Research in Veterinary Science 97 (2014) 333–340

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Mutations in the 3c and 7b genes of feline coronavirus in spontaneously affected FIP cats

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ARTICLE INFO

Article history:
Received 7 December 2013
Accepted 25 July 2014

Keywords:
Cat
Coronavirus
Mutation
3c gene
7b gene

ABSTRACT

Feline infectious peritonitis (FIP) is the most frequent lethal infectious disease in cats. However, understanding of FIP pathogenesis is still incomplete. Mutations in the ORF 3c/ORF 7b genes are proposed to play a role in the occurrence of the fatal FIPV biotype. Here, we investigated 282 tissue specimens from 28 cats that succumbed to FIP. Within one cat, viral sequences from different organs were similar or identical, whereas greater discrepancies were found comparing sequences from various cats. Eleven of the cats exhibited deletions in the 3c gene, resulting in truncated amino acid sequences. The 7b gene was affected by deletions only in one cat. In three of the FIP cats, coronavirus isolates with both intact 3c genes as well as 7b genes of full length could also be detected. Thus, deletions or stop codons in the 3c sequence seem to be a frequent but not compelling feature of FIPVs.

1. Introduction

Feline infectious peritonitis is a widely distributed, fatal disease in felidae occurring as two clinical forms: effusive and granulomatous. An efficient vaccine or therapy does not exist. The aetiologic agent is the feline coronavirus (FCoV) that occurs as two different biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). It has been assumed that the FIPV biotype arises by as yet unidentified mutation(s) of FECV strains in individual cats (Chang et al., 2010, 2012; Pedersen et al., 2009; Poland et al., 1996; Vennema, 1999; Vennema et al., 1998). In contrast to the fatal course of FIPV infections, infection with FECV only contributes to a subclinical stage of disease where cats exhibit mild diarrhoea or no clinical signs at all. Morphologically or antigenetically, the different biotypes cannot be distinguished from each other. Another classification subdivides feline coronaviruses into serotype I and serotype II strains. This classification system is based on the relationship to canine coronavirus (CCoV). FCoV type II strains developed from recombination of a type I strain with a CCoV (Herrewegh et al., 1998; Motokawa et al., 1996). Classification as type I or type II strains does not give any information about the virulence. Both type I and II strains contain highly virulent FIPVs as well as low virulent FECVs (Pedersen et al., 1984). In vitro cultivation of type I strains has proven to be very difficult, whereas type II strains grow well in cell culture. Unfortunately, in this respect field infections are predominantly caused by serotype I isolates (Benetka et al., 2004; Hohdatsu et al., 1992; Kummrow et al., 2005; Lin et al., 2009a). Independent from bio- and serotypes, FCoVs show a relatively high mutational rate during the course of virus replication (Lai et al., 2007). Therefore, complex virus populations or so-called quasi species could be detected in FCoV isolates (Battilani et al., 2003; Gunn-Moore et al., 1999). To date, the mechanisms of FIP pathogenesis are not fully understood. After oropharyngeal uptake of FCoVs, they predominantly infect the epithelial cells of the gut (Herrewegh et al., 1997; Kipar et al., 2010; Stoddart et al., 1988). The infection of monocytes/macrophages and thereby the systemic spread of the virus had been considered to be the determining step in FIP development in the past (Pedersen et al., 1981). Today, it is known that FCoV genome can be detected in the blood even in clinically healthy cats (Gunn-Moore et al., 1998; Herrewegh et al., 1995a, 1997; Kipar et al., 1999). Indeed, FIPVs seem to replicate more effectively in macrophages than FECVs (Dewerchin et al., 2005; Stoddart and Scott, 1989). The viral spike protein was identified to play an important role for infection and macrophage tropism (Rottier et al., 2005). Chang et al. (2012) identified two alternative amino acid differences in the spike protein that are supposed to distinguish FECV and FIPV in a high percentage of cases. Recently, Porter et al. (2014) put into perspective these findings by detecting the assumed FIP-specific mutations also in coronavirus-infected cats without any signs of FIP.

Furthermore, Balint et al. (2012) observed a difference in the replication efficiency of FIPV strains with intact and deleted ORF 3abc genes. The latter exhibited more effective replication in macrophages than do FIPVs with intact ORF 3abc. In contrast, another study detected a crucial role of ORF 7ab in FIPV replication in monocytes/macrophages and not in ORF 3abc (Dedeurwaerder et al., 2013). Additionally, the ORF 7a unit combined with ORF 3-encoded proteins seems to be an effective antagonist of IFN-alpha-induced anti-viral response (Dedeurwaerder et al., 2014). The above

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http://dx.doi.org/10.1016/j.rvsc.2014.07.016
0034-5288/

mentioned genomic regions belong to FCoV nonstructural or accessory genes. Products of the 7a and 7b genes are small, secretory glycoproteins whereas the function of ORF 3a, 3b or 3c is not known (De Groot et al., 1988; Vennema et al., 1992a, 1992b). In several studies, sequence variation could be detected in the nonstructural protein genes of FIPVs compared to FECVs (Chang et al., 2010; Pedersen et al., 2009; Vennema et al., 1992b, 1998). Most frequently, deletions in the 3c gene were found in FIPVs that were not present in FECVs. This led to the assumption that deletions in ORF 3c may result in an increase of virulence even if FIPV strains with intact ORF 3c could be found as well. On the other hand, deletions in ORF 7b were considered to play a role in virus attenuation (Dedeurwaerder et al., 2013; Herrewegh et al., 1995b; Vennema et al., 1998). The relevance of all these findings is still controversial (Balint et al., 2012; Brown et al., 2009; Chang et al., 2010; Dedeurwaerder et al., 2013, 2014; Kennedy et al., 2001, 2006; Kiss et al., 2000; Lin et al., 2009b; Pedersen et al., 2009, 2012; Porter et al., 2014; Tekes et al., 2012; Vennema et al., 1998).

To obtain an overview of the presence of mutations in ORF 3c and ORF 7b in naturally occurring FIP cases and to study the characteristics of FCoVs in different organs within one cat, tissue specimens from spontaneously affected and necropsied cats with FIP were investigated in the present study.

2. Material and methods

A total of 28 cats that died because of FIP were included in the study. All of them were necropsied at the Department of Veterinary Pathology of the University of Giessen from 2008 to 2010. The cats originated from different husbandry forms, but all of them developed FIP spontaneously. None of the cats had been infected experimentally. With the exception of three cats, the following tissue specimens were taken from each animal: abdominal wall, omentum, liver, intestine, diaphragm, mesenteric lymph node, spleen, pancreas, kidney, lung, and brain. From two of the cats, sampling was restricted to liver and kidney, and from a third one to liver, kidney and pleura. Summing up, 282 tissues were investigated.

2.1. Histopathology and immunohistochemistry

After necropsy, histopathological and immunohistochemical examination were carried out to confirm the diagnosis of FIP. The determining factor for histopathological diagnosis was the presence of a severe, pyogranulomatous or necrotising inflammatory response with involvement of small veins.

For histopathology and immunohistochemistry, the tissues were fixed in 10% non-buffered formalin for 24 to 72 hours and were embedded routinely in paraffin wax.

For the immunohistochemical examination, paraffin slides were dried at room temperature, deparaffinised with Roti-Histol® and rehydrated in a graded alcohol series. Blocking of endogenous peroxidase was performed for a period of 30 minutes at room temperature using pure methanol with 0.5% H_2O_2. After washing in Tris buffered saline (TBS) the slides were pretreated with citrate buffer at 95 °C for 25 minutes. Afterwards, they were incubated in rat serum (10% in TBS) for 10 minutes at room temperature on Coverplates™ (Shandon Racks, Thermo Scientific, Dreieich, Germany). Incubation of the primary antibody for 12 to 18 hours at 4 °C followed. As primary antibody, FCV 3–70 (1:80 in TBS) (Custom Monoclonals Int., Sacramento, CA, USA) was used. Alternating with three washing steps in TBS each, incubation with the secondary antibody rat anti-mouse IgG (1:100) (Dianova, Hamburg, Germany) and with the mouse PAP complex (1:500) (Dianova) was carried out for 30 minutes at room temperature. Under continuous stirring, the slides were incubated in 0.05% 3,3′-Diaminobenzidintetrahydrochloride (DAB) in 0.1 M imidazole buffer (pH 7.1) with 0.01% H_2O_2 for 10 minutes at room temperature. After threefold washing in TBS and a final washing step in distilled water, the slides were incubated for 5 minutes in Kardasewitsch to remove formalin pigment. Counterstaining was performed using Papanicolaou's haematoxylin (1:10 in distilled water) for approximately 30 seconds and a 5-minute blueing in water. Finally, the slides were dehydrated in a graded alcohol series and cleared with Roti-Histol® for 10 minutes.

2.2. RNA extraction and cDNA synthesis

After necropsy of the FIP cats, tissue cubes with a size of approximately $5 \times 5 \times 5$ mm were prepared from the abdominal wall, omentum, liver, intestine, diaphragm, mesenteric lymph node, spleen, pancreas, kidney, lung and brain. Areas with grossly visible granulomas were selected where possible. The specimens were frozen in liquid nitrogen and stored at –80 °C until further preparation. For RNA isolation, Qiagen RNeasy® Mini Kit (Qiagen, Hilden, Germany) was used. Thirty milligrams of the frozen tissue samples were homogenised with a mortar and pestle, or an automatic homogeniser (Bullet Blender® Blue 50, Next Advance, Averill Park, NY, USA), respectively. The RNA isolation procedure was carried out according to the kit's instructions.

The transcription of RNA into cDNA was performed using the RT Sensiscript® Kit (Qiagen). Additionally, random hexamers (Promega, Mannheim, Germany) were used as primers, and to protect RNA from degradation an RNase inhibitor (Invitrogen, Karlsruhe, Germany) was added.

2.3. Amplification of ORF 3c

Specific amplification of ORF 3c was accomplished by polymerase chain reaction with Multi Cycler PTC 200 (Biozym, Hess. Oldendorf, Germany). Because the amplification was insufficient after a single PCR reaction, a semi-nested PCR was required. The following temperature–time scheme served as amplification protocol: unique heating at 95 °C for 2 min, 94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s and finally 72 °C for 10 min. Steps 2–4 were repeated in 40 cycles.

Published sequences of feline coronaviruses (NCBI GenBank®, accession no.: NC_007025 and EU186072) were used for the development of primers. Because of the sequence variation between serotype I and serotype II strains at the 5′-end of the 3c gene, two different forward primers (FIP1.1F and FIP2.1F), one consistent with serotype I sequence data, the other matching to serotype II were created. FIP2.1R served as reverse primer for both type I and type II strains. For the semi-nested PCR additional primers were synthesised (FIPn_T1F, FIPn_T2F, FIPn_T1R) and combined as follows with the primers of the first PCR: FIP1.1F – FIPn_T1R and FIPn_T1F, FIPn_T2F – FIP2.1R. Because of sequence variation between type I and II strains at the site of the internal forward primers, two serotype-specific primers were used (FIPn_T1F and FIPn_T2F). The sequence data of all primers are listed in Table 1.

The web-based programme GeneFisher (University of Bielefeld, Germany) was used for the selection of primers with optimal binding characteristics.

To control the process of RNA isolation and cDNA synthesis, from each sample an amplification of feline GAPDH gene was performed in parallel.

2.4. Amplification of ORF 7b

In contrast to the 3c gene, sequence variation between published serotype I and II strains in the 7b gene is not present. Thus, a single forward and reverse primer were designed (FIP7bF and FIP7bR; see Table 1). For primer development, as with the

Table 1
Sequence data of primers.

Primer	Gene	Sequence	T_m	Position	FCoV strain
FIP1.1F	3c	accttttgcagtcagattgc	55.3 °C	25065	FIPV Black
FIP2.1F	3c	aaacacacaagacccaaagc	55.3 °C	24917	FIPV 79–1146
FIP2.1R	3c	ttatcaacaggagccagaag	55.3 °C	25800/25904	FIPV 79–1146/ FIPV Black
FIPn_T1F	3c	tggttactatatagatggaattg	53.5 °C	25395	FIPV Black
FIPn_T2F	3c	tggttactacattgatggcattg	57.1 °C	25288	FIPV 79–1146
FIPn_T1R	3c	gcagctctgtcatgtacaaa	55.3 °C	25538	FIPV Black
FIP7bF	7b	ggtgtgcctgacagtagtctgcg	66.0 °C	28153	FIPV Black
FIP7bR	7b	tgcttgtacctcctattacacgtgctt	63.4 °C	29030	FIPV Black

T_m: melting temperature.

amplification of ORF 3c, published coronavirus sequences were used. The following PCR protocol was carried out: unique heating at 95 °C for 2 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and finally 72 °C for 10 min. Steps 2–4 were repeated in 35 cycles.

2.5. Agarose gel electrophoresis

Visualisation of PCR products was carried out using a 2% agarose gel with ethidium bromide (Roth, Karlsruhe, Germany) or, alternatively, Midori Green (Nippon Genetics Europe, Düren, Germany) applying a voltage of 4.5 V/cm and a maximum amperage of 70 mA. Ten microlitres of each PCR product were mixed with 2 µl loading dye (Orange 6x, Fermentas, St. Leon-Rot, Germany). For standardisation of the base pair length, 5 µl of a DNA ladder (O'GeneRuler™ 50 bp DNA ladder or pUC Mix Marker 8, respectively; Fermentas) were included in each run.

2.6. DNA sequencing and analysis of sequence data

DNA sequencing was performed by GATC Biotech (Konstanz, Germany) with DNA analyser 3730xl (Applied Biosystems, Carlsbad, CA, USA). PCR products were purified using exonuclease I and shrimp alkaline phosphatase (Fermentas) during a 30 minute incubation step in a Multi Cycler PTC 200 according to the manufacturer's instructions. The purified PCR products were used directly for sequencing.

The sequence data analysis was carried out with the help of BLAST® (NCBI, Bethesda, MD, USA), ClustalW (EMBL-EBI, Cambridge, GB), Reverse Complement® (http://www.bioinformatics.org/sms/rev_comp.html) and Bioedit (Ibis Biosciences, Carlsbad, CA, USA). For translation of nucleic acids into the corresponding amino acid sequences ExPASy translate tool (Swiss Institute of Bioinformatics, Switzerland) was used.

3. Results

3.1. Sequence data of reference strains

The comparison of sequence data of published FCoV strains exhibited differences between serotype I and II strains in ORF 3c. In ORF 7b, no serotype-specific variation could be found. As reference for the study of the FIPV isolates, a FIPV type I strain (Black, GenBank® accession no.: EU186072), a FIPV type II strain (79–1146, GenBank® accession no.: NC_007025) and, for the 3c sequences, an additional type I FECV (FECV-6, Department of Virology, University of Giessen) were used. In the 3c gene, the nucleic acid sequences of both type I strains were more closely related than those of type I and II strains, even though the Black strain was a FIPV and the FECV-6 is a member of enteric coronaviruses.

The total length of the 3c gene was 714 bases in FECV-6, 695 bases in FIPV Black – due to a deletion of 62 nucleotides between ORF 3b and ORF 3c as described in prior studies (Tekes et al., 2008; Vennema et al., 1998) – and 733 bases in FIPV 79–1146. While type II FIPV 79–1146 exhibited a length of 621 bases in ORF 7b, the type I strain Black was only 618 bases long due to a small deletion.

3.2. Nonstructural protein gene 3c

As described above, the yield of PCR product after one round of amplification was very low or undetectable in gel electrophoresis. To increase detection sensitivity, the PCR products (diluted 1:100 in distilled water) from the first PCR were used for a further amplification step. The outer primers were maintained and combined each with an internal one. The product of the first PCR (with primers FIP1.1F, FIP2.1F and FIP2.1R) was expected to be approximately 900 bp. Only one cat showed distinct bands of this size on gel electrophoresis after a single reaction procedure in 9/11 examined tissues. In 10/28 cats only faint bands were visible in between 1–6 tissues of the 11 tested tissues of each cat. Amplification products in the tissues of the remaining 17/28 cats could not be detected.

The results of the semi-nested PCR were more successful: detection of 3c sequences was possible in a higher number of cats (21/28). From these 21 cats, 171/231 tested tissues were positive (approximately 75%). In seven of the cats, amplification was not detectable in any of the tissues, even after nested PCR.

3.3. Nonstructural protein gene 7b

The size of PCR products of primers FIP7bF and FIP7bR was around 900 bp. In contrast to the low detection sensitivity of 3c sequences, distinct bands were recognisable after a single PCR procedure in 26/28 cats with positive results in 194 tissues. Additionally, successful amplification of ORF 7b was possible in six of the seven cats where amplification of ORF 3c had failed. In two of the cats, successful amplification of ORF 7b was not possible with primers FIP7bF and FIP7bR.

3.4. Immunohistochemical signal, detection of ORF 3c/7b and pathomorphological changes

Immunohistochemistry was carried out only in tissues with FIP-typical histopathological alterations. For this reason, the immunohistochemically examined tissues were not completely identical with the spectrum of organs investigated by RT-PCR. Nevertheless, from a total of 72 immunohistochemically positive tissues, 66 were tested in PCR, too. In 45/66 tissues, ORF 3c and in 62/66 tissues ORF 7b were detectable using PCR. In contrast, amplification of the 3c gene by PCR was successful in 31 and of the 7b gene in 38 tissues that were all histopathologically and immunohistochemically negative.

An association between the results of immunohistochemistry and PCR with the grossly visible FIP form (effusive or granulomatous) was not obvious. FCoV genome or antigen could be detected in both, effusive and granulomatous forms, similarly.

3.5. Frequency of 3c/7b detection in different organ systems, sensitivity of PCR

Using PCR, ORF 3c was detectable in 171/282 tissues (detection sensitivity = 61%), whereas ORF 7b was positive in 194/282 tissues (detection sensitivity = 69%). Isolates from the omentum and mesenteric lymph nodes could be successfully amplified more frequently than from the other investigated organs (omentum: 80% positive 3c and 84% positive 7b results, respectively; mesenteric lymph node: 68% positive 3c and 92% positive 7b results, respectively).

3.6. Sequencing

3c sequences of different tissues within one cat were very similar or identical, whereas isolates from different cats exhibited greater discrepancies (see Fig. 1a). If sequence aberration occurred in the isolates of one cat, PCR and sequencing were repeated.

Final evaluation of sequences resulted in the following possibilities: the sequences of all isolates of one cat were identical, ≤10 positions were not identical, >10 positions showed differences in the base pair pattern.

In two of the cats, sequencing of the full-length gene was not possible. Because of this, sequence analysis of the complete 3c gene was only feasible in 19/21 cats with successful amplification of ORF 3c. In 5/19 cats, an amplification of ORF 3c was possible in all examined tissues (55/55). In the remaining 14 cats, detection was only possible in a fraction of the examined tissue spectrum (102/154).

Beside single nucleotide polymorphism, the 3c sequences were investigated for the presence of deletions. From the 19 cats with complete sequence data from the 3c gene 11 showed deletions with a size of one up to 56 bases. The position of the deletions varied between isolates from different cats.

Comparing the 3c gene of the FIPV isolates with reference strains FIPV 79–1146 and FIPV Black, respectively, an average sequence identity of 88% to type II strain 79–1146 and of 95% to type I strain Black could be found.

Sequencing of the 7b gene revealed a high rate of identity in the isolates of one cat. Nevertheless, single nucleotide changes occurred in 12 of the cats. Comparing isolates of different cats, more variations could be found (see Fig. 1b). In 26 of the cats investigated in this study the amplification of the 7b gene was successful. Only three animals yielded amplification bands in all examined tissues. In the remaining 23 cats, 161/236 tissues were positive. All

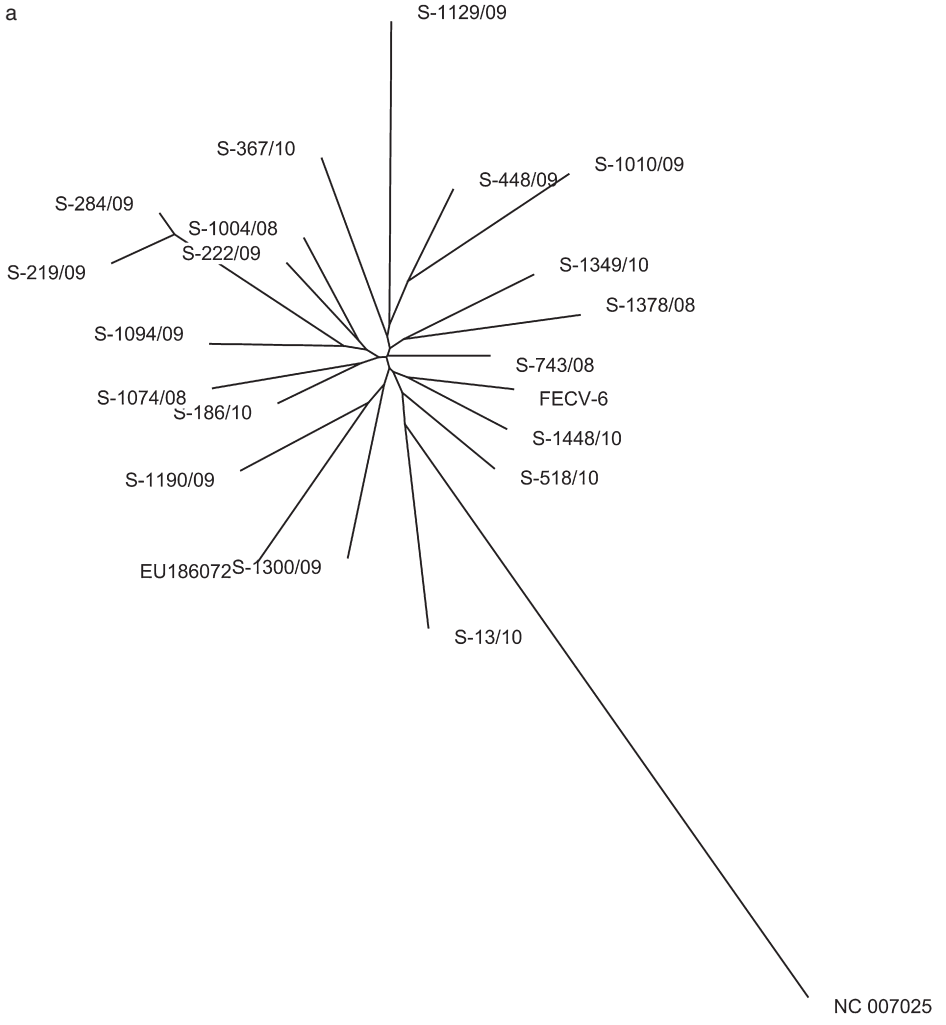

Fig. 1. (a) Phylogenetic tree to demonstrate sequence variations in the 3c gene between FCoV strains from different cats. (b) Phylogenetic tree to demonstrate sequence variations in the 7b gene between FCoV strains from different cats. (Created by ClustalW, visualised by TreeView). The further the branches of the diagram are from each other, the more variations exist between sequences of different cats. The bar length gives information about the relative number of sequence variations between adjacent strains.

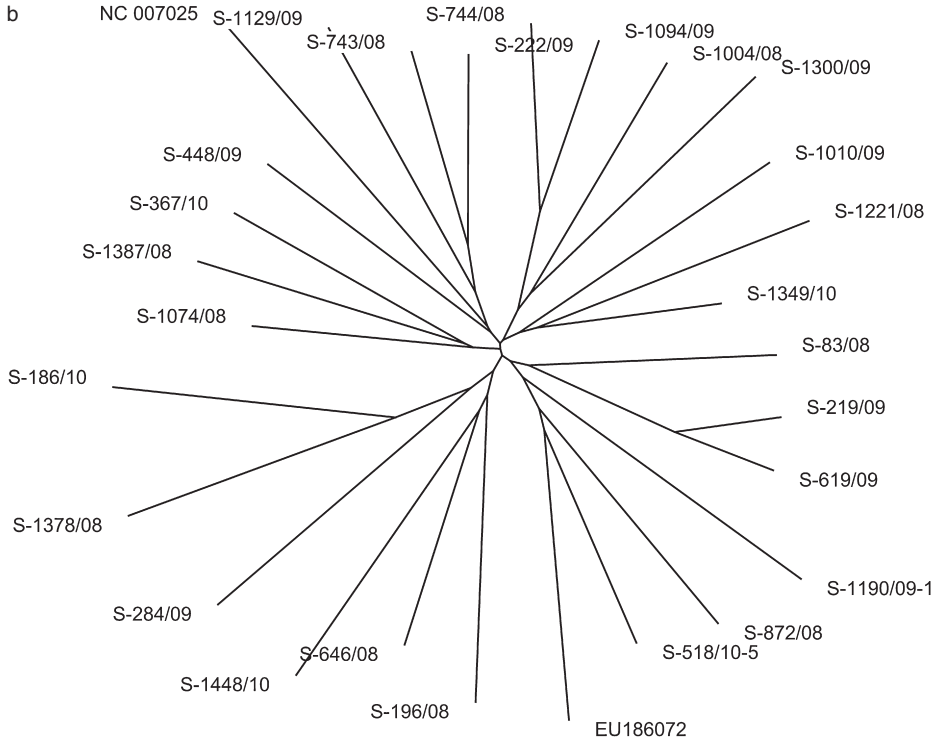

Fig. 1. (*continued*)

but one of the 26 positive cats exhibited an identical length of ORF 7b of 621 bases. Only one cat showed two deletions of three bases each resulting in a length of 615 bases.

There was no difference in the average sequence identity of the isolated 7b sequences compared to reference strains FIPV 79–1146 and FIPV Black.

3.7. Amino acid sequences

All of the 3c and 7b sequences, respectively, were translated into the corresponding amino acid sequences. In the case of ORF 3c, a 238-length amino acid sequence could be expected. This length was found in 10/19 investigated cats. In 9/19 cats, sequences of 219–237 amino acids were present. Premature stop codons occurred in sequences of 14/19 of the cats. The 7b gene encodes a protein consisting of 207 amino acids. This length was found in 25/26 7b-positive cats. The isolates of the cat with deletions exhibited a sequence of only 205 amino acids. Premature stop codons did not occur in any of the 7b isolates.

The frequency of premature stop codons in the 3c sequences of the FIPV isolates was compared with 3c sequences of 35 FECVs (NCBI GenBank®, Table 2). None of the FECV 3c sequences exhibited premature stop codons. Fisher's exact test (http://www.langsrud.com/fisher.htm) revealed a highly significant accumulation of stop codons in the 3c sequence of FIPVs compared to FECVs ($p < 0.0001$).

4. Discussion

The main difficulty in the development of an effective vaccination or treatment against FIP is the fact that the crucial step leading to a disease outbreak is still enigmatic. It is not completely understood if the virus itself is responsible for the fatal course of FCoV infection or if an individual immunological dysfunction contributes to FIP. The current understanding of the occurrence of two

Table 2
Source of FECV strains used for comparison with isolates of the present study.

Designation	ORF 3c length	GenBank® accession no./source
FECV-6	714	Department of Virologie, JLU Giessen
FECV-53	714	Department of Virologie, JLU Giessen
FECV-UCD 3a	714	FJ943761
FECV-UCD 3b	714	FJ943762
FECV-UCD 4	714	FJ943763
FECV-UCD 5	714	FJ917522
FECV-UCD 6	714	FJ943771
FCoV-RM	714	FJ938051
DSKUU113F	714	GU053613
DSKUU1F	714	GU053614
DSKUU2F	714	GU053615
DSKUU9F	714	GU053616
DSKUU25F	714	GU053617
DSKUU33F	714	GU053618
DSKUU54F	714	GU053619
DSKUU55F	714	GU053620
DSKUU101F	714	GU053621
DSKUU102F	714	GU053622
DSKUU103F	714	GU053623
DSKUU104F	714	GU053624
DSKUU123F	714	GU053625
DSKUU152F	714	GU053626
DSKUU160F	714	GU053627
DSKUU165F	714	GU053628
DSKUU166F	714	GU053629
DSKUU167F	714	GU053630
DSKUU168F	714	GU053631
DSKUU169F	714	GU053632
DSKUU174F	714	GU053633
DSKUU176F	714	GU053634
DSKUU179F	714	GU053635
DSKUU185F	714	GU053636
DSKUU193F	714	GU053637
DSKUU194F	714	GU053638
DSKUU197F	714	GU053639

different biotypes (FECV and FIPV) primarily focuses on the virus properties as a principal cause. But despite numerous investigations of the FCoV genome, no definite discrimination between FECV and FIPV on the molecular level has been described until now. Nevertheless, an involvement of nonstructural protein genes 3c and 7b is widely accepted in the literature (Chang et al., 2010; Herrewegh et al., 1995b; Pedersen et al., 2009; Vennema et al., 1998).

The selection of published FCoV strains (NCBI GenBank®) for comparison to the FIPV isolates revealed high sequence variation in the 3c and lower variation in the 7b gene. This reflects the high mutation rate of coronaviruses in general. Regarding the gene length, ORF 7b showed 621 bases except in two cell culture-adapted strains. ORF 3c exhibits 714 bases in all feline coronaviruses described as FECV. According to the hypothesis, FIPVs are thought to carry deletions in the 3c gene. This is the case in FIPV Black (acc. no.: EU186072) and an isolate from a cat with FIP (acc. no.: DQ848578) that showed a gene length of 695 and 694 bases, respectively. In contrast, FIPV 79–1146 (acc. no.: NC__007025) and another FIPV strain (acc. no.: AY994055) exhibit a gene length of 733 and 735 bases, respectively. Therefore, ORF 3c of FIPV can also be affected by insertions. These insertions, however, occurred in cell culture-adapted type II strains which potentially carry mutations that are not present in naturally occurring FIPVs.

With the exception of one cat, a semi-nested PCR was necessary for successful amplification of the 3c gene. In 7/28 cats, ORF 3c was not amplifiable, even after repeated PCR. Regarding the 7b gene, amplification was not successful in only 2/28 cats. In both genes, there was no difference in the success of detection comparing virus isolation from granulomas or tissue cubes with no grossly visible granulomas. Additionally, amplification was also possible in tissues without any histopathological lesions. This argues for the possibility of the isolation of strains systemically spread in the blood.

The absence of detectable isolates can be explained by three alternatives: The amount of FCoVs in the tissue material was only marginal. Because of the high detection sensitivity of PCR, and especially nested PCR, this possibility is rather unlikely. More likely are sequence variations in the binding sites of the primers. Furthermore, progressive degradation of the tissue material might have contributed to fragmented virus RNA.

The quasi species hypothesis describes the occurrence of many different FCoVs as so called “mutational clouds” (Battilani et al., 2003; Gunn-Moore et al., 1999; Herrewegh et al., 1997; Kiss et al., 2000). Because of the direct sequencing of purified PCR products, all potential variants are included into one sample. In the case of greater discrepancies in the base pair pattern of the isolates of one sample, a mixture of sequences or non-evaluable sequence data should be expected. Sequencing, however, yielded overall identical results. Thus, one organ/tissue specimen seemed to harbour only one predominant variant. Studies of Gunn-Moore et al. (1999), Kiss et al. (2000) and Battilani et al. (2003) discovered a high variability between strains from different organs of one cat. In those investigations, the S, N, and 7b genes were tested with predominant variation in the S and N genes and information about the 3c gene is not given.

Comparing the 3c sequences to serotype I and II reference strains, there was higher sequence identity to type I than type II strains. Thus, the isolates from the FIP cats used in this study seemed to be members of serotype I which is in accordance with the predominant type referred to in literature (Benetka et al., 2004; Hohdatsu et al., 1992; Kummrow et al., 2005; Lin et al., 2009a).

Whereas sequence data of individual cats revealed identical or very similar results, greater sequence variation was evident when isolates from different cats were compared. A similar phenomenon was previously observed by Vennema et al. (1998) who compared FCoV sequences from cats of one living group with cats from geographically distinct areas. This again reflects the high mutation rate of coronaviruses in general, because it can be assumed that with each replication new variants will be generated. The results of the present study indicate that of these newly arisen variants only one type or very few strains become manifest in one cat systemically.

The deletions found in the 3c sequences of 11/19 cats were situated at different sites and were of variable lengths from one up to 56 bases. Deletions in the 3c gene seemed to be much more frequent than in the 7b gene, in accordance with prior studies.

The fact that 3c sequences from 8/19 cats were without any deletions and thereby 714 bases long shows that deletions in ORF 3c cannot be used as a tool for the definition of a FIPV in contrast to FECV. It remains questionable if the increased frequency of mutations in ORF 3c in FIPVs is one of the primary causes for the occurrence of the fatal FIPV biotype or developed secondary during a FIP outbreak.

Vennema et al. (1998) hypothesised that the virulence of FCoV might be determined by an interaction of the nonstructural 3c and 7b genes. Whereas an intact 7b gene is thought to play a role for virulence, an intact 3c gene (which is the case in FECV) is considered to prevent the virulent potential of 7b. In case of deleted 3c (what is postulated to be the case in FIPV), inhibition of virulence encoded by 7b should not be possible. These considerations are complicated by the fact that only little is known about the function and products of ORF 3c and ORF 7b. To gain more insight into the amino acid sequences of putative proteins, all nucleotide sequences were translated. The occurrence of deletions usually resulted in disruption of the amino acid sequences. In all PCR-positive tissues of 14/19 cats with complete 3c sequence data, premature stop codons could be observed. However, not all of the nucleic acid deletions resulted in the generation of stop codons. On the other hand, stop codons also occurred in sequences without deletions, but with single nucleotide exchanges. Of the remaining 5/19 cats without premature stop codons two cats exhibited deletions in their sequences. Three out of 19 cats showed complete sequences of 714 bases and no premature stop codons. Summing up, the occurrence of premature stop codons is not inevitably associated with deletions in the nucleic acid sequence.

Even if the 7b isolate of one of the cats exhibited two small deletions in the nucleic acid sequence, none of the 7b sequences showed premature stop codons. All sequences carried a continuous reading frame.

In conclusion, deletions and stop codons in the 3c gene were frequent whereas ORF 7b did not show premature stop codons and the isolates of only one cat were affected by deletions. Nevertheless, three cats with isolates containing both intact 3c and intact 7b genes occurred in this study. Because of this, 3c and 7b genes cannot be the only factor determining the virulence potential of a FCoV strain. Presumably, mutations in other genes or a complex interaction between the feline immune system and as yet unidentified feline coronavirus properties take place. The attempt to ascribe one or several defined mutations to the FIP-causing FCoV failed until now. The results of some earlier studies in which presumable FIP-specific mutations were found in the spike or 3c gene, respectively, sounded promising (Chang et al., 2012; Pedersen et al., 2009). The present study and other research groups relativised these findings by detecting FIPV strains that do not carry those mutations (Porter et al., 2014).

ORF 7b could be detected in more cats with FIP than ORF 3c, although the latter had been amplified more intensely in a nested PCR. Generally, two mechanisms may have contributed to this result:

1 The amount of 3c RNA in the tissue specimens was lower than that of ORF 7b.
2 Identical RNA amounts of both genes were present, but amplification of ORF 7b was accomplished more successfully than ORF 3c. A possible explanation of the latter of the two hypotheses

could be that high sequence variability in the primer binding sites exists. The results gained after semi-nested PCR were 3c-specific and exhibited distinct bands on gel electrophoresis. Thus, large deletions at primer binding sites can be ruled out. The observed differences in detectability of both nonstructural protein genes therefore led to the assumption of different RNA amounts of ORF 3c and ORF 7b. During virus replication of coronaviruses several subgenomic mRNAs are synthesised (Lai and Cavanagh, 1997; Masters, 2006). Whether the isolates amplified in the present study originated from genomic RNA or mRNA is not known. Presumably, both variants were present in the tissues. Different amounts of 3c and 7b RNA can only be explained by quantitative differences in the subgenomic mRNAs because genomic RNA presumably contains all viral genes in equal parts. Different amounts of subgenomic mRNAs might be the result of a regulatory mechanism restricting the 3c mRNA synthesis or fostering degradation. Another possibility might be that the 3c gene is not translated into a corresponding protein. These considerations are speculative and have to be investigated in further studies.

Acknowledgements

The authors would like to thank Prof. Dr. Heinz-Jürgen Thiel and the Department of Virology, University of Giessen, for providing isolated FCoV RNA to test the PCR procedure. Additionally, many thanks to Dr. Werner Hecht, Department of Veterinary Pathology, University of Giessen, for his excellent technical support.

The authors are grateful to the H. Wilhelm Schaumann Stiftung for financial support.

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