Recombination of *Feline calicivirus* within an endemically infected cat colony

K. P. Coyne, F. C. Reed, C. J. Porter, S. Dawson, R. M. Gaskell and A. D. Radford

Departments of Veterinary Clinical Sciences and Veterinary Pathology, University of Liverpool Veterinary Teaching Hospital, Leahurst, Chester High Road, Neston, South Wirral CH64 7TE, UK

To understand the evolution of the family *Caliciviridae*, the persistence of *Feline calicivirus* (FCV) was studied within an endemically infected cat colony. Polymerase and capsid sequences were analysed for 34 FCV isolates obtained over a 4 year period. Initially, the colony was infected with one strain of virus, but a second distinct strain was later identified. Subsequently, the emergence of a recombinant virus was observed, containing elements of both of the strains circulating within the colony. The recombination event mapped close to the ORF1/ORF2 junction. This is consistent with recombination in other caliciviruses, suggesting a common mechanism within this family. This is the first report of recombination within the genus *Vesivirus* in the family *Caliciviridae* and the first time that a recombination event has been observed where the parental strains have also been identified.

The family *Caliciviridae* comprises an important group of human and animal RNA pathogens, causing a range of diseases including acute gastroenteritis in humans and vascular disease in animals. Members of the family *Caliciviridae* have a single-stranded, positive-sense RNA genome encoding the non-structural proteins (NSPs) at the 5' end and the major structural capsid protein (CAP) towards the 3' end (Clarke & Lambden, 1997). The family comprises four well-defined genera: *Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus*. In noro- and vesiviruses, the NSPs and CAP are expressed from separate open reading frames, ORF 1 and ORF 2. In contrast, in sapo- and lagoviruses, the NSPs and CAP are expressed from a single ORF. In addition to the genomic RNA, the CAP is expressed from a 3' co-terminal subgenomic RNA (Neill et al., 1991).

There is considerable antigenic and genetic diversity both among and within the four calicivirus genera. This diversity has led to the emergence of new variants, some of which are highly transmissible, such as human norovirus strain GII.4 (Noel et al., 1999; Lopman et al., 2004), and others with increased virulence, such as *Rabbit hemorrhagic disease virus* and virulent systemic *Feline calicivirus* (FCV) (Moss et al., 2002; Hurley & Sykes, 2003). However, the mechanisms underlying the generation of such diversity and the evolution of more virulent strains generally are not well understood.

FCV provides a useful model for studying the evolution and diversification of the family *Caliciviridae* in a natural host population. FCV belongs to the genus *Vesivirus* and is a highly infectious oral and respiratory pathogen of domestic cats (Gaskell et al., 2004). More recently, virulent strains of FCV that cause systemic febrile disease with high mortality have been identified following isolated outbreaks in groups of cats (Pedersen et al., 2000; Schorr-Evans et al., 2003; Hurley et al., 2004; Coyne et al., 2006). In each outbreak, these virulent viruses appeared be genetically distinct and to have evolved independently.

Infection with FCV does not invoke a sterilizing immune response and therefore may result in persistent infection in the host (Povey et al., 1973). Within such persistently infected cats, the virus CAP has been shown to evolve by a process of immune-mediated positive selection, and it has been suggested that this enables the virus to escape the host’s immune response (Kreutz et al., 1998; Radford et al., 1998). Within endemically infected colonies, we have shown that this evolution leads, over time, to a high level of virus diversity (Radford et al., 2003).

Although immune-mediated positive selection is important in generating FCV diversity, other possible mechanisms have not yet been identified. This is in contrast to members of the genera *Norovirus* and *Sapovirus*, where analysis of both RNA-dependent RNA polymerase (POL) and CAP sequences has shown homologous (copy-choice) recombination at the junction between the NSP and CAP regions of the genome (Jiang et al., 1999; Katayama et al., 2004; Oliver et al., 2004; Bull et al., 2005). Homologous recombination
occurs during virus replication when the viral polymerase switches from the RNA template of one virus to another during a mixed infection; the resulting product usually retains full functionality and contains elements of the genetic sequences of both parental viruses (Lai, 1992; Worobey & Holmes, 1999). However, within the family *Caliciviridae*, the immediate parental strains of such recombinants have not been identified and the epidemiological circumstances in which they are generated have not been defined. In this paper, we report the identification of a recombinant FCV in a cat colony in which the parental strains were also present.

Partial POL and CAP sequences for 34 FCV isolates were obtained from a 30-cat household over a 4 year period. Within this colony, the prevalence of FCV ranged from 9 to 65% (Coyne, 2005). Briefly, viruses were isolated and passaged no more than twice in feline embryo cells line A or Crandell–Reese feline kidney cells (European Collection of Cell Cultures). RNA was extracted (QIAmp Viral RNA Mini kit; Qiagen) and transcribed into cDNA (Superscript III; Invitrogen) by using primer Percp2 (Table 1) according to the manufacturers’ instructions. A 529 nt region of the CAP gene containing immunodominant regions C–E (Neill, 1992; Seal et al., 1993) was amplified by using Pfu DNA polymerase (Stratagene) according to the manufacturer’s instructions. Each 50 μl reaction contained 2 μl cDNA and 100 ng each of primers Percp1 and Percp2 (Table 1). Thermal-cycling conditions consisted of DNA denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s and primer extension at 72 °C for 90 s. A final extension was performed at 72 °C for 5 min. In addition to this CAP region, a 486 nt region corresponding to the 3’ end of the POL region of the FCV genome was amplified from the same cDNA template by using ReddyMix (ABgene), according to the manufacturer’s instructions, in 50 μl reactions containing 100 ng each of primers M13-53D and T7-33D (Sommerville, 2001). Thermal-cycling conditions consisted of DNA denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min and primer extension at 72 °C for 3 min. A final extension was performed at 72 °C for 5 min. Both POL and CAP amplicons were purified (QIAquick PCR Purification kit; Qiagen) and sequenced bidirectionally by using M13/T7 and PCR primers, respectively (Table 1) (ABI Prism BigDye Terminator v3.0 Cycle Sequencing kit; Applied Biosystems) according to standard protocols. Consensus sequences were analysed by using programs available in the GCG package (Devereux et al., 1984).

Phylogenetic analysis showed that all of the viruses belonged to one of two clusters, designated the Major and Minor strains (Fig. 1). These were defined based on a previous analysis suggesting that distinct strains of FCV are >20% divergent in the CAP region amplified (Radford et al., 2000). Using CAP phylogeny, 28 of 34 sequences grouped within the Major strain, with the remaining six grouping within the Minor strain. In contrast, based on POL phylogeny, 32 of 34 sequences grouped within the Major strain, with only two grouping within the Minor strain. The discrepancy was associated with four FCV isolates for which the POL sequence was consistent with the Major strain viruses, whilst their CAP sequence was congruent with the Minor strains (Fig. 1). This finding suggested that recombination had occurred between the two circulating Major and Minor strains. However, as the POL and CAP sequences were obtained from independent amplicons, the existence of a recombinant FCV could not be proved conclusively by these findings.

In order to characterize this potential recombination event further and to rule out the possibility that it was an artefact, five of these isolates were selected for further analysis. These consisted of three Major viruses (V024, V037 and W104), one Minor virus (S298) and one putative recombinant (W112) (Fig. 1). RNA was isolated as described previously and transcribed by using SuperScript III and random hexamers according to the manufacturer’s instructions. A 2168 nt region of the FCV genome, spanning both the POL and CAP regions sequenced previously, was amplified by using 100 ng each of primers M13-53D and Percp2 and Extensor Hi-Fidelity PCR Master Mix (ABgene) in 50 μl

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Binding site (nt)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percp1</td>
<td>CCGTTTGTGTTTCAAGCAACCG</td>
<td>6406–6428</td>
</tr>
<tr>
<td>Percp2</td>
<td>CCTACCTATACCCGGGTGAACCC</td>
<td>6934–6913</td>
</tr>
<tr>
<td>M13-53D</td>
<td>CAGGAAACACGTATACGAGAYATGATAGACYTAYGGKGAYGAYGG</td>
<td>4766–4791</td>
</tr>
<tr>
<td>T7-33D</td>
<td>TAATACGACTCTTATAGGGCGCGCGYTCACCACRCCRTTARAYTG</td>
<td>5252–5228</td>
</tr>
</tbody>
</table>

*Nucleotide numbers of the binding sites relate to FCV vaccine strain F9 (GenBank accession no. M86379) (Carter et al., 1992).*
reactions, according to the manufacturer's instructions. PCR thermal-cycling conditions comprised 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s and primer extension at 68 °C for 5 min. Amplicons were purified and sequenced bidirectionally by using the PCR primers and subsequent primer walking. Consensus sequences were assembled by using ChromasPro v1.32 (Technelysium Pty) and the putative recombinant W112 was compared with the Major and Minor viruses and with the vaccine strain F9 (GenBank accession no. M86379) by using GCG and SimPlot (Lole et al., 1999).

The results of the SimPlot analysis are shown in Fig. 2(a). This analysis clearly illustrated that the ORF1/POL region of W112 was highly similar to the Major strain viruses (V024, V037 and W104), but very different from the Minor strain (S298). In contrast, the ORF2/CAP region had a high percentage similarity to the Minor virus (S298) alone. Analysis of the similarity suggested that a recombination event had occurred between nt 5227 and 5373, in a highly conserved region spanning the ORF1/ORF2 junction and including the start of the subgenomic mRNA (Fig. 2b). This finding was supported by bootstrap analysis (Fig. 2c), which suggested that the recombination site was close to the ORF1/ORF2 junction.

Although recombination has been shown to be a common mechanism of virus evolution, few studies have documented this process in natural populations. In this study, we have shown the existence of a recombinant virus circulating within a naturally infected population of cats. This colony was endemically infected with the Major strain of the virus at the beginning of the study (T = 0 months). It is likely that this virus had been present within the colony for many
months previously, as at time $T=0$ it already contained a large amount of diversity [colony D of Radford et al. (2003)]. The Minor strain was first observed at $T=6$ months with minimal diversity, suggesting that it was a recent introduction (Fig. 1); the recombinant appeared 27 months later. The recombinant virus was most closely related in the POL region to Major strain viruses isolated in the colony at 20 months (Fig. 2a), suggesting that the recombination event occurred closer to this time.

Sequence analysis indicated that recombination was likely to have occurred within the area of the ORF1/ORF2 junction and was consistent with previous recombinants identified in the genera Norovirus and Sapovirus of the family Caliciviridae (Jiang et al., 1999; Katayama et al., 2004; Oliver et al., 2004; Bull et al., 2005). This is the first time that recombination has been observed in the genus Vesivirus and, to our knowledge, the first time that the parental strains have been identified following recombination in the family Caliciviridae. As such, this allowed us to conclude that recombination occurred within this colony in the 27 month period between the first identification of the Minor parent strain and the subsequent identification of the recombinant. We suggest that the high prevalence of infection, often with more than one strain of virus, together with the occasional mixed infection of an individual cat (Coyne, 2005; Radford et al., 2000), make such colonies the ideal environment from which recombinants can emerge.

As we observed the emergence of a recombination event within a natural environment, we can speculate on the mechanisms by which such recombinants are selected. We have shown previously that the CAP of the Major strain of the virus is likely to be evolving by immune-mediated
positive selection [colony D of Radford et al. (2003)]. This may allow the selection of antigenic variants able to escape the collective immune response within the colony, thereby enabling endemic infection. It has also been suggested that the replication of pathogens within such an environment may lead to the selection of viruses with increased fitness/ virulence (Gandon et al., 2001). If this occurs in FCV, such evolutionary pressures are likely to be exerted through mutations in the NSP/ORF1 region. It is therefore possible that the recombinant that we observed combines the replicative potential of the Major strain ORF1 with the antigenic diversity of the Minor strain ORF2. This hypothesis is supported by the observation that, following its emergence, the recombinant appeared to replace its Minor strain parent within the colony (parenticide) (Fig. 1).

The predominant mechanism involved in the recombin- tion of RNA viruses is widely accepted to be the copy-choice mechanism (Lai, 1992; Oberste et al., 2005). In this model, the plus-strand genomic RNA synthesis would be initiated at the 3′ end of the minus-strand genomic RNA. Predicted secondary structure at the ORF1/ORF2 junction might then be responsible for the premature termination of replication, with the replication complex reinitiating RNA synthesis on a second template. The conserved nature of the ORF1/ORF2 junction (Neill et al., 1991; Clarke & Lambden, 1997; Glenn et al., 1999) and the predicted secondary structure at this site (Fig. 2d; Jiang et al., 1993; Porter, 2004) may therefore be responsible for the apparent predilection for recombination at this location within the family Caliciviridae. However, we cannot exclude the possibility that recombinants are produced at other regions in the genome but are selected against due to some inherent reduced fitness (Worobey & Holmes, 1999). The apparent ‘hot spot’ for recombination in the caliciviruses is in contrast to the related picorna-viruses. These viruses do not use a subgenomic RNA during replication and, although recombination in the CAP appears to be relatively rare, recombination can occur throughout the genome (Lai, 1992; Oberste et al., 2004a, b; Lukashev, 2005).

In conclusion, we observed the emergence of a recombi-nant FCV within a cat colony infected endemically with the two parental strains. The recombinant emerged during a 27 month period, which may have allowed the virus to combine both antigenic and replicative fitness.

Acknowledgements
F.C.R. was funded by the Department of the Environment, Food and Rural Affairs as part of the Veterinary Training and Research Initiative MSc in Veterinary Infection and Disease Control. F.C.R. is grateful to Dr Paul Wigley for his support during the MSc. K.P.C. received funding from the PetPlan Charitable Trust. The authors wish to thank Miss P. for allowing us to sample her cats. The authors also extend their thanks to Professor Evans for helpful comments on the manuscript.

References


