Sites of Feline Coronavirus persistence in healthy cats

Anja Kipar¹, Marina L. Meli², Keith E. Baptiste³, Laurel J. Bowker⁴, Hans Lutz²

1 Veterinary Pathology, School of Veterinary Science, University of Liverpool, Crown Street, Liverpool L69 7ZJ, UK
2 Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland
3 Department of Large Animal Sciences, Faculty of Life Sciences, University of Copenhagen, Højbakkegård Allé 5, 2630 Tåstrup, Denmark
4 South Beech Veterinary Surgery, 40 Southend Road, Wickford, Essex, SS11 8DU, UK

Corresponding author:
Anja Kipar
Veterinary Pathology
School of Veterinary Science
University of Liverpool
Crown Street
Liverpool, L69 7ZJ
UK
Tel. +44 151 794 4260 (Secr. 4265), Fax +44 151 794 4268
Electronic mail address: akipar@liverpool.ac.uk

Running title: Sites of FCoV persistence in healthy cats

Words (main text): 5452
Words (summary): 164 words.
Number of figures: 3
Number of tables: 2
Summary

Feline coronavirus (FCoV) is transmitted via the faecal-oral route and primarily infects enterocytes, but subsequently spreads by monocyte-associated viraemia. In some infected cats, virulent virus mutants induce feline infectious peritonitis (FIP), a fatal systemic disease that can develop in association with viraemia. Persistently infected, healthy carriers are believed to be most important in the epidemiology of FIP, since they represent a constant source of FCoV, either persistently or intermittently shedded in faeces. So far, the sites of viral persistence have not been determined definitely. The purpose of this study was to examine virus distribution and viral load in organs and gut compartments of specified pathogen-free cats, orally infected with non-virulent type I FCoV, over different time periods and with or without detectable viraemia. The colon was identified as the major site of FCoV persistence and likely source for recurrent shedding, but the virus was shown to also persist in several other organs, mainly in tissue macrophages. These might represent additional sources for recurrent viraemia.
Introduction

Feline infectious peritonitis (FIP) is a fatal disease of cats, caused by feline coronavirus (FCoV). FIP is currently the leading infectious cause of death in cats (Pedersen, 2009). Despite the generally high prevalence of FCoV infection among the cat population (up to 90% seropositive animals depending on environment and geographical area), FIP morbidity is low and rarely surpasses 5% of infected cats, mostly affecting young animals (Pedersen, 2009). This is likely due to the fact that FIP only develops with the occurrence of virulent FCoV mutants, possibly either generated within the individual infected host or externally acquired (Poland et al., 1996; Vennema et al., 1998; Brown et al., 2009; Chang et al., 2009; Pedersen et al., 2009). Two FCoV serotypes, I and II, can be distinguished; these show different geographical prevalence but, so far, no evident differences in their pathogenic potential (Kummrow et al., 2005; Lin et al., 2009). FCoV is transmitted via the faecal-oral route and infects primarily enterocytes (Pedersen, 1995). In the scientific literature, two FCoV biotypes are distinguished, Feline Enteric Coronaviruses (FECV) that are endemic in cat populations and generally not associated with clinical disease, and FIP viruses (FIPV) that might arise from endemic FECV either as an in vivo mutation or as virulent strains and are responsible for the development of FIP (Vennema, 1999; Brown et al., 2009; Chang et al., 2009; Pedersen et al., 2009). Regardless of the development of FIP, however, FCoV spreads from its initial site of infection within the intestine via monocyte-associated viraemia (Gunn-Moore et al., 1998; Kipar et al., 1999, 2005; Meli et al., 2004) and can replicate within monocytes in healthy cats (Can-Sahna et al., 2007). The pathogenesis of FIP is not fully understood, but it has been shown that a monocyte-triggered vasculitis, in association with systemic monocyte and endothelial cell activation, represents the crucial event (Kipar et al., 2005), likely in combination with some antibody-mediated enhancement and complement activation (Dewerchin et al., 2006). Cats can be FCoV carriers and commonly remain healthy...
despite systemic infection (Herrewegh et al., 1995; Addie et al., 1996; Gunn-Moore et al.,
1998; Kipar et al., 1999; Meli et al., 2004). Previous studies indicate that FCoV persists
within the intestine and is shed persistently or intermittently with the faeces (Foley et al.,
1997; Herrewegh et al., 1997; Harpold et al., 1999; Meli et al., 2004). Therefore, persistently
infected, healthy carriers are believed to play the key role in the epidemiology of FIP (Foley
et al., 1997; Meli et al., 2004).

The aim of the present study was to identify the sites of FCoV persistence in healthy carriers.
As FCoV can spread systemically within monocytes, we surmised that the virus could persist
in both intestinal and extra-intestinal sites. To investigate this hypothesis, specified pathogen-
free (SPF) cats were experimentally infected via the oral route with high doses of infectious,
non-pathogenic FCoV serotype I field isolates. Cats were examined at different time points,
between 14 and 80 days post infection (p.i.) for viraemia, viral shedding and viral loads as
well as viral antigen in selected organs.

Results

Oral infection with non-virulent FCoV serotype I field strains remains clinically inapparent
but leads to viraemia.

FCoV I is known to be the most common serotype in field cases (Hohdatsu et al., 1992;
Addie et al., 2003; Benetka et al., 2004; Kummrow et al., 2005; Lin et al., 2009). Different
from serotype II FCoV, they hardly grow in tissue culture (Jacobse-Geels & Horzinek, 1983);
however, oral administration of faeces or gut homogenates from shedding cats leads to
intestinal infection and monocyte-associated viraemia (Meli et al., 2004).

For the present study, 30 SPF cats were orally infected with different doses of previously
described non-virulent FCoV serotype I field strains (FECV biotype) prepared from faeces or gut homogenates of infected, clinically healthy cats (Acc.-Nos. DQ256137 to DQ256140; Table 1; Meli et al., 2004). The different virus isolates and infectious doses lead to similar relative viral loads in all organs tested (see below), as confirmed by an ANOVA comparison. Viraemia was confirmed by FCoV real-time RT-PCR, performed weekly and at the time of euthanasia on whole blood, plasma and/or monocytes (Meli et al., 2004). Viraemia was detected one week p.i., and most cats (20/30; 67%) were positive at one to several, often not consecutive time points, with a peak at day 7 p.i. (14/30; 47%) and decline thereafter (day 14 p.i.: 12/30 (40%), day 17 p.i.: 4/28 (14%), day 28 p.i.: 1/25 (4%), day 35 p.i.: 1/21 (5%), day 42 p.i.: 0/21, day 48 p.i.: 3/21 (14%); days 58, 65 and 80 p.i.: 0/6). No cat proved to be viraemic over the entire test period, but three showed recurrent viraemia. A total of four cats were viraemic at the time of death (day 17 p.i. (n=1), day 48 p.i. (n=3), 13.3%). There were no differences in the course and level of viraemia depending on virus isolate or infectious doses. These results show that the virus isolates generally lead to viraemia within a week after infection, which can recur at later time points.

Cats were necropsied and examined grossly and histologically to identify any potential pathological effects of the infection. There were no changes indicative of feline infectious peritonitis (FIP; Kipar et al., 2005). Histological findings were restricted to moderate lymphatic hyperplasia in spleen and mesenteric lymph nodes, some degree of lymph node sinus histiocytosis, none or minimal thymic involution and moderate to high bone marrow activity. This was consistent with previous findings in clinically healthy, FCoV infected cats and confirms that FCoV infection induces an intense systemic immune response (Kipar et al., 1999, 2001; Meli et al., 2004).

After oral infection FCoV can spread to all organs and persist in the absence of detectable
Having confirmed that oral infection did generally induce viraemia, we wanted to assess
whether all intestinal compartments become infected and where in the gut the virus
persists, and ii) whether the virus establishes itself in other organs. For this purpose, we
performed FCoV real-time RT-PCR on a large range of tissues (see below) collected
immediately after death, a time when only four cats (13.3%) were confirmed to be viraemic.

FCoV RNA was detected in organs of all animals. All organs were positive, regardless of
detectable viraemia, in at least some cats (181 positive samples; 41.9%), with the following
frequency: colon 28 (15.5%), liver 21 (11.6%), mesenteric lymph nodes 19 (10.5%), ileum 14
(7.7%), thymus 13 (7.2%), jejunum 12 (6.6%), kidney 12 (6.6%), tonsil 11 (6.1%), lung 11
(6.1%), spleen 10 (5.5%), duodenum 9 (5.0%), bone marrow 8 (4.4%), brain 6 (3.3%), skin 5
(2.8%) and skeletal muscle 2 (1.1%) (Fig. 1). Overall, the colon tested positive significantly
more frequently than any other tissue. The liver was positive significantly more often than all
other organs, except the mesenteric lymph nodes. The frequency of a test positive organ or
tissue outside the alimentary tract and the lymphatic tissues was as follows: liver 64%
(16/25), kidney 40% (10/25), lung 36% (9/25), brain 20% (5/25), skin 22% (4/18) and
skeletal muscle 11% (2/18). These findings confirm that FCoV can infect the entire intestine,
but indicate the colon as the main site of viral persistence. They also show that FCoV
viraemia generally leads to widespread organ infection and viral persistence in organs and
tissues when virus cannot be detected in the blood.

Viral shedding is consistent in the early phase of infection and seen with infection of several
intestinal compartments; the colon is the main site of viral persistence.

Having found evidence of viral persistence within the intestine, we wanted to identify any
possible association with viral shedding and tested faecal samples from all cats by FCoV
PCR over the time periods between infection and death (Meli et al., 2004). All animals started to shed on day 2 or 3 p.i. and shed relatively consistently up to day 17, followed by intermittent shedding (data not shown). Independent of the level of viral shedding, the cats remained clinically healthy, confirming the FCoV isolates as non-pathogenic FECV biotypes. Also, virus isolates and infectious dose did not have any effect on onset and duration of viral shedding (Meli et. al 2004).

At the time of death, all cats euthanased at days 14, 17 and 28 p.i. did shed virus. On days 14 and 17 p.i., colon and ileum were always positive, duodenum and jejunum each in most (4/5) cats. On day 28 p.i., duodenum, ileum and colon were always positive, jejunum in most (3/4) cats. On day 48 p.i., cats did not shed virus. Duodenum, jejunum and ileum were only positive in one, two and three of the 15 cats, respectively, but the colon yielded a positive result in 14 cats. On day 80, 50% (3/6) of cats did shed virus. While the duodenum was always negative, the jejunum was positive in three, the ileum in two and the colon in five of the six cats. One cat tested negative in the entire intestine. Comparison of relative viral loads in each intestinal compartment over the entire study identified the significantly highest loads in the colon on day 14 (Fig. 2B), and in jejunum and ileum on day 17.

We looked at the association between viral shedding and the presence of viral RNA in the different intestinal compartments at the time of death (Table 2). From the 12 cats that exhibited FCoV RNA only in the colon, only the one with the highest relative viral load was shown to shed virus. When the colon plus at least two small intestinal compartments were positive, most cats (8/10) did shed virus. The two non-shedders (day 28 and day 48 p.i., respectively) even tested positive in all intestinal compartments, but with relatively lower overall intestinal viral loads than the five shedding animals that showed viral RNA in all intestinal compartments.

Overall, relative viral loads in the colon were significantly higher (on average $1.66 \times 10^4$
[range: 11 – 1.3 x 10^5] fold higher) than in any other organ; on average 122 [range: 11 - 327] fold higher than in the other intestinal compartments. Also, there was a significant difference between positive and negative faecal shedders in terms of the cumulative viral intestinal load, whereby cats that were found to shed virus had, on average, ten times higher cumulative viral loads than cats that did not shed.

The results indicate that oral FCoV uptake leads to initial infection of all intestinal compartments. The virus is cleared frequently from all compartments but the colon at the later stages (Fig. 1) which confirms the colon as the main site of virus persistence and the source of recurrent shedding. However, virus spread to and colonisation of the small intestine appears to be essential for viral shedding, even when the colon exhibits relatively high viral loads.

At different time points post infection, the range of organs that harbour FCoV varies.

Having identified FCoV in a wide range of organs in infected cats, we evaluated whether the extent and pattern of organ infection varied over time (Fig. 1). Overall, colon, liver and mesenteric lymph nodes were positive with the highest frequency at any time point.

Cats examined on days 14, 17 and 28 p.i. all exhibited systemic infection since FCoV RNA was demonstrated in several organs. On days 14 (n=2) and 17 (n=3), 6 to 11 of the 13 organs tested were positive (average: 70.8%), with intestines and mesenteric lymph nodes positive in all cats. On day 28 (n=4), 8 to 12 of the 13 organs were positive (average: 73.1%).

On day 48 (n=15), the number of positive organs had significantly dropped (average: 26.2%; 1 to 10 of 15). In one cat, only the colon was positive, at that time point without detectable viraemia; thus, this cat was most likely not systemically infected at this stage, although it had been viraemic on days 7 and 14 p.i. (data not shown).

On day 80 (n=6), the number of organs harbouring virus increased again (average:
42.2%; 3 to 10 of 15). The one cat which did not harbour virus in its intestine (see above) was nonetheless systemically infected, since tonsils, lung and kidney were positive.

These results show that oral FCoV infection with non-pathogenic strains leads to viraemia and subsequent persistent systemic infection in the absence of detectable viraemia. There was no distinct organ infection pattern or a correlation between the number and distribution of positive organs and the presence of viraemia. The higher number of FCoV positive organs at day 80 p.i. compared to day 40 p.i. indicates that organs can become re-infected with recurrent viraemia. In general, however, at later time points (days 48 and 80 p.i.), colon, liver, mesenteric lymph nodes and tonsils were the organs positive with the highest frequency (Fig. 1).

Organs exhibit highest relative viral loads at earlier time points after infection but can remain persistently infected for a longer period.

The presence of FCoV in organs without detectable viraemia indicates that the virus can infect and persist in other cells apart from monocytes and enterocytes. As a basis to identify candidate host cells, we identified the organs with the relative highest viral loads by means of FCoV real-time RT-PCR. In general, average relative viral loads varied considerably (Fig. 2A). However, there were significant differences in overall relative viral loads between the different time points after infection (14, 17, 28, 48 and 80 days p.i.; p = 0.002) and Tukey's grouping for multiple comparisons identified significant differences in cats euthanized at days 14 and 17 compared to other time points, but not significantly different from each other. Thus, there was a trend for higher viral recoveries at 14 and 17 days p.i. compared to the later time points. For example, viral recoveries at day 14 were on average 41 (range: 1.1 – 152) fold higher than at other time points. Day 17 viral recoveries were 49 (range: 3.9 – 138) fold higher than days 28, 48 and 80 recoveries.
There were significant differences between viral recovery and the organ examined (p<0.001). Particularly, the colon and jejunum showed significantly higher viral recoveries than all other organs but not from each other. When the jejunum with a particularly high viral load (day 17 p.i.) was excluded the colon showed the significantly highest loads. An organ group comparison on the lymphatic tissues revealed a significantly higher relative viral load in mesenteric lymph nodes compared to other lymphatic tissues [e.g. spleen (p = 0.021; 25-fold higher), tonsil (p = 0.017; 74-fold higher), thymus (p = 0.016; 146-fold higher)]. These findings indicate viral spread from the intestine to the regional lymph nodes via the lymphatics, with only limited further spread. No other organ group comparison revealed significant differences. Relative viral loads within the liver, which was the organ with the second highest frequency of test positives, were not particularly high, and on average lower than, for example, in the lungs (9.3-fold lower) (Fig. 2A).

We compared relative viral loads in selected organs (liver, mesenteric lymph nodes, lung) and the colon over time. Relative viral loads in the colon and liver were significantly highest on day 14. For the mesenteric lymph nodes, day 14 displayed significantly higher viral loads than days 48 and 80, but not days 17 and 28 (Fig. 2B). The lung showed no significant differences in viral loads over time. These results reveal a general peak in organ virus loads in the first weeks after infection and ebbing off with time, similar to the intestine.

Viral persistence is mediated by columnar epithelial cells in the colon and tissue macrophages in other organs.

FCoV is known to infect intestinal epithelial cells (Pedersen, 1983; Herrewegh et al., 1997; Kipar et al., 1998). In an attempt to identify the cells in which FCoV persist in the gut, we performed immunohistology for FCoV antigen (Kipar et al., 1998, 2005) on all intestinal compartments (FCoV-RNA-positive or -negative) of the 15 cats euthanized at day 48 p.i.
without evidence of virus shedding. FCoV antigen was detected in epithelial cells of the colon in two animals. Staining was restricted to several columnar epithelial cells on the mucosal surface in one animal (Fig. 3A) and a single columnar epithelial cell on the mucosal surface of the second. In both cats the colon had been the only intestinal compartment tested positive for FCoV RNA, with comparatively high relative viral loads. These results confirm the colonic columnar epithelial cells as the site of viral persistence in the intestine of non-shedding cats.

Circulating monocytes mediate FCoV viraemia and are responsible for the granulomatous vasculitis and focal granulomatous lesions in organs that are characteristic for FIP and harbour virus in macrophages (Kipar et al., 2005). Having demonstrated viral RNA in organs regardless of detectable viraemia, we hypothesised that the virus can also infect resident tissue macrophages and performed immunohistology for FCoV antigen on some tissues that had exhibited comparatively high relative virus loads or were consistently FCoV RNA-positive, the mesenteric lymph nodes since they drain the intestines, as well as liver and lung, which both contain specific tissue macrophages in constant contact with blood, i.e. hepatic Kupffer cells and pulmonary intravascular macrophages (Brain et al., 1999; Bilzer et al., 2006). FCoV antigen was detected in several sinus macrophages in the mesenteric lymph nodes with the highest relative viral load (day 28 p.i.), in association with intense sinus histiocytosis with several macrophage aggregates (Fig. 3B). In those with the 2nd and 4th highest viral loads (day 14 and 17 p.i. respectively), each one sinus macrophage was found to express viral antigen. All three cats exhibited high viral loads within all intestinal compartments and virus shedding which suggests virus uptake by macrophages in the intestine and subsequent transport to the regional lymph nodes. Among the three examined lungs was one (day 28 p.i.), where viral antigen was observed in scattered pulmonary intravascular macrophages (Fig. 3C). FCoV antigen was not demonstrated in the livers,
suggesting that the amount of virus per cell was too low to be detected by immunohistology, a relatively insensitive method compared to in particular the real-time RT-PCR.

Discussion

The present study investigated SPF cats that had been orally infected with high doses of non-virulent FCoV type I field strains, for evidence of systemic viral infection, virus persistence and viral shedding in relation to viraemia over a period of up to 80 days after infection.

All animals remained clinically healthy. Initially, viral RNA was found in all intestinal compartments, the blood and several organs of most animals, confirming viraemic spread and systemic infection.

In the intestine FCoV was detected most consistently and at highest levels in the colon. Virus shedding, however, was generally observed when both colon and small intestine harboured virus, with significantly higher cumulative intestinal viral loads, and in the early phase after infection. It was occasionally seen also at later time points and in cats with recurrent shedding. These findings suggest that the virus persists in the colon from which it can re-infect the small intestine at any time. The immunohistological demonstration of viral antigen within columnar epithelial cells in the colon of cats that did not shed virus, but harboured FCoV RNA in the intestine confirms the colonic enterocytes as the major site of FCoV persistence in the gut (Herrewegh et al., 1997). Presence of viral antigen in intestinal epithelial cells without evidence of cell loss and faecal shedding suggests that non-virulent FCoV persist by establishing a non-lytic infection in epithelial cells, different from FCoV that induce enteritis in cats with enterocyte degeneration (Kipar et al., 1998). With regard to persistence in epithelial cells, FCoV shows similarities to foot-and-mouth disease virus and
Coxsackievirus B3 (Harrath et al., 2004; Zhang & Alexandersen, 2004). The question arises as to how the colonic columnar epithelium remains infected, considering that viral antigen appears only to be present in the superficial epithelium, which is sloughed off in the course of normal epithelial turnover, and not within replicating cells. Cell-to-cell spread between epithelial cells may be the mode of transmission to ensure persistence. Furthermore, the mechanism of FCoV entry into monocytes/macrophages within the gut has so far not been identified, or if it occurs within specific intestinal compartments. The latter seems unlikely since resident macrophages are generally numerous throughout the intestine (Platt & Mowat, 2008).

The mesenteric lymph nodes were shown to frequently harbour FCoV RNA, and at relatively high levels. Considering that some also exhibited viral antigen within sinus macrophages, it appears very likely that virus from enterocytes is taken up by macrophages in the intestinal mucosa and is transported to the regional lymph nodes. From there, further spread via the lymphatics and ultimately the blood, as a potential additional mode of viral distribution, is possible.

We also identified FCoV RNA in a large range of organs and tissues, which must be a consequence of systemic viral spread via the blood, i.e. monocyte-associated viraemia, at some point. In viraemic cats, organs could harbour viral RNA in infected monocytes within the vasculature. In addition and in the absence of detectable viraemia, however, infection of parenchymal cells and/or resident macrophages has to be considered. Previous in vitro studies showed that FCoV can infect specialised macrophages, such as peritoneal macrophages (Stoddard & Scott, 1989). Indeed, we were able to identify viral antigen in scattered pulmonary intravascular macrophages (PIM) in a non-viraemic cat with a relatively high viral titre in the lung. PIM, like hepatic Kupffer cells (HKC) are bone marrow derived macrophages that form part of the mononuclear phagocyte system. They are resident
macrophages anchored to endothelial cells within pulmonary capillaries. Together with HKC, the resident macrophages of the liver that adhere to endothelial cells in the sinusoids, PIM have been shown in cats to be the main cells to rapidly phagocytose particles carried in the blood (Brain et al., 1999). PIM have been identified as target cells for viruses, such as classical swine fever virus, porcine reproductive and respiratory syndrome virus and African Horse Sickness virus, in acute infections (Thanawongnuwech et al., 1997; Carrasco et al., 1999, 2001), while HKC can become infected with human and feline immunodeficiency virus and African swine fever virus (Bingen et al., 2002; Ciborowski & Gendelman, 2006; Sanchez-Cordon et al., 2008). Although we were not able to demonstrate FCoV antigen within HKC, HCK may be responsible for the presence of FCoV RNA in the liver particularly of non-viraemic cats. This is supported by a previous study which provided ultrastructural evidence of FCoV in HKC in cats that had developed FIP after intra-peritoneal application of highly virulent FIPV (Pedersen, 1976). Consequently, PIM and HKC could represent sources of recurrent viraemia in FCoV infected cats, via release of virus into the blood and/or transmission to monocytes. In acute African swine fever, PIM have been shown to become activated (Carrasco et al., 2002) and it needs to be determined whether in FCoV infection PIM, due to their location in pulmonary capillaries, can contribute directly to the development of the granulomatous vasculitis that is often seen in the lung in cats with FIP (Kipar et al., 2005). FCoV was demonstrated in all organs/tissues tested both in viraemic cats and cats without detectable viraemia. The number of infected organs and overall viral loads were significantly higher in the early phase after infection (days 14 and 17). However, over time, most animals remained systemically infected and FCoV persisted in several organs, although in a more sporadic manner and with evidence of re-infection in the course of intermittent viraemia. The latter is a well known feature of FCoV infection (Foley et al., 1997a; Herrewegh et al., 1997;
Harpold et al., 1999; Meli et al., 2004) and is also suggested by our real-time RT-PCR results on the blood. Alternatively, infected cats might exhibit highly variable blood viral loads over time which drop below detection levels in several cases. In the present study, beside the mesenteric lymph nodes, the liver was shown to harbour the virus most frequently and at comparatively high levels. This finding is not surprising considering that the liver directly drains the blood from the intestines via the portal vein. Also, HKC, should they indeed become infected with FCoV, are very numerous in the liver and represent 80-90% of tissue macrophages in the body (Bilzer et al., 2006), which renders them a potential source of high viral burdens in the liver. The presence of FCoV RNA in tissues without specialised vessel-associated resident macrophages, such as the skeletal muscle, the brain or the skin, in non-viraemic cats cannot readily be explained. However, circulating monocytes have been shown to leave the blood stream and differentiate to a large proportion into long-lived tissue macrophages (Randolph et al., 1999). It might therefore be possible that FCoV-infected monocytes migrate into any tissue where they then differentiate into (persistently infected) resident macrophages.

In conclusion, our data identifies the colon as the major site of FCoV persistence and provides convincing evidence that the differentiated columnar epithelial cell is the target cell of persistence. However, our results also identify other organs/tissues as sites of persistent infection and potential sources for recurrent viraemia. Organs with specialised resident macrophages that either directly drain the gut, such as the mesenteric lymph nodes, or serve to filter the blood, such as liver (HKC) and lung (PIM), are the main candidates for this. These findings imply that clearance of the virus from the gut does not necessarily protect FCoV-infected cats from recurrent viraemia and, ultimately, the development of FIP at some stage.
Methods

Virus preparation

For experimental infections, FCoV serotype I field strains isolated from faecal samples of naturally infected cats (FCoVZu1 (Acc. No. DQ256137), FCoVZu2 (Acc. No. DQ256138), FCoVZu3 (Acc. No. DQ256139) and FCoVZu5 (Acc. No. DQ256140)) or from the intestines (gut homogenates) of cats experimentally infected with FCoVZu1 (NCBI Acc. No. DQ256137) were used. All isolates have been shown to have similar effects with regard to infectivity, induction and rate of viral shedding, development of viraemia and pathological effects (Meli et al., 2004).

Briefly, faecal samples were prepared by dilution in RPMI 1640 medium containing L-glutamine and 10% fetal calf serum, incubation for 10 min at 4°C with occasional shaking, and centrifugation (2 x 10 min at 900 x g). Gut homogenates were prepared from snap-frozen intestinal segments of kittens experimentally infected with the FCoVZu1 strain, using an Ultra Tourax instrument (Kinematica AG, Lucerne, Switzerland) at 20,000 rpm for 15 min at 5-6 intervals. Faecal supernatants and intestinal homogenates were analysed to determine the FCoV load (RNA copy numbers; see below; Meli et al., 2004).

Animals

In total, 30 specified pathogen-free (SPF) kittens were provided by IFFA-Credo (Saint-Germain sur l’Arbresle, France) or Liberty Research Labs (Waverly, NY, USA) at either six or 16 weeks of age. The animals were first acclimatized by keeping them together for four days and later separated into groups (Table 1). After infection, all cats underwent daily clinical examinations. At the end of each experiment, all animals were euthanized and
Experimental infection

Experimental studies were officially approved by the Swiss veterinary office (66/2000). Cats were kept in groups under optimal ethological conditions. Each animal was infected orally twice within 24 h, under anaesthesia, by application of 2 ml of the total infectious dose using an oesophageal tube. Different virus isolates, infection doses and formulations were used (Table 1).

Sample preparation for determination of viraemia, faecal shedding and viral loads

Viral shedding was identified from purified viral RNA extracted from faecal samples collected during the examination period and immediately after death. Viraemia was detected from purified viral RNA extracted from whole blood (cats infected for 14, 17, 21 and 28 days) and monocytes (cats infected for 48 days) every 7th day p.i. and the day of euthanasia as described previously (Meli et al., 2004).

For RNA isolation from tissues, samples were collected from the duodenum, jejunum, ileum, colon, mesenteric lymph nodes, liver, spleen, bone marrow, kidney, thymus, lung, tonsil and brain (frontal cortex), as well as from skin and skeletal muscle (cats euthanased 48 and 80 days p.i. only), under sterile, RNase-free conditions and frozen directly in liquid nitrogen. The tissues were then stored at -80°C. RNA was purified starting with about 30 mg of frozen tissue by means of the ABI Prism™ 6700 Automated Nucleic Acid Workstation (Applied Biosystems (ABI), Rotkreuz, Switzerland) (Meli et al., 2004). As a control for the efficacy of RNA purification, the RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) and the expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was measured by real-time PCR.
(Leutenegger et al., 1999).

Tissue processing for histological examination

At necropsy, samples were collected from the duodenum, jejunum, ileum, caecum (with caecal lymph nodes), colon, rectum, spleen, mesenteric lymph nodes, tonsils, bone marrow and thymus as well as liver, kidneys, lung and brain. Tissues were fixed in 10% non-buffered formalin and routinely embedded in paraffin wax. Sections (3-5 μm) were cut and stained with haematoxylin-eosin or processed for immunohistological examination.

Determination of viral loads

Relative FCoV viral loads were determined by one-tube real-time RT-PCR, using an automated fluorometer (TaqMan, ABI 7700, Applied Biosystems) to detect a 102 bp fragment of the conserved FCoV 7b gene (Gut et al., 1999). The measured expression of the house-keeping gene GAPDH was used to normalize the extracted RNA to the same cell number in all samples and the viral loads were recalibrated to the GAPDH content in the respective tissues. For samples where an FCoV signal was not observed after 45 cycles, a plausible viral load was created. This value was ascertained from the lowest RNA signal for GAPDH found in this study. Thus, a test result in an organ was defined as positive with a viral load value of > 0.000000000027776.

The infectious doses for the experimental infection were calculated as the RNA copy number present in the volume of the infectious inoculum given to the cats (Table 1). RNA copy number was determined based on a standard RNA template (Gut et al., 1999).

Immunohistological demonstration of FCoV antigen in tissues

The intestines (duodenum, jejunum, ileum, caecum, colon, and rectum) from cats euthanized
at day 48 p.i. (5.1-5.15) and the seven livers (cats 1.1 and 1.2 (day 14 p.i.), 3.1 and 4.2 (day 28 p.i.), 5.4, 5.14 and 5.15 (day 48 p.i.)), ten mesenteric lymph nodes (cats 1.1 and 1.2 (day 14 p.i.), 2.1 - 2.3 (day 17 p.i.), 3.1, 4.1 and 4.2 (day 28 p.i.), 5.4 and 5.14 (day 48 p.i.)) and three lungs (cats 1.1 (day 14 p.i.), 2.3 (day 17 p.i.) and 4.1 (day 28 p.i.)) with the highest relative FCoV viral loads were examined for the presence of FCoV antigen by immunohistology, using a mouse monoclonal antibody (clone FCV3-70, Custom Monoclonals Int., West Sacramento, USA) as described previously (Kipar et al., 1998, 1999, 2001, 2005).

Statistical analysis

All statistics were performed using SAS 9.1 computer software (SAS Institute, Cary, NC, USA). Statistical associations were explored between the viral loads in cats after infection and characteristics of the experiment (e.g. dose of virus, viral load in organs and days from challenge to euthanasia). Initial assessment of the data was done using descriptive statistics. Comparisons between the two groups of cats were done using a Mann-Whitney test with unpaired ties. Comparisons between organs (i.e. more than two groups) were done using a Kruskal-Wallis analysis of variance. For instances of four or more group comparisons, then an additional statistical test (e.g. two-way analysis of variance and multiple comparisons using Tukey’s test for unequal groups) was also employed and results were compared. Comparisons between the frequency of positive organs was performed using a Fisher’s exact test. The exact p-values for all non-parametric statistics were calculated using Monte Carlo estimation.
Acknowledgements

The authors would like to gratefully acknowledge Armin Rüdimann, Claudia Müller, Karoline Jenal and Nicole Borel for expert assistance with the cats as well as Enikő Gönczi, Edith Rhiner, Elizabeth Rogg, Beatrice Weibel and Anne Griffiths for excellent technical support. They are grateful to Prof James P Stewart, University of Liverpool, for expert advice and discussions. The study was performed using the logistics of the Centre for Clinical Studies at the Vetsuisse Faculty of the University of Zurich and the Histology Laboratory, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool.
References


Path 121, 25-38.


tissues from cats without feline infectious peritonitis (FIP), cats with FIP virus infection but no FIP, and cats with no infection. *J Comp Path* **125**, 182-191.


323-335.


**Table 1:** Groups of cats, infectious doses and time points of euthanasia after oral infection with FCoV serotype I strains

<table>
<thead>
<tr>
<th>Cat Group and No.</th>
<th>Virus strain and origin</th>
<th>Infectious dose (RNA copy numbers)</th>
<th>Day post infection of euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1, 1.2</td>
<td>FCoVZu1 (FE*)</td>
<td>$8 \times 10^5$</td>
<td>14</td>
</tr>
<tr>
<td>2.1-2.3</td>
<td>FCoVZu1 (FE)</td>
<td>$8 \times 10^6$</td>
<td>17</td>
</tr>
<tr>
<td>3.1, 3.2</td>
<td>FCoVZu2 + Zu5 (FE*)</td>
<td>$1 \times 10^5$</td>
<td>28</td>
</tr>
<tr>
<td>4.1, 4.2</td>
<td>FCoVZu3 (FE*)</td>
<td>$3 \times 10^5$</td>
<td>28</td>
</tr>
<tr>
<td>5.1-5.15</td>
<td>FCoVZu1 (GH)</td>
<td>$1.5 \times 10^7$</td>
<td>48</td>
</tr>
<tr>
<td>6.1, 6.2</td>
<td>FCoVZu1 (GH)</td>
<td>$3 \times 10^7$</td>
<td>80</td>
</tr>
<tr>
<td>7.1, 7.2</td>
<td>FCoVZu1 (GH)</td>
<td>$3 \times 10^8$</td>
<td>80</td>
</tr>
<tr>
<td>8.1, 8.2</td>
<td>FCoVZu1 (GH)</td>
<td>$3 \times 10^7$</td>
<td>80</td>
</tr>
</tbody>
</table>

FE* - faecal extract from naturally infected cat; FE - faecal extract from experimentally infected cat; GH - gut homogenate (Meli et al., 2004)
**Table 2:** Correlation between viral shedding (faecal swabs, detection of FCoV by real-time RT-PCR) and the detection of FCoV RNA (real-time RT-PCR) in different intestinal compartments of FCoV serotype I infected, healthy cats at the day of euthanasia (14 to 80 days post infection)

<table>
<thead>
<tr>
<th>Intestinal compartments positive for FCoV RNA (n)</th>
<th>Faecal swab: FCoV RNA positive</th>
<th>Faecal swab: FCoV RNA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (2)</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Colon (12)</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Colon, jejunum (2)</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Colon, ileum (2)</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Colon, jejunum, ileum (3)</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Colon, duodenum, ileum (2)</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Colon, duodenum, jejunum, ileum (7)</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure Legends:

**Fig. 1:** Detection of FCoV RNA (real-time RT-PCR) in organs of FCoV serotype I infected, healthy cats sacrificed at different time points after infection (days 14, 17, 28, 48 and 80 post infection; post mortem tissue samples).

**Fig. 2:** Average relative viral loads (in relation to GAPDH) in organs of healthy cats after experimental oral infection with FCoV type I strains for 14 to 80 days. **A.** Box and whisker plots demonstrating overall average relative viral loads in the different organs tested at 14 to 80 days post infection. **B.** Box and whisker plots demonstrating overall average relative viral loads in selected organs at days 14, 17, 28, 48 and 80 after infection. Y-axis: Viral load relative to GAPDH.

**Fig. 3:** Demonstration of FCoV antigen in organs of healthy SPF cats after oral infection with FCoV serotype I strains. **A.** Cat 5.2. Day 48 p.i.. Colon. Several intact superficial columnar epithelial cells express FCoV antigen (arrows). **B, C.** Cat 4.1. Day 28 p.i.. **B.** Mesenteric lymph node. Several sinus macrophages express FCoV antigen (arrows). **C.** Lung. Individual cells within capillaries (arrows; pulmonary intravascular macrophages) express FCoV antigen. Peroxidase anti-peroxidase method. Papanicolaou’s haematoxylin counterstain.