et al*., 1981). Viral RNA has, however, also been detected by RT-PCR in blood and some (haemolymphatic) tissues of infected animals (Gunn-Moore *et al*., 1998; Herrewegh *et al*., 1995,1997; Kipar *et al*., 2006a, b; Meli *et al*., 2004; Simons *et al*., 2005). The significance of this apparent viremia is still poorly understood. While cells of the monocyte lineage are the prime targets of FIPV, these cells are poorly susceptible to FECV and support FECV replication and spread only very inefficiently, at least *in vitro* (Stoddart and Scott, 1989; Rottier *et al*., 2005). Studies aimed to detect FCoV replication,

by using an RT-PCR specifically designed to identify viral mRNA, suggest this to be the case as well *in vivo* (Herrewegh *et al.*, 1995; Simons *et al.*, 2005). Actually, viral mRNA or infected cells was not observed in organs other than the intestinal tract. Apparently, non-replicating FECV is acquired by mononuclear cells in the gut and carried to organs and tissues with the blood. Consistently, while studying haemolymphatic tissues, a major site for the accumulation of monocytes/macrophages, Kipar and co-workers found significantly higher levels of viral RNA in cats with FIP than in healthy FCoV positive cats (Kipar *et al.*, 2006a). Moreover, FCoV antigen was detectable by immunohistology in these tissues only in FIP cats (Kipar *et al.*, 2006a, b), consistent with the low FECV replication activity seen in the mononuclear cells.

Our observations give important new insights into the biology and epidemiology of feline coronaviruses and provide an attractive explanation for the typically rare occurrence of FIP outbreaks in the field. Based on our data we arrive at the following scenario. Cats become infected by circulating FECVs that home to and replicate in the gut, establishing a low grade chronic infection apparently kept in check by the immune system. Replication in this compartment and efficient fecal shedding strictly requires an intact viral gene repertoire, most notably a fully functional 3c gene. Inherent to the nature of RNA viruses, mutations continually occur, one or more of which incidentally provides the virus with the ability to replicate in macrophages and monocytes, which then spread the - now FIPV - infection to organs throughout the body. Once in this new environment viral propagation no longer depends on all gene functions and some accessory proteins that are crucial for enteric replication may become dispensable. Mutations such as we observed in the 3c gene may not only be tolerated, they may even enhance viral fitness in

the new biotope. Ironically, but importantly, while providing a selective advantage during systemic replication, such mutations may effectively prevent the resulting FIPVs from returning back and recolonizing the gut, where an intact 3c gene is apparently essential. Fecal shedding of FIPV may occur only in rare circumstances, e.g. as a result of extensive intestinal lesions, an event that might have caused Dye and Siddell detecting identical 3c-mutated viruses in gut and liver of an FIP cat (Dye and Siddell, 2007) and that might explain the single instance in which we found a virus with mutated 3c both in FIP lesions and in the feces (cat #12).

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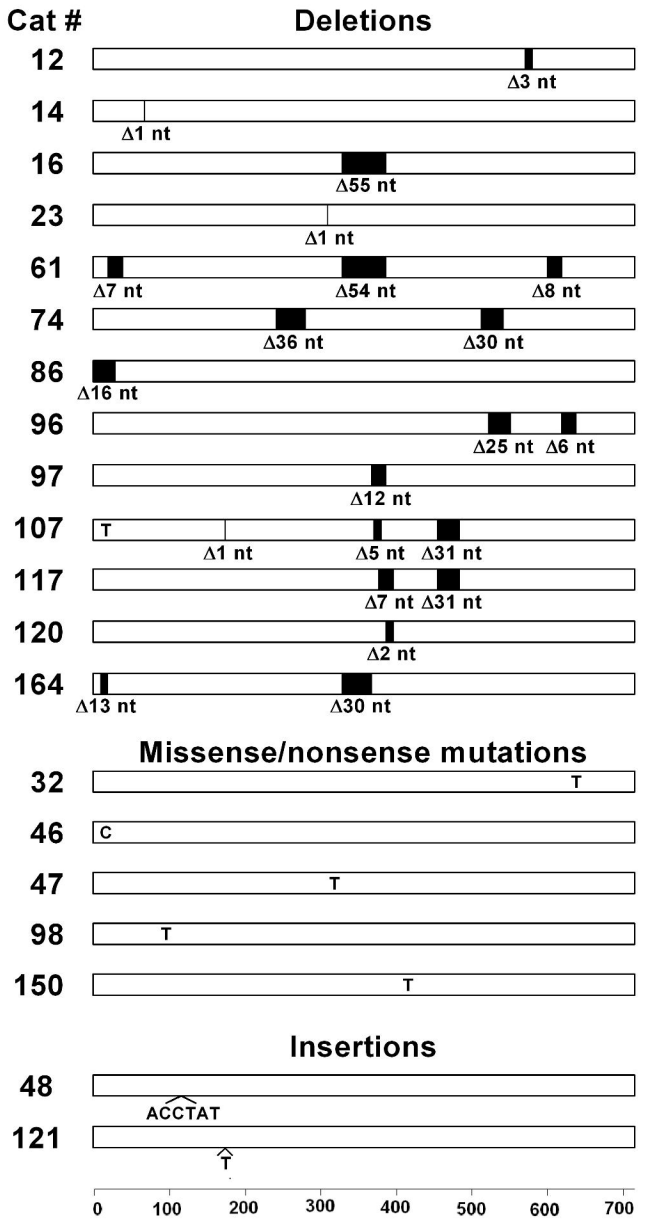
FIGURE LEGENDS

Fig. 1. Schematic representation of the 3c gene of lesion-derived FCoVs from 20 FIP confirmed cats showing deletions, nonsense/missense mutations, and insertions. The 3c sequences are indicated by white boxes. Deletions are indicated by black bars (Δ nt, number of nucleotides deleted). In the column labeled "lesion", the effects of the mutations on 3c translation are indicated; 'PT': premature termination. The column labeled "feces" summarizes the results of the analysis of fecal samples. Intact: intact full-length 3c gene; '– ': no FCoV detected; 'NA': no fecal samples available.

Fig. 2. (A) Nucleotide sequence identities among 3c sequences from enteric (feces-derived, F) and non-enteric viruses (derived from lymph node, LN, or omentum, O) in FIP cats. (B) Alignment of the predicted 3c polypeptides from enteric and non-enteric viruses in FIP cats. Shading indicates residues identical to the FECV 3c consensus sequence. Dots are shown when polypeptides terminate prematurely. (C) Phylogenetic relationships among feline coronaviruses isolated from fecal samples and from FIP lesions. A gapless alignment of the 3c nucleotide sequences was used to generate a rooted Neighbor-Joining tree with the 3c sequence of canine coronavirus strain 119/08 (Genbank EU 924791) serving as outgroup. Confidence values are indicated at the relevant branching points. Branch lengths are drawn to scale; the scale bar represents 0.01 nucleotide substitutions per site. Virus pairs in feces and lesions of individual cats are marked by shading. Viruses from FIP cats # 23, 150, and 107 and from contact animals in the same households are indicated by A, B, and C, respectively.

Cat No.	Age	Sample	Type of FIP
12	2y10m	ascites	wet form
14	8m	liver	wet form
16	2y6m	ascites	wet form
23	4m	mesenteric LN	wet form
29	1y	mesenteric LN	wet form
32	9m	mesenteric LN	wet form
38	10m	mesenteric LN	wet form
46	2y6m	ascites	wet form
47	1y3m	mesenteric LN	dry form
48	4y	mesenteric LN	wet form
61	2y	ascites	wet form
68	1y	ascites	wet form
74	8m	mesenteric LN	wet form
83	1y	liver	wet form
86	3y	kidney	dry form
96	1y	liver	wet form
97	4m	mediastinal LN	wet form
98	2y	kidney	dry form
107	9m	omentum	wet form
108	3y5m	mesenteric LN	dry form
110	6y	mesenteric LN	dry form
117	11m	mesenteric LN	wet form
120	5m	mesenteric LN	wet form
121	6m	liver	wet form
150	9m	mesenteric LN	wet form
164	5m	mesenteric LN	wet form
190	1y	mesenteric LN	wet form
206	7m	ascites	wet form

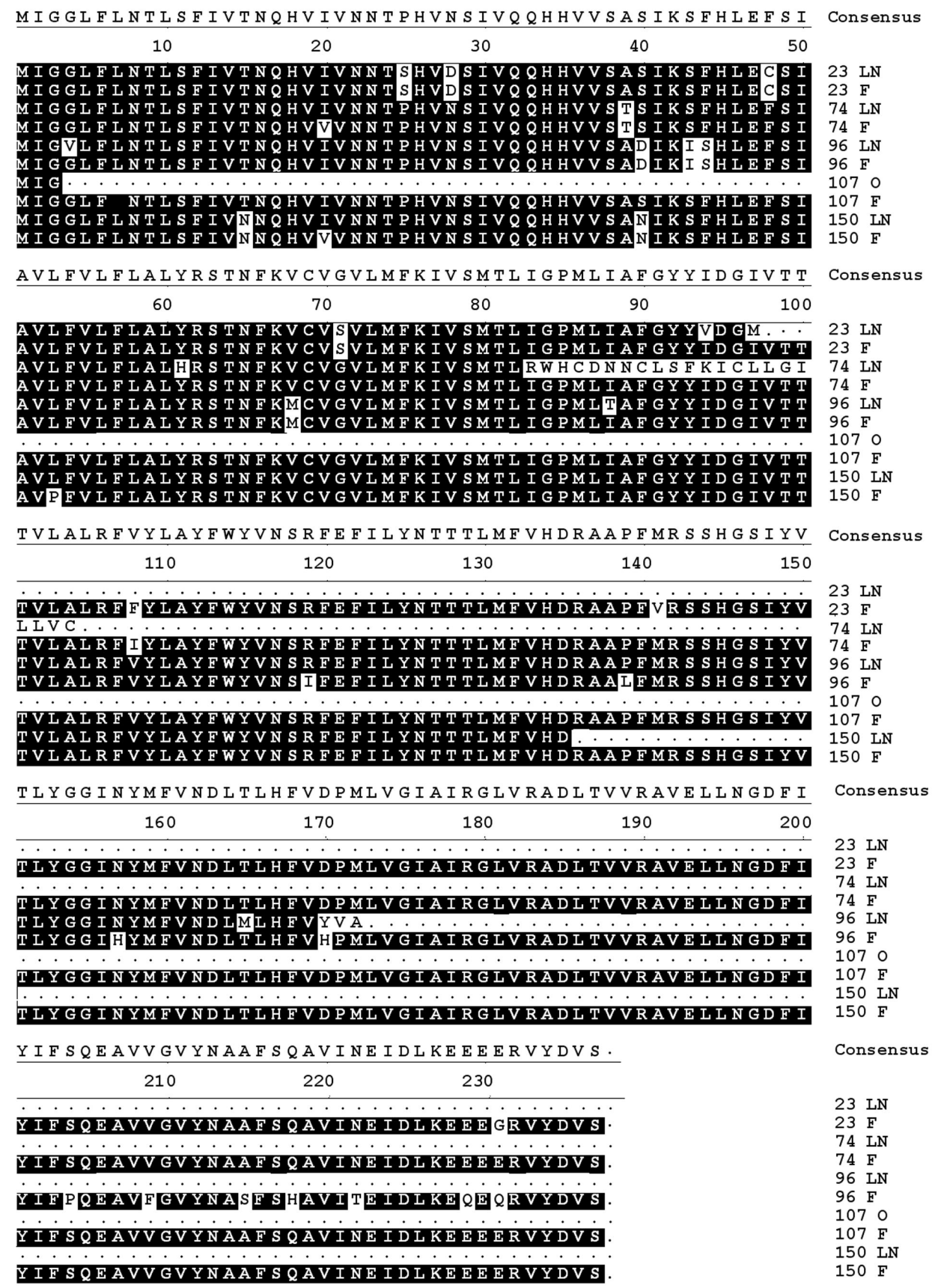
Table 1. FIP cats used, their ages, the clinical material taken for study, and the form of FIP (dry or wet) the animal was diagnosed with. LN: lymph node.


Cat #
Deletions
Lesion
Feces
12
Δ3 nt
1 aa deletion
1 aa deletion
14
Δ1 nt
Frame shift/PT
-
16
Δ55 nt
Frame shift/PT
-
23
Δ1 nt
Frame shift/PT
intact
61
Δ7 nt
Δ54 nt
Δ8 nt
Frame shift/PT
-
74
Δ36 nt
Δ30 nt
Frame shift/PT
intact
86
Δ16 nt
Frame shift/PT
-
96
Δ25 nt
Δ6 nt
Frame shift/PT
intact
97
Δ12 nt
4 aa deletion
NA
107
T
Δ1 nt
Δ5 nt
Δ31 nt
Nonsense /PT
intact
117
Δ7 nt
Δ31 nt
Frame shift/PT
-
120
Δ2 nt
Frame shift/PT
-
164
Δ13 nt
Δ30 nt
Frame shift/PT
-
Missense/nonsense mutations
32
T
PT
-
46
C
PT
-
47
T
PT
NA
98
T
PT
NA
150
T
PT
intact
Insertions
48
ACCTAT
2 aa insertion
-
121
T
Frame shift/PT
-
0
100
200
300
400
500
600
700

A

	150 F	150 LN	107 F	107 O	96 F	96 LN	74F	74 LN	23 F	23 LN
23 LN	94.7	94.1	92.9	91.9	90.6	91.1	93.1	92.3	99.2	
23 F	95.0	94.4	93.2	92.2	90.9	91.4	93.2	92.4		
74 LN	94.1	94.1	86.7	85.8	90.9	91.8	97.4			
74 F	95.1	93.8	79.5	87.2	90.9	91.5				
96 LN	93.7	93.0	84.9	83.9	96.6					
96 F	93.1	92.2	84.6	83.6						
107 O	93.6	92.9	97.9							
107 F	95.4	94.7								
150 LN	98.3									
150 F										

B



C