Short Communication

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

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

Correspondence
Peter J. M. Rottier
p.rottier@uu.nl

Virology Division, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Utrecht, The Netherlands

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

Feline coronaviruses (FCoVs; family *Coronaviridae*, order *Nidovirales*), important pathogens of cats, occur as two distinctly different pathotypes. Feline enteric coronavirus (FECV), the pathotype most common in the field, seems to be confined mainly to the intestinal tract and causes mild, often unapparent enteritis. The virus spreads efficiently via the faecal–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; reviewed by de Groot & Horzinek,1995; Haijema *et al.*, 2007; Pedersen, 2009).

There is genetic and animal experimental evidence to indicate that the virulent pathotype evolves time and time again from the avirulent one by mutation in individual infected cats. Comparative sequence analysis of FCoV

laboratory strains and field variants revealed that FECVs and FIPVs come in genetically closely related pairs, more identical to each other than to other FCoVs (Herrewegh *et al.*, 1995b; 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 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, induced FIP readily 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 convert apathogenic FECV into FIPV have not been identified in the huge 29 kb FCoV RNA genome. Several genes, including the spike gene and the so-called group-specific genes 3c, 7a and 7b, have been suggested to be associated with the pathotypic switch (Kennedy *et al.*, 2001; Rottier *et al.*, 2005; Vennema *et al.*, 1998). In particular, it has been noted 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 Dye &

Siddell (2007). Departing from the assumption that cats with FIP should harbour distinct enteric and non-enteric FCoV populations, these authors determined and compared the sequences of the most 3′ one-third of viral genomic RNAs isolated from the jejunum and liver of a cat with FIP. The approximately 10 kb sequences of the two viruses, including gene 3c, appeared to be completely identical, an observation that the authors considered to be in flat violation of the mutation hypothesis.

To try to resolve this controversy and to gain more insight into FCoV epidemiology, we compared naturally occurring FECV and FIPV variants with respect to the 3c gene. We screened 198 cats to identify animals positive for FCoV by using a nested RT-PCR targeting the highly conserved 3′-untranslated region of the viral genome (Herrewegh et al., 1995a) and then amplified gene 3c by RT-PCR using specific primers (sense, 5′-CAAGTACTATAAAACGTAGAAGMAG-3′; antisense, 5′-CAGGAGCCAGAAGAAGACACTAA-3′), applying 30 cycles of 94 °C for 60 s, 50 °C for 30 s and 72 °C for 1 min, and an additional extension at 72 °C for 7 min at the end of amplification. Thus, gene 3c sequences were obtained from the faeces of 27 apparently healthy FECV-infected cats as well as from organs, ascites or typical pyogranulomatous lesions of 28 cats with pathologically

confirmed FIP (see Supplementary Table S1, available in JGV Online) collected during 2007–2008 in the Netherlands. In addition, from 17 of the 28 cats with confirmed FIP, faecal material was also obtained and used for 3c gene analysis. The sequences of the FCoV 3c gene were deposited in GenBank under accession numbers GU053607–GU053667 (see Supplementary Table S2, available in JGV Online).

Without exception, the FECVs possessed an intact 3c gene, specifying a 238 aa polypeptide. In contrast, 3c gene sequences amplified from FIPV-affected tissues fell into two categories. Of the 28 sequences, only eight had an intact open reading frame (ORF) the size of that in FECV; the majority (71.4 %) exhibited various aberrations, as depicted in Fig. 1. Some had small in-frame deletions (cats 12 and 97) and insertions (cat 48); as these mutations preserve the reading frame and result in the loss or gain of just 1–3 aa, it is difficult to assess to what extent 3c protein function is affected. Importantly, however, such changes, minor as they might seem, were never observed in any of the FECV-derived ORF3c sequences. The remaining 3c sequences amplified from FIP lesions displayed more serious aberrations, including various out-of-frame deletions ($n=12$) or insertions ($n=2$), nonsense mutations ($n=5$) and combinations of deletions and point mutations
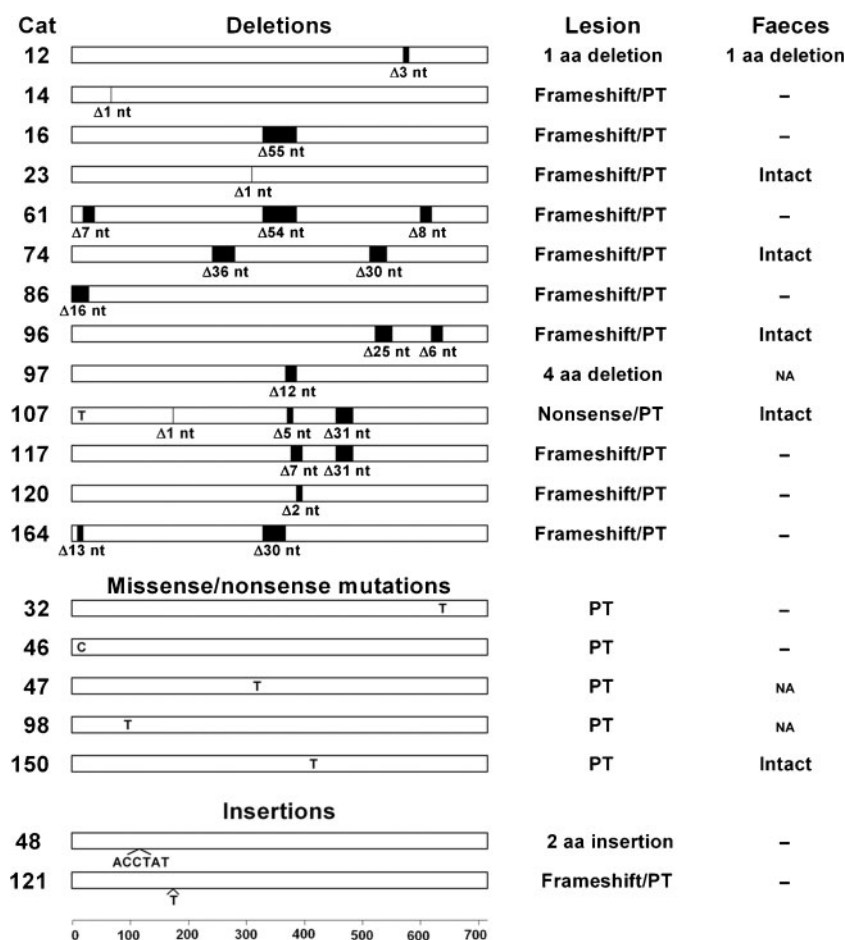
Fig. 1. Schematic representation of the 3c gene of lesion-derived FCoVs from 20 cats with confirmed FIP, showing deletions, nonsense/missense mutations and insertions. 3c sequences are indicated by white boxes. Deletions are indicated by black bars (Δ nt, number of nucleotides deleted). In the column labelled 'Lesion', the effects of the mutations on 3c translation are indicated (PT, premature termination). The column labelled 'Faeces' summarizes the results of the analysis of faecal samples (Intact, intact full-length 3c gene; −, no FCoV detected; NA, no faecal sample available).

(n=1), as depicted in Fig. 1. Most of these mutations will result in premature termination of translation and severe 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 FCoV replication in the gut, but dispensable for systemic propagation.

From 17 of the 28 cats with FIP, faecal 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 faecal viruses, their 3c sequence and the pathotypic signature thereof would provide crucial insight into both FIPV aetiology 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., 1995a). 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 of six animals where we did succeed in detecting FCoV and amplifying 3c sequences from the faeces, the 3c ORF 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 that 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 harboured 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 cats with FIP (Table 1; Fig. 2a). 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 %; Table 1) was higher than expected for such close relatives; the data rather suggested that the cats with FIP were doubly infected with genetically closely related FECVs that they might have acquired in their multi-cat environments. To study this possibility, we sampled faeces from apparently healthy contacts of the cats with FIP 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., 1995b, 1997), the FCoVs in the three multi-cat households form separate clades, each with a distinctive genetic signature (Fig. 2b). Our findings are best illustrated by the results for cat 107 with FIP. This animal harboured a systemic virus (FIPV 107) that, with respect to its 3c sequence, was related most closely to FECV isolated from cat 179, whilst the virus from its bowels (Faeces 107) was virtually identical to FECVs from companion animals 113 and 176. Similarly, for cats 23 and 150, the viruses in the faeces were related more closely to FECVs from healthy contacts (FECV 25 and FECV 152, respectively) than to their systemic viruses (FIPV 23 and 150, respectively; Fig. 2b). 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 cats with FIP where we did detect FCoV in the faeces, the virus represented neither the systemic FIPV nor its FECV predecessor, but rather a superinfecting 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 immune dysregulation and collapse of key effectors of the immune system in cats with end-stage FIP (de Groot-Mijnes et al., 2005; Haagmans et al., 1996) might then create an opportunity for FECVs circulating in surrounding healthy carriers to cause the superinfections that we apparently observe in a fraction of the animals.

In view of its supposed role in the pathogenesis of FIP, the aim of the present study was to sequence and compare the

Table 1. Nucleotide sequence identities (%) among 3c gene sequences from enteric (faeces-derived) and non-enteric (derived from lymph node or omentum) viruses in cats with FIP

Abbreviations: F, faeces; LN, lymph node; O, omentum.

Cat/sample	150 F	150 LN	107 F	107 O	96 F	96 LN	74 F	74 LN	23 F
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								
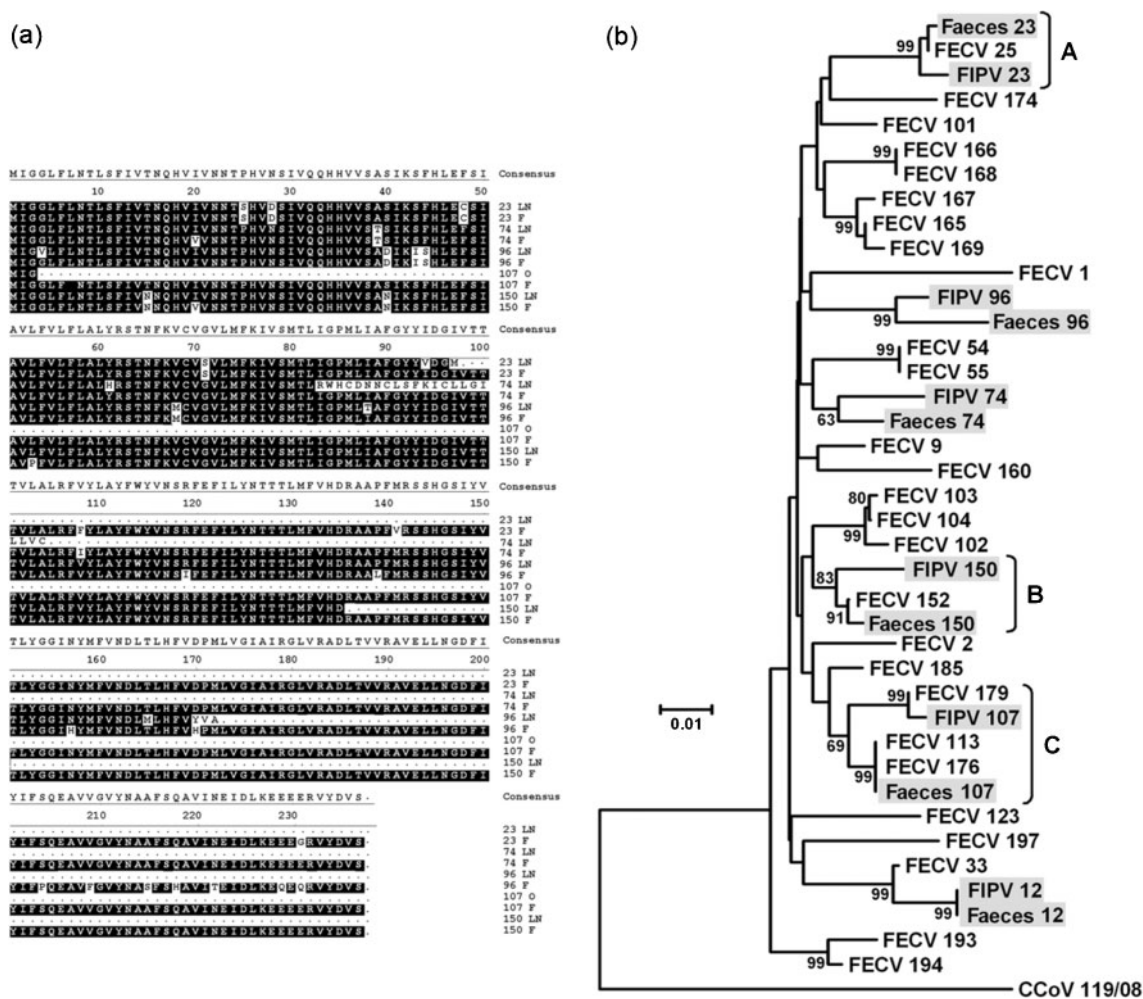
Fig. 2. (a) Alignment of the predicted 3c polypeptides from enteric and non-enteric viruses in cats with FIP. Shading indicates residues identical to the FECV 3c consensus sequence. Dots are shown when polypeptides terminate prematurely. (b) Phylogenetic relationships among FCoVs isolated from faecal samples and from FIP lesions. A gapless alignment of the 3c nucleotide sequences was used to generate a rooted neighbour-joining tree, with the 3c sequence of canine coronavirus strain 119/08 (GenBank accession no. EU924791) serving as outgroup. Bootstrap confidence values (percentages of 500 replicates) are indicated at the relevant branching points. Virus pairs in faeces and lesions of individual cats are marked by shading. Viruses from cats 23, 150 and 107 with FIP and from contact animals in the same households are indicated by A, B and C, respectively. Branch lengths are drawn to scale; bar, 0.01 nucleotide substitutions per site.

3c gene of FCoVs in a large number (n=55) of symptomatic and asymptomatic infected cats. The results show that FECVs invariably carry an intact 3c gene, whereas in the majority (71.4 %) of FIPVs, the gene has mutations unique for each virus, consistent with earlier studies (Pedersen *et al.*, 2009; Vennema *et al.*, 1998) and with the internal mutation theory. Our key observation, however, is that the viruses replicating in the gut invariably had an intact 3c gene, whereas those replicating outside the gut mostly did not. Importantly, intact 3c genes were also found in the intestines of all those cats with FIP that carried a mutated 3c in their lesion-derived FCoV genome, except in one case, cat 12. It is, however, conceivable that the single-residue deletion found in ascitic and intestinal virus

of this cat does not affect the functionality of the 3c protein, hence leaving the gene actually intact; alternatively, the faecal appearance of the virus might just have resulted from passive leakage of systemic FIPV into the gut.

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

virulent pathotype. We favour 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 non-essential 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 even enhance the fitness of the mutant virus in its new biotope.

Sequence comparisons of our collection of 3c genes initially seemed to confirm the expected relatedness in each cat with FIP between the faecal 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 faecal 3c sequences found in surrounding healthy FECV carriers than to that in the respective cat with FIP. Combined with the remarkable lack of detectable faecal FCoV in a large fraction of cats with FIP, it seems as though the original FECV infection is generally cleared from the gut following the pathotype switch; the cats sometimes become 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.*, 1995a, 1997; Kipar *et al.*, 2006a, b; Meli *et al.*, 2004; Simons *et al.*, 2005). The significance of this apparent viraemia is still poorly understood. Whilst 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* (Rottier *et al.*, 2005; Stoddart & Scott, 1989). Studies aimed at detecting FCoV replication, by using an RT-PCR designed specifically to identify viral mRNA, suggest this to also be the case *in vivo* (Simons *et al.*, 2005). Actually, viral mRNA or infected cells were 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 *et al.* (2006a) found significantly higher levels of viral RNA in cats with FIP than in healthy FCoV-positive cats. Moreover, FCoV antigen was detectable by immunohistology in these tissues only in cats with FIP (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 FCoVs 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 faecal shedding strictly require an intact viral gene repertoire, most notably a fully functional 3c gene. Inherent to the nature of RNA viruses, mutations occur continually, 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, virus 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, but 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 to recolonize the gut, where an intact 3c gene is apparently essential. Faecal 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 & Siddell (2007) to detect identical 3c-mutated viruses in gut and liver of a cat with FIP; this might explain the single instance in which we found a virus with mutated 3c both in FIP lesions and in the faeces (cat 12).

During the submission process of this manuscript, two publications relating to the subject appeared, one by Pedersen *et al.* (2009), which is largely in agreement with the present paper; another by Brown *et al.* (2009) proposed 'distinctive circulating virulent and avirulent strains in natural populations'.

Acknowledgements

References

Brown, M. A., Troyer, J. L., Pecon-Slattery, J., Roelke, M. E. & O'Brien, S. J. (2009). Genetics and pathogenesis of feline infectious peritonitis virus. *Emerg Infect Dis* **15**, 1445–1452.

de Groot, R. J. & Horzinek, M. C. (1995). Feline infectious peritonitis. In *The Coronaviridae*, pp. 293–309. Edited by S. G. Siddell. New York: Plenum Press.

de Groot-Mijnes, J. D., van Dun, J. M., van der Most, R. G. & de Groot, R. J. (2005). Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. *J Virol* **79**, 1036–1044.

Dye, C. & Siddell, S. G. (2007). Genomic RNA sequence of feline coronavirus strain FCoV C1Je. *J Feline Med Surg* **9**, 202–213.

Gunn-Moore, D. A., Gruffydd-Jones, T. J. & Harbour, D. A. (1998). Detection of feline coronaviruses by culture and reverse transcriptase-polymerase chain reaction of blood samples from healthy cats and cats with clinical feline infectious peritonitis. *Vet Microbiol* **62**, 193–205.

Haagmans, B. L., Egberink, H. F. & Horzinek, M. C. (1996). Apoptosis and T-cell depletion during feline infectious peritonitis. *J Virol* **70**, 8977–8983.

Haijema, B. J., Volders, H. & Rottier, P. J. (2004). Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. *J Virol* **78**, 3863–3871.

Haijema, B. J., Rottier, P. J. M. & de Groot, R. J. (2007). Feline coronaviruses: a tale of two-faced types. In *Coronaviruses: Molecular and Cellular Biology*, pp. 183–203. Edited by V. Thiel. Norfolk, UK: Caister Academic Press.

Herrewegh, A. A. P. M., de Groot, R. J., Cepica, A., Egberink, H. F., Horzinek, M. C. & Rottier, P. J. (1995a). Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. *J Clin Microbiol* **33**, 684–689.

Herrewegh, A. A., Vennema, H., Horzinek, M. C., Rottier, P. J. & de Groot, R. J. (1995b). The molecular genetics of feline corona-viruses: comparative sequence analysis of the ORF7a/7b transcription unit of different biotypes. *Virology* **212**, 622–631.

Herrewegh, A. A. P. M., Mähler, M., Hedrich, H. J., Haagmans, B. L., Egberink, H. F., Horzinek, M. C., Rottier, P. J. M. & de Groot, R. J. (1997). Persistence and evolution of feline coronavirus in a closed cat-breeding colony. *Virology* **234**, 349–363.

Kennedy, M., Boedeker, N., Gibbs, P. & Kania, S. (2001). Deletions in the 7a ORF of feline coronavirus associated with an epidemic of feline infectious peritonitis. *Vet Microbiol* **81**, 227–234.

Kipar, A., Baptiste, K., Barth, A. & Reinacher, M. (2006a). Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats. *J Feline Med Surg* **8**, 69–72.

Kipar, A., Meli, M. L., Failing, K., Euler, T., Gomes-Keller, M. A., Schwartz, D., Lutz, H. & Reinacher, M. (2006b). Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection. *Vet Immunol Immunopathol* **112**, 141–155.

Meli, M., Kipar, A., Müller, C., Jenal, K., Gönczi, E., Borel, N., Gunn-Moore, D., Chalmers, S., Lin, F. & other authors (2004). High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. *J Feline Med Surg* **6**, 69–81.

Oostra, M., de Haan, C. A., de Groot, R. J. & Rottier, P. J. (2006). Glycosylation of the severe acute respiratory syndrome coronavirus triple-spanning membrane proteins 3a and M. *J Virol* **80**, 2326–2336.

Pedersen, N. C. (2009). A review of feline infectious peritonitis virus infection: 1963–2008. *J Feline Med Surg* **11**, 225–258.

Pedersen, N. C., Boyle, J. F., Floyd, K., Fudge, A. & Barker, J. (1981). An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. *Am J Vet Res* **42**, 368–377.

Pedersen, N. C., Allen, C. E. & Lyons, L. A. (2008). Pathogenesis of feline enteric coronavirus infection. *J Feline Med Surg* **10**, 529–541.

Pedersen, N. C., Liu, H., Dodd, K. A. & Pesavento, P. A. (2009). Significance of coronavirus mutants in feces and diseased tissues of cats suffering from feline infectious peritonitis. *Viruses* **1**, 166–184.

Poland, A. M., Vennema, H., Foley, J. E. & Pedersen, N. C. (1996). Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. *J Clin Microbiol* **34**, 3180–3184.

Rottier, P. J., Nakamura, K., Schellen, P., Volders, H. & Haijema, B. J. (2005). Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *J Virol* **79**, 14122–14130.

Simons, F. A., Vennema, H., Rofina, J. E., Pol, J. M., Horzinek, M. C., Rottier, P. J. & Egberink, H. F. (2005). A mRNA PCR for the diagnosis of feline infectious peritonitis. *J Virol Methods* **124**, 111–116.

Stoddart, C. A. & Scott, F. W. (1989). Intrinsic resistance of feline peritoneal macrophages to coronavirus infection correlates with *in vivo* virulence. *J Virol* **63**, 436–440.

Vennema, H., Poland, A., Foley, J. & Pedersen, N. C. (1998). Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology* **243**, 150–157.