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

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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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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