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SHORT COMMUNICATION
Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats

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Natural feline coronavirus (FCoV) infection has been shown to not only induce intestinal infection with viral shedding, but also systemic infection which either remains without clinical signs or leads to feline infectious peritonitis (FIP). As systemic infection is not the key event in the development of FIP, the question arises as to whether a potential difference in viral load might be of importance. Therefore, the purpose of this study was to quantitatively assess feline coronavirus (FCoV) RNA loads in haemolymphatic tissues of healthy, long-term FCoV-infected cats and cats with FIP. In cats that died from FIP, viral loads were significantly higher, indicating a higher rate of viral replication or a reduced capacity for viral clearance in cats developing and/or suffering from FIP.

Feline infectious peritonitis (FIP) is a well-known and widely distributed coronavirus (CoV)-induced systemic disease in cats, characterised by fibrinous-granulomatous serositis with protein-rich effusions into body cavities, granulomatous-necrotising phlebitis and periphlebitis and granulomatous inflammatory lesions in several organs (Hayashi et al 1977, Weiss and Scott, 1981a, b, Kipar et al 1998, 2005).

Feline CoV (FCoV) is transmitted via the faecal-oral route and infects primarily enterocytes (Pedersen 1995), but subsequently spreads systemically via a monocyte-associated viraemia (Gunn-Moore et al 1998, Kipar et al 1999, 2005, Meli et al 2004, de Groot-Mijnes et al 2005). FCoV-infected circulating monocytes are responsible for both viral dissemination and, in an activated state, development of vasculitis (Weiss and Scott 1981a, b, Pedersen 1995, Kipar et al 2005). Also, regardless of the development of FIP, FCoV infection is associated with increased numbers of (proliferating) monocytes/macrophages in haemolymphatic tissues (Kipar et al 1999, 2001a). On the other hand, it has been suspected that higher levels of viral replication could be a prerequisite for the occurrence of the virulent viral mutants among the quasispecies cloud generally seen in infected animals as a consequence of the high rate of viral mutation (Vennema et al 1998, Gunn-Moore et al 1999). Considering also the role of monocytes/macrophages as mediators of both viral replication and formation of granulomatous lesions in FIP, the question arises as to whether the long-term outcome of FCoV infection might relate to the degree of viral replication within an infected individual. We, therefore, performed a relative quantitation of FCoV RNA loads in haemolymphatic tissues in naturally FCoV-infected cats with and without FIP.

The study was performed on 15 routinely necropsied cats which had died, with or without euthanasia, due to FIP (group 1). Animals ranged from 5 months to 4 years of age and all exhibited lesions typical for FIP, namely fibrinous to granulomatous peritonitis and pleuritis, often associated with effusion, and/or granulomatous lesions in various organs, lymph nodes and the central nervous system. Brain and spinal cord

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involvement was characterised by a granuloma-tous leptomeningitis, often with granulomatous phlebitis and periphlebitis. The latter was also observed occasionally in eyes, renal cortices and lungs. Diagnosis of FIP was confirmed by immunohistological demonstration of FCoV antigen within macrophages in lesions (Kipar et al 1998). Group 2 consisted of 13 apparently healthy specific pathogen-free cats, aged 1 year, who had been housed for 30 weeks with cats developing FIP (Kipar et al 1999). All group 2 cats had shown positive results in tests for CoV antibodies, circulating FCoV-specific immune complexes and monocyte-associated FCoV vir-aemia (Kipar et al 1999).

Samples from spleen, mesenteric lymph nodes and bone marrow were collected immediately after death from all cats in areas without macroscopic FIP lesions, and frozen at $-80°C$ for RNA extraction. Additional tissue samples were fixed in 10% buffered formalin, routinely embedded in paraffin and tested by immuno-histology for the presence of FCoV antigen (Kipar et al 1998, 2005).

For RNA extraction, approximately 100 mg of tissue was homogenised with a tissue homo-geniser (PCR Tissue Homogenizing Kit, Süd-Laborbedarf GmbH, Gauting, Germany) and lysed in each 700 µl lysis buffer. Total RNA was extracted with a commercially available kit (RNeasy Mini kit; QIAGEN, Hilden, Germany) according to the manufacturer's protocol and dissolved in 30 µl RNase-free water. A one-tube real-time TaqMan-RT-PCR, detecting a conserved 102 bp fragment of the FCoV 7b gene, using primers and probes already described (Gut et al 1999, Meli et al 2004), was performed, using an automated fluorometer (ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, Weiterstadt, Germany). RNA extracts from in vitro FIPV-infected monolayers of feline embryonal cells served as positive controls. Adequate negative controls were included into each TaqMan PCR run. In parallel, cDNA was synthesised and real-time TaqMan-PCR for feline GAPDH was performed according to previously published protocols (Kipar et al 2001b).

Relative quantification of FCoV transcripts was undertaken by the comparative C_T method and is reported as relative transcription or the n-fold differences of FCoV transcripts relative to the calibrator cDNA (fGAPDH) (Kipar et al 2001b). For samples where an FCoV-RNA signal was not observed after 45 cycles, an approxi-mated C_T value was created. In order to get this value, the lowest C_T for fGAPDH obtained from the sample with the highest expression of GAPDH was subtracted from 45 (number of cycles). The resulting value was rounded to the next higher integer value. For each sample, relative quantitation of FCoV amounts was achieved by calculating the difference between the fGAPDH C_T value and the FCoV C_T value (ΔC_T). These served to both normalise for differ-ences in the amount of total nucleic acid added to each reaction and to compare the amount of FCoV RNA in the different tissue samples, and expressed as $2^{-\Delta C_T}$.

Initially, all data were assessed using descrip-tive statistics. Data analysis involved compar-isons between cats in groups 1 and 2. First, comparisons were made between groups 1 and 2 according to the organ examined (eg, bone marrow, spleen, mesenteric lymph nodes) using a Mann—Whitney test with unpaired ties. For instance, the relative virus quantitation in group 1 cats' bone marrow was compared to relative virus quantitation in group 2 cats' bone marrow. Finally, the relative virus quantitation of each organ within groups was compared using a Kruskal—Wallis analysis of variance. Signifi-cance was defined as a P-value <0.05.

In all cats with FIP (100%) and 11 of the 13 FCoV-infected cats without FIP (85%), FCoV RNA was detected in at least one of the tissues examined. In cats with FIP, test positive samples included spleen in 60% (9/15), mesenteric lymph nodes in 87% (13/15) and bone marrow in 67% (10/15). In five cases (33%), only one organ tested positive, whereas two organs were posi-tive in three cases (20%) and all organs tested positive in the remaining seven cases (47%). In FCoV-infected cats without FIP, test positive samples included spleen in 38% (5/13), mesen-teric lymph nodes in 33% (4/12) and bone marrow in 46% (6/13) each. In seven cases, only one organ tested positive, in the remaining four cases, two organs tested positive.

The relative FCoV genome content in haemo-lymphatic tissues was variable but overall significantly higher in cats with FIP than in FCoV-infected cats without FIP (bone marrow: $P = 0.0273$, mesenteric lymph nodes: $P = 0.0029$, spleen: $P = 0.0037$; Fig 1). There was no signif-icant difference in the relative virus load between the different organs in each group of animals.

FCoV antigen was not detected by immuno-histology in tissues from FCoV-infected cats without FIP and in lesion-free areas of tissues from FIP cats.
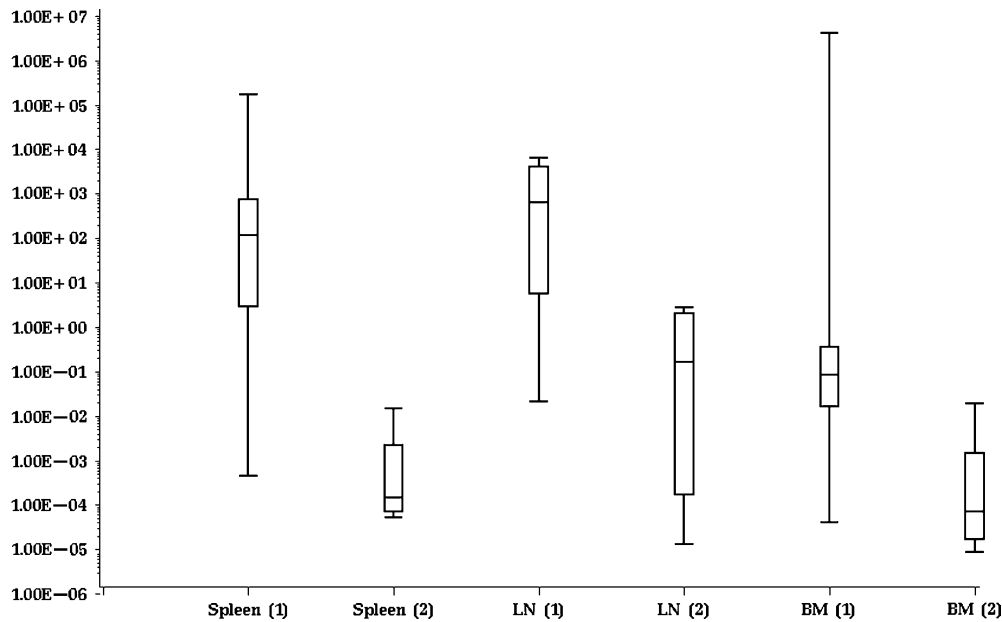
Fig 1. Relative FCoV loads in haemolymphatic tissues of FCoV-infected cats and cats with FIP (box and whisker plots). (1) = group 1: cats with FIP ($n = 15$), (2) = group 2: FCoV-infected cats without FIP ($n = 13$), BM = bone marrow, LN = lymph nodes.

This study represents the first attempt to quantify the viral load in FCoV-infected cats with and without FIP. We have chosen to examine the haemolymphatic tissues, as these represent a major site for the accumulation of monocytes/macrophages (ie, cells responsible for viral replication) (Stoddart and Scott 1989; Gunn-Moore et al 1998, Kipar et al 2005, Simons et al 2005) and likely best reflect the actual viral load.

All cats with FIP and the vast majority of FCoV-infected cats without FIP (85%) exhibited systemic infection, demonstrated by the highly sensitive real-time-RT-PCR method (Gut et al 1999). Findings confirm other studies, showing FCoV long-term, persistent viraemia and systemic spread regardless of the development of FIP (Gunn-Moore et al 1998, Kipar et al 1999, Meli et al 2004, de Groot-Mijnes et al 2005). However, our study identified generally higher viral loads in the haemolymphatic tissues of cats with FIP than in FCoV-infected cats without disease.

Previous in vitro studies provided evidence that FCoV virulence is associated with higher effectiveness of infecting and replicating in peritoneal macrophages (Stoddart and Scott 1989). This is further supported by a recent study which showed by means of a reverse transcriptase-polymerase chain reaction specific for FCoV

mRNA that replication of FCoV in circulating monocytes occurs mainly in cats with FIP (Simons et al 2005). We could show that cats with FIP have higher viral loads in spleen, lymph nodes and bone marrow, the major organs harbouring monocytes/macrophages, the cells mediating FCoV viraemia. Taken together, all these findings indicate that enhanced viral replicative capacity could be a key feature in the development of FIP. This could be characteristic for the virulent variants of FCoV which are predominantly formed within the individual infected host and characterised by deletions in genes encoding non-structural proteins of yet unknown function, which develop during replication (Vennema et al 1998, Kennedy et al 2001). On the other hand, it is possible that reduced capacity for viral clearance in cats developing and/or suffering from FIP, possibly overall or only in monocytes/macrophages, is responsible for the higher viral loads in cats with FIP. Evidence for this was provided by a recent study which observed rise in blood viral RNA levels together with immunosuppression in cats with end-stage FIP (de Groot-Mijnes et al 2005). This supports the idea that the viral loads in infected animals are a consequence of individual host factors, particularly the immune response (Herrewegh et al 1997, de Groot-Mijnes et al 2005).

Our study did not examine viral characteristics, particularly viral strains and viral mutations, in the two groups of cats. Therefore, further genetic studies are required to assess the role of viral variation and quasispecies clouds in individual and groups of cats, the replicative capacity of individual FCoV isolates and their direct impact on the outcome of FCoV infection (Vennema et al 1998, Gunn-Moore et al 1999, Kennedy et al 2001). However, together with other recent studies on the development of FIP lesions and the immunological changes associated with FCoV infection and FIP (de Groot-Mijnes et al 2005, Kipar et al 2005), this study provides another important piece of information confirming that FIP develops as a consequence of an interplay between virulent FCoVs and the immune system of the individual infected cat.

Acknowledgements

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