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Preliminary studies on feline coronavirus distribution in naturally and experimentally infected cats

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SUMMARY

The shedding, tissue distribution and quasispecies composition of feline coronaviruses were studied in naturally and experimentally infected cats. The infection remained subclinical, but the majority of the animals shed the virus via faeces throughout the experiment. Sequences corresponding to the viral nucleocapsid region were amplified by reverse-transcription polymerase chain reaction from the cortex, dura mater, pancreas, lungs, third eyelid, and the heart muscle in four cases. Interestingly, the ORF7b viral region – a supposed virulence factor – was detected in fewer organs, raising the possibility that this region can be affected by deletions during virus replication in vivo.

It is demonstrated that the composition of the viral quasispecies differs between organs, and that genomic regions with different functions undergo distinct processes of selection, which should be considered during the evolution of feline coronaviruses.

FELINE CORONAVIRUSES (FCoV) are members of the family *Coronaviridae*. Their genome consists of a single-stranded positive-sense RNA (de Groot et al 1987, Luytjes 1995). Regarding their pathogenicity, FCoV comprise two biotypes: feline infectious peritonitis (FIP) viruses (FIPV) and feline enteric coronaviruses (FECV; Pedersen 1987). Based on in-vitro neutralisation using monoclonal antibodies, the existence of at least two serotypes of FCoV (type I and II) has been shown. Each serotype contains viruses of both the FIPV and FECV biotype (Hohdatsu et al 1991a,b). Type I and II viruses differ in their in vitro growth characteristics (Pedersen 1987) and importantly, in the composition of their spike protein (Herrewegh et al 1998). In the field, the prevalence of FCoV type I seems to be higher, since about 70 per cent of FIP cases are due to infection with type I viruses (Hohdatsu et al 1992).

FIP is a highly fatal disease in *Felidae* (Foley et al 1997a). The pathogenesis of the disease is still not fully understood. The diagnosis of the disease is rather complicated, and therefore it represents one of the major pet animal health problems that still remains to be solved. Thus, serological data on their own are not always informative (Olsen 1993) in diagnosis. Vaccines may not provide protection since they may even induce accelerated pathogenesis and early death, associated with antibody-dependent enhancement of infection (Vennema et al 1990, de Groot and Horzinek 1995, Harvey et al 1996). Nevertheless, a commercially available vaccine has shown promising results (Fehr et al 1997).

FECV is not considered a direct cause of morbidity in cats. It may produce mild enteritis but most experimental and natural infections remain subclinical. However, it presents a considerable health risk to cats because it has been revealed that it is the parent of FIPV (Foley et al 1997b, Vennema et al 1998).

Recent findings suggest that genetic changes frequently occur in feline coronaviruses (Herrewegh et al 1995, 1997, 1998, Vennema et al 1998). For instance, it has been demonstrated that type II feline coronaviruses have arisen from an RNA recombination event between canine coronavirus and a type I feline coronavirus (Herrewegh et al 1998). Further observations indicate that deletions in different parts of the FECV genome may be responsible for the formation of FIP viruses (Vennema et al 1998). However, the mechanism of these events is not totally understood. It is known that type I FECV can establish persistent infection in cats when the virus replicates in the enterocytes of the crypts of Lieberkühn in the large intestine (Herrewegh et al 1997). During virus replication, the specific changes in the viral genome may lead to the development of the highly pathogenic FIP viruses and consequently, manifestation of the disease. Possibly a malfunction of the immune system contributes to this process (Poland et al 1996).

The laboratory diagnosis and control of FIP is seriously hampered by the failure of most FCoV to replicate in cell cultures (de Groot and Horzinek 1995). Considering this fact, the aim of this study was to develop and apply efficient approaches for the detection and differentiation of FCoV subpopulations. Using these techniques, we investigated the persistence, distribution and quasispecies composition of FCoV in naturally and experimentally infected cats.

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MATERIALS AND METHODS

Experimental animals

In order to mimic natural conditions, 12 randomly selected mixed-breed domestic cats of both genders were used. The animals originated from a vendor and were clinically healthy, and between 4–6 months of age. The cats had not been immunised with any vaccine. The faeces of cats Nos 6, 8, 9 and 10 were positive for FCoV using a reverse-transcription polymerase chain reaction (RT-PCR). Therefore these animals were regarded as carriers, while the remaining eight cats were repeatedly negative for FCoV before starting the experiment. Six FCoV free animals (Nos 1, 2, 3, 4, 5 and 7) were experimentally infected with FCoV, and two cats (Nos 11 and 12) were used as negative controls. The animals were kept in the same room but in separate cages and had no physical contact with each other. They were fed with commercially available cat food, milk and fresh water.

Experimental infection

Faecal samples were collected from cats Nos 6, 8, 9 and 10 at the beginning of the experiment. The fresh samples were pooled, homogenised in PBS (1:10, w/v), centrifuged at $3100 \times g$ for 20 minutes and the supernatant fluid was given orally to six FCoV free cats (Nos 1, 2, 3, 4, 5 and 7) at a dose of 1 ml cat^{-1} . The negative controls and the FCoV carriers were mock-infected with PBS.

Clinical examinations and sample preparation

The cats were monitored daily for clinical signs. Rectal swab samples were collected 7 days before starting the experiment, on the day of experimental inoculation, and on post-infection days (PID): 10, 20, 30, 40, 50, 60, 70 and 80. The swabs were processed as described by Foley et al (1997b). Briefly, the swabs were vortexed in 1.5 ml double-distilled water and then the suspensions were centrifuged at $5000 \times g$ for 5 minutes. On PID 80 the cats were killed and gross and histopathological examinations were carried out. The following tissue samples were collected for RT-PCR: brain cortex, medulla oblongata, dura mater, liver, kidneys, spleen, lymph nodes, lungs, small and large intestines, pancreas, third eyelid and heart muscle. From the organ samples, 10 per cent (w/v) suspensions were prepared in double-distilled water and then centrifuged at $7000 \times g$ for 10 minutes. The supernatants were used immediately for RNA extraction or stored at -70°C .

RNA extraction

The supernatants of the faeces or tissue homogenates were used for RNA preparation by the procedures of Boom et al (1990) and Cheung et al (1994) as follows: 100 μl suspension was added to 20 μl size-fractionated silica in 900 μl lysis buffer (120 g guanidine thiocyanate dissolved in 100 ml 0.1 M Tris-HCl, [pH 6.4], and 22 ml 0.2 M EDTA, [pH 8.0], and 2.6 g Triton X-100). The samples were vortexed and left for 10 minutes at room temperature. The supernatant was removed after a quick spin ($12000 \times g$ for 1 minute), and the silica-RNA pellet was washed twice with

450 μl washing buffer (120 g guanidine thiocyanate dissolved in 100 ml 0.1 M Tris-HCl, [pH 6.4]) and twice with 1 ml of 70% (v/v) ethanol. After a final wash with 1 ml acetone, the pellet was dried at 56°C for 10 minutes. The RNA was eluted by resuspending the pellet in 40 μl diethyl pyrocarbonate-treated (DEPC) water at 56°C for 10 minutes. After pelleting the silica particles ($12000 \times g$ for 3 minutes), the supernatant fluid containing the RNA was used for reverse transcription.

Reverse transcription

The cDNA synthesis was performed in 28 μl reaction mixtures. A preliminary mixture containing 5 μl RNA, 5 μl DEPC-treated water, and 1 μl random hexamers (0.02 U; Pharmacia Biotech, Uppsala, Sweden) was incubated at 65°C for 5 minutes for RNA denaturation. The tube was placed on ice, and 17 μl RT-mix containing 2.5 μl of each deoxynucleotide triphosphate (2 mM; Pharmacia), 5 μl of $5 \times$ reaction buffer (0.25 M Tris-HCl, [pH 8.3], 0.375 M KCl, 15 mM MgCl_2), 1 μl RNAGuard (24 U; Pharmacia), and 1 μl Moloney murine leukaemia virus reverse transcriptase (200 U; Gibco BRL, Bethesda, MD, USA) was added. The reaction mixture was incubated at 37°C for 90 minutes, followed by incubation at 98°C for 5 minutes to inactivate the enzyme.

Primer selection

Primers were selected from the highly conserved nucleocapsid and ORF7b regions of the FIP virus strain 79-1146 genome (Vennema et al 1991) using the OLIGO 4.0 program (National Biosciences Inc., Plymouth, MN, USA). Sequences of the primers are shown in Table 1. It should be noted that the N-PCR has been applied for more than 6 years in our routine diagnostic laboratory for the diagnosis of FIP and several hundreds of samples from clinical cases were found to be positive (unpublished data).

PCR

The two PCR assays were carried out in 50 μl volumes. The reaction mixtures contained 5 μl 10 \times GeneAmp PCR buffer II (Perkin-Elmer Cetus, Norwalk, CT, USA), 15-pmol OFIP1A and OFIP2A primers for the first nucleocapsid PCR, 15 pmol P399 and P997 primers for the first ORF7b PCR, 2.5 μl of each deoxynucleotide triphosphate (10 mM; Pharmacia), 2.5 mM MgCl_2 , AmpliTaq DNA polymerase (1 U; Perkin-Elmer Cetus), and 5 μl cDNA. Two droplets of mineral oil (Sigma Chemical, St. Louis, MO, USA) were added to prevent evaporation. The second (nested) PCR assays were performed on 2 μl amounts of the first PCR products with 15–15 pmol of primer OFIP4 and OFIP3 for the nucleocapsid and 15–15 pmol of primer P503 and P970 for the ORF7b. The following temperature profile was used: 94°C for 45 seconds, 45°C for 60 seconds (50°C for 60 seconds for the first five cycles), and 72°C for 2 minutes. For the second round of amplification, 96°C for 45 seconds, 52°C for 1.5 minutes, and 72°C for 3 minutes was applied. This cycle profile was repeated 30 times followed by a final extension step at 72°C for 7 minutes.

TABLE 1: Primers used for the RT-PCR of feline coronaviruses

Primers for the nucleocapsid region	Sequence (5'-3')	5' position
OFIP1A (F)	ATT TTG GAA TTT ATG TCC GAG AGA	990
OFIP2A (R)	CTA GCA CCA TAG AAA GTT GTC ACA	1598
OFIP3 (F)	CGC TGA GAG GTG GTT CTT TTA CTT C	1110
OFIP4 (R)	CTT CCA GGT GTG TTT GTT GGC ATT C	1554
Primers for the ORF7b region		
P399 (F)	TGT GTA TCT TTT TGG CTA ATG GA	399
P997 (R)	TTT TAG ACA TCG GGT TGC CTT AT	997
P503 (F)	ACC CTC TAC ATT ACA ACA CAC CA	503
P970 (R)	CAG TTT TAT GAT GTT GGT TGA TT	970

F, forward primer; R, reverse primer. The expected lengths of the amplified products are as follows: OFIP1A-OFIP2A: 680 bp; OFIP3-OFIP4: 444 bp; P399-P997: 621 bp; P503-P970: 490 bp.

The amplified DNA products were detected by standard 2 per cent agarose gel-electrophoresis. To avoid false positive and negative results, the precautions of our routine diagnostic PCR laboratory were used throughout the experiments (Belák and Ballagi-Pordány 1993).

The sensitivity of the two PCR assays was evaluated and compared by testing 10-fold dilutions of the tissue culture supernatants of reference virus strains (DF-2, NW-1, 79-1683, Black, UCD-2, and UCD-3) and clinical samples, such as rectal swab and heart muscle homogenate originating from cat No 9. The concentration of the nucleic acid solutions was determined by a spectrophotometer (GeneQuant RNA/DNA Calculator, Pharmacia, Uppsala, Sweden).

In serial 10-fold dilution tests, the two PCR assays showed the same sensitivity. As calculated, 3.2 pg ml⁻¹ total RNA was the minimal target where visible PCR product was obtained (not shown).

Single strand conformational polymorphism (SSCP) analysis

In order to generate single stranded PCR products for the SSCP analysis, asymmetric PCR was performed on 2 µl amounts of the completed first PCR mixtures by using the nested primers at a ratio of 1:10 (5:50 pmol). The same cycle profile was applied as for the second round of amplification. Five microlitre amounts of the single stranded PCR products were added to 15 µl SSCP loading buffer (95 per cent formamide, 0.05 per cent xylene cyanol, 0.05 per cent bromophenol blue), kept at 95°C for 5 minutes, put on ice for 5 minutes and finally loaded onto 10 per cent polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed in a Mini Protean Cell (Bio-Rad) at 140 V constant power for 4 hours at room temperature. Subsequently, the gels were submerged into a 0.5 µg ml⁻¹ ethidium-bromide solution for 5 minutes and visualised under UV light.

RESULTS

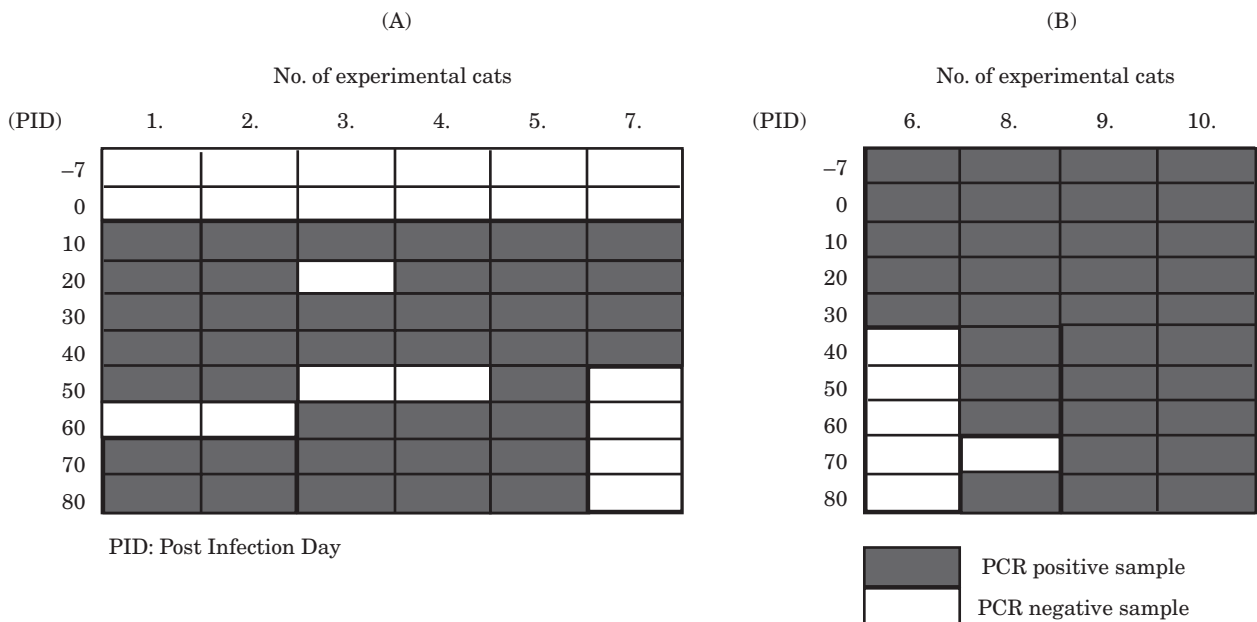
Clinical observations

After experimental infection, the clinical status of the cats was not severely affected. Only a mild diarrhoea was observed among the experimentally infected animals at PID 3-6. The appetite, behaviour and body temperature remained normal, while the body weight of the cats increased by 15 to 25 per cent up to the end of the experiment.

Detection of FCoV

By testing the swab samples, the N-PCR and the ORF7b-PCR assays gave consistent results. In cat Nos 5, 9 and 10, FCoV was detected at each occasion of testing. In other animals, the virus was detected at various occasions, as shown in

TABLE 2: PCR results of the rectal swabs of cats experimentally (A) and naturally (B) infected with FCoV



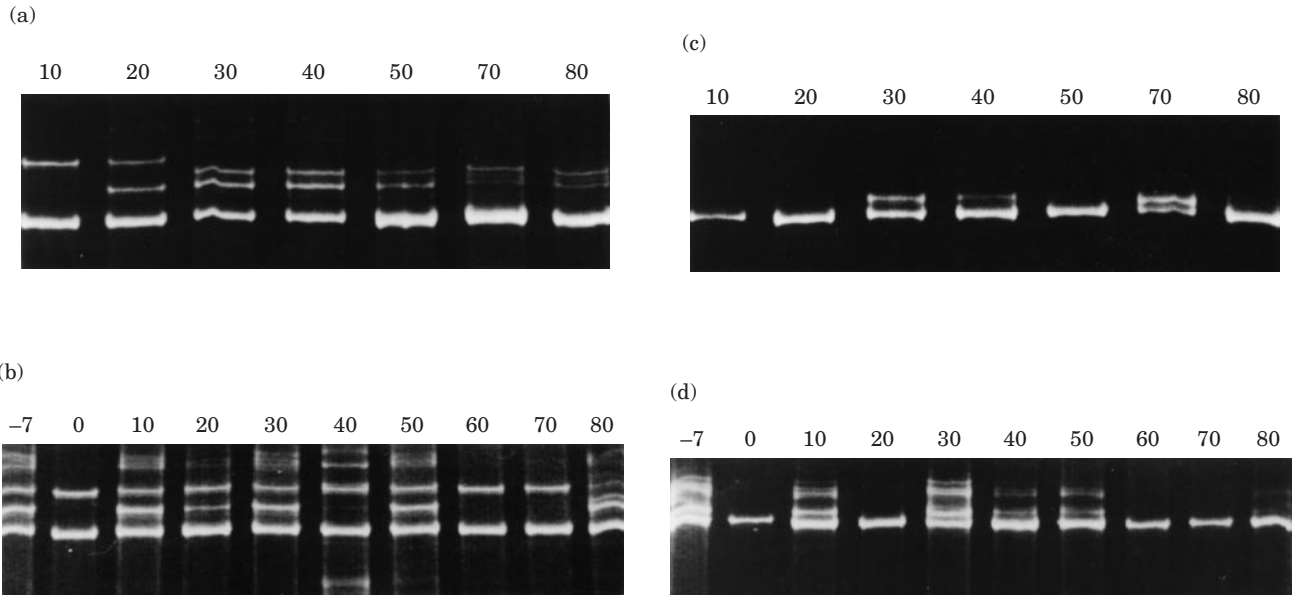


FIG 1. SSCP patterns of the products amplified by the nucleocapsid (a and b) and the ORF7b (c and d) specific PCR assays from the rectal swab samples of cat Nos 1 and 9, respectively. The lanes represent the consecutive occasions of sampling displayed in post infection days (PID).

Table 2. The samples obtained from the control animals were negative throughout the experiment (not shown).

By testing the large intestines in the N-PCR assay, virus was detected in eight animals. Cat Nos 6 and 7 were negative. In cat Nos 1, 5, 9 and 10, the virus was also demonstrated in the following organs; cat No 1: cortex, dura mater, lungs, pancreas, third eyelid and heart muscle; cat No 5: third eyelid and heart muscle; and cat Nos 9 and 10: heart muscle. The ORF7b region was detected in the large intestines of the same eight animals as in the N-PCR assay. It was also demonstrated in the pancreas of cat No 1 and in the heart muscle of cat Nos 5 and 9.

SSCP patterns of the amplified sequences

In the SSCP assays, the quasispecies nature of the detected viruses was revealed as illustrated in Fig 1. The bands in the same lane represent viral subpopulations with slight nucleotide sequence differences (Lázaro and Estivill 1992). Generally the N region showed higher variability than the ORF7b region. The consistency of the patterns was confirmed by repeated PCR-SSCP analyses (data not shown).

The changes of the viral quasispecies in the large intestine of cat No 1 (Fig 1a,c) could be characterised as follows. At 10 days after infection, there appeared to be one major and one minor variant of virus subpopulation. At PID 20 there was an additional minor variant. The latter remained until termination of the experiment at PID 80. The first minor variant appeared to be replaced by a third minor variant at PID 30 which also remained present to the end. The major variant was always present throughout the experiment and was never replaced. The third minor variant sequence in the N gene that appeared at PID 30 might correlate with a variant in the 7b gene sequence (Fig 1c). In cat No 9 (Fig 1b,d) the major and minor N variants present at day 0 were most likely the same as the variants present throughout the experiment

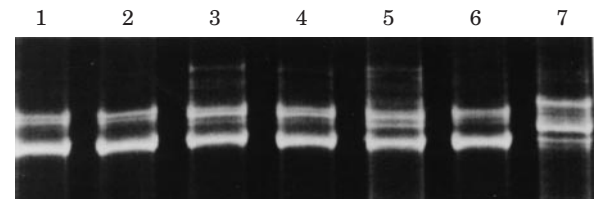


FIG 2. SSCP patterns of the products amplified by the nucleocapsid region specific PCR from the organ samples of cat No. 1. The lanes represent the following organs: 1. cortex; 2. dura mater; 3. lung; 4. large intestine; 5. pancreas; 6. heart muscle; 7. third eyelid.

from day -7 to PID 70, with the appearance and disappearance of additional minor subpopulations. Changes of the 7b region showed similar characteristics but to a more moderate extent.

The N-SSCP patterns of the organs were more homogeneous than those of the swab samples, as demonstrated in Fig 2. Apparently each organ consistently contained three predominant variants. The ORF7b-SSCP patterns of the organs had substantially the same pattern as the swabs (not shown).

DISCUSSION

Recent studies suggest that FECV is the parent of FIPV, and the alterations in the genome of the 'harmless' parent lead to the development of the fatal disease of infectious peritonitis (Vennema et al 1992, Herrewegh et al 1995, Poland et al 1996, Vennema et al 1998). Considering that FECV is extremely widespread in the cat populations (Foley et al 1997b), detailed studies are required to investigate the biology of the two virus biotypes, with particular regard to the genetic stability of FECV and the genomic regions

responsible for the development of viruses of high pathogenicity. These investigations will rely on the ability to detect and characterise the different viruses at various stages of the infection.

In the present study we investigated the invasion, the tissue distribution, and the variability of FCoV. Our aim was to establish and compare two PCR assays with respect to their applicability for monitoring purposes. By applying these assays to amplify two conserved regions of the FCoV genome, we found that eight of 10 cats shed the virus in the faeces throughout the 80-day course of the experiment. This finding supports the observations of Herrewegh et al (1997) who reported that the FCoV infection is maintained by chronically infected carriers. The short-lasting and mild diarrhoea that was observed among the experimentally infected animals at the early stage of infection could be the clinical symptom of FCEV infection, or it may have been the consequence of the massive exposure of the animals to the FCoV, as suggested by Foley et al (1997a). The two cats (Nos 6 and 7), which became and remained PCR-negative at the relatively early stage of the experiment, might have had strong and effective immune response against FCoV.

A crucial step in the pathogenesis of FIP is when certain virus variants leave the intestines and reach different organs (Pedersen et al 1981). It is not known how long this process takes or which conditions are required for this event. The present experiment showed that despite the relatively short observation period of 80 days, in one cat FCoV apparently invaded different organs. Considering the suggestions of Pedersen et al (1981), this may be an important step in the development of FIP.

It is interesting that the N and ORF7b-PCR assays gave identical results when the swab and large intestine samples were tested. However, in the case of additional organs, the N-PCR scored considerably more positive samples. An obvious explanation would be the different sensitivity of the two systems. This is not likely, however, considering that the two assays yielded identical results not only on the swab and large intestine samples, but also when serial dilutions of FCoV and clinical specimens were tested. We therefore hypothesise that the viruses detected in the cats could presumably have their ORF7b region affected by deletion(s). Such a phenomenon was reported for avirulent, tissue culture adapted feline coronaviruses and it was concluded that the loss of virulence was associated with the deletions in the ORF7b region (de Groot and Horzinek 1995). According to Vennema et al (1998), as well as being a virulence factor the presence of this region is an advantage for the virus replication. Therefore, this phenomenon should be investigated further, both in vitro and in vivo, and studies should also involve the ORF3c region which is considered to exert a regulatory effect on the ORF7b (Vennema et al 1998).

The quasispecies nature of the members of the *Coronaviridae* family has been well documented, mainly for murine coronaviruses (Makino et al 1986, Baric et al 1990, Holland et al 1992, Lai 1996). The present study revealed that FCoV also comprised virus subpopulations. However, there were differences in the prevalence of viral subpopulations between body compartments, i.e., not all variants present in the swab samples were present in the organ samples, and some variants were more prevalent in the organ samples than in the large intestine. Additionally, during

in vivo replication, the SSCP patterns of the N region showed higher variability than the ORF7b region. These findings suggest that presumably only selected virus variants became able to leave the large intestine and invade the blood circulation and different organs. It appears, however, that the selection pressure is not on the N gene, since the predominant subpopulations (the master sequences) could be demonstrated in each N-PCR positive organ specimen. Nevertheless, presumably a new selection process takes place in every infected organ that may result in a virus quasispecies that is different in composition from that which originally replicated in the large intestine. This process, in conjunction with the relatively impaired reaction of the respective immune response of particular cats could eventually lead to the development of FIP.

The present study demonstrated that FCoV is able to establish chronic enteric infection in cats. The data indicate that the viruses undergo continuous but subtle changes during the chronic replication, and that the extent of variation and the composition of the viral quasispecies is not the same in the different organs. Additionally, even neighbouring genomic regions showed distinct variability: the N region varied more than the ORF7b region.

The possible role of ORF7b in virus replication is indicated by the highly conservative SSCP pattern of this region exhibited throughout the experiment. The exact information on ORF7b and its product, the gp7b, should reveal the role of the region in the development of FIP.

In conclusion, this study provided information concerning alternations in the genome of FCoV during in vivo replication. It was shown that the composition of the viral quasispecies differs between organs, and that genomic regions with different functions undergo distinct processes of selection, which should be considered during the evolution of feline coronaviruses. Future investigations will aim to determine the significance of the changes of particular viral genomic regions. Beyond this, the immune responses against FCoV infections and the probable impairment of these functions, that eventually participate in the pathogenesis of FIP, will be elaborated.

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