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Detection of FCoV quasispecies using denaturing gradient gel electrophoresis

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Feline infectious peritonitis (FIP) is usually a fatal, immune-mediated disease, triggered by infection with feline coronavirus (FCoV). While it is not as yet known what triggers FIP to develop within a particular individual, a relatively new theory suggests that, within an already immune-compromised individual, the development of extensive viral quasispecies may overwhelm the immune system, allowing FIP to develop (Horzinek et al., 1995; Horzinek, 1997). The aim of this study was to determine whether FCoV infection involved the generation of quasispecies, and if quasispecies did exist, to determine what role they may play in the development of FIP.

FCoV was detected by RT-PCR using primers designed to either the C-terminus of the spike (S) gene (De Groot et al., 1987) or the nucleocapsid (N) gene (Vennema et al., 1991) (Table 1). Positive and negative controls were incorporated in each set of reactions. The PCR protocol consisted of an initial incubation of 94°C (10 min), followed by 35 cycles of melting 94°C (1 min), annealing 55°C (1 min) and extension 72°C (2 min), then a final extension of 72°C (10 min). PCR products were Southern blotted and hybridised with specific probes to ensure that the RT-PCR assay produced only specific products which ran on agarose gels as a single band (Gunn-Moore et al., 1998). The presence of quasispecies within the PCR products was assessed by denaturing gradient gel electrophoresis (DGGE), then constant denaturant gel electrophoresis (CDGE), using the denaturing gel gradient electrophoresis system (DGENE, BioRad), according to the manufacturer's recommendations. The acrylamide gels were stained with silver stain. Experiments were run in duplicate to ensure repeatability.

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Table 1
Details of FCoV primers and probes[a]

Oligos.	Size (nt)	TTm (°C)	G + C (%)	Priming site	Priming site	Size (nt)	Sequence 5′ 3′
Primers							
Spike							
No. 66 (F)	18	50	39	2720 → 2737		800	CTG CAT GTC AAA CTA TTG
No. 67 (R)	17	52	53	3521 ← 3504			CTT GTG CAT CAG CAC TC
A–F	24	61	54	2537 → 2560		839	TCA CAC ATT CTG ACG GAG ACG TGC
A–R	24	61	54	3375 ← 3352			CGC TTT AGC AAC AGT GGC AAG ACC
N/capsid							
NF	28	74	50	436 → 463			CCA CCA CAA TTC CAG CTT GAA GTG AAC
NR2	45	100	73	809 ← 785		373	GCG CGC GCG CGC GCG CGC GCA GCA TTT GGC AGC GTT ACC ATT GGC
Probes							
Spike	18	66	50	2816 → 2834			ATT GGC ATC TGT TGA GGC
N/capsid	26	70	54	641 → 666			AAC GTA GTC AGT CCA AGT CCA GGG AC

[a] nt: nucleotides; TTm: theoretical annealing temperature based on 100% identity; F: forward primer; R: reverse primer.

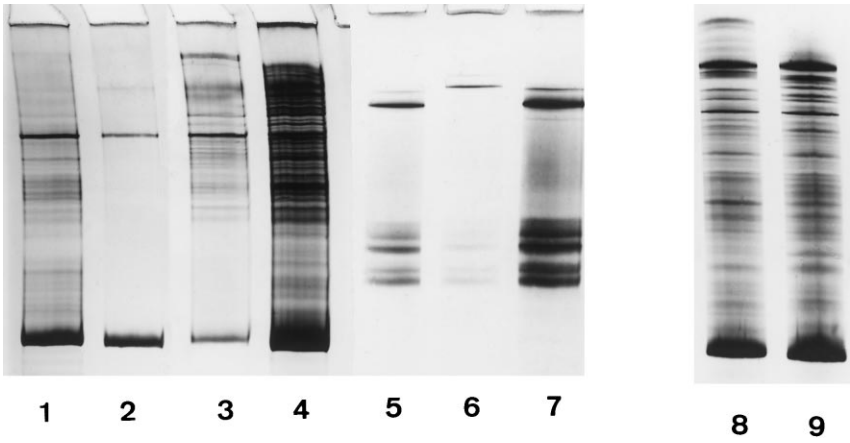

Fig. 1. CDGE analysis. **1–4, 8&9** primers A-F/A-R (S); **5–7** primers NF/NR2 (N). **1&5:** FIPV 79-1146. **2&6:** FIPV Wellcome. **3&7:** FIPV Primucell. **4:** FECV 79-1683. **8&9:** FIPV 79-1146 biological clones; **8:** grown in whole feline embryo (WFE) cells. **9:** grown in Crandell feline kidney cells (CrFK).

The presence of FCoV quasispecies was assessed in vitro and in vivo. A number of cell culture-adapted strains of FCoV were available ($n = 8$), and blood was collected from healthy cats living within households with endemic FCoV ($n = 34$), and cats with clinical FIP ($n = 20$). To assess how quasispecies changed on ex vivo culture a number of samples were assessed both prior to culture and after variable periods of culture in different cell types (Gunn-Moore et al., 1998). Quasispecies were demonstrated within FCoV extracted directly from blood. They were also evident when field-strain viruses were grown in cell culture, and although they showed some degree of simplification over time, they maintained complex patterns, irrespective of whether they were grown in heterogeneous or homogeneous cell lines (data not shown). Quasispecies were also found in cell-culture adapted FCoV, and even in biological clones generated by serial dilution (Fig. 1).

The two regions of FCoV demonstrated different levels of variation (Fig. 1). Only limited evidence of quasispecies development was found in the N region and the variant patterns were similar for most samples assessed. In contrast, considerable variation was seen in the S region, both between individual cats (Fig. 2), within the same cat over time, and even within separate blood fractions from the same cat (data not shown).

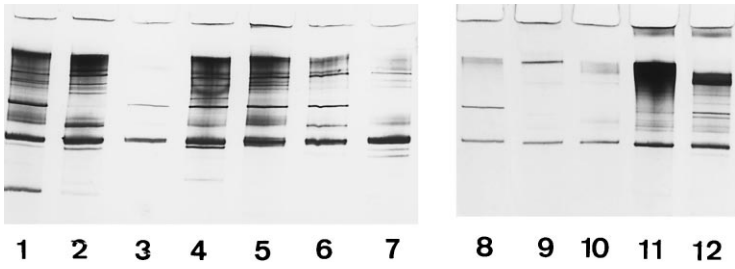

Fig. 2. CDGE analysis. Primers No. 66/67 (S). **1–7:** Healthy cats. **8–12:** Cats with FIP.

Little difference was found in the quasispecies patterns attributable to the age of the cat. Overall, cats with FIP demonstrated rather more variability in their quasispecies patterns than healthy cats, with occasional sick cats having very complex populations, but most having very few variants (Fig. 2). Increased diversity of exposure appeared to increase viral heterogeneity (data not shown).

From this study we see that the combination of RT-PCR and DGGE or CDGE provides a rapid and relatively inexpensive method of assessing genetic differences within single viral isolates. The technique can be used to assess large sections of genome, identifying areas with significant variation for more detailed study, for example, by sequence analysis.

The results of the current study demonstrate that FCoV exists within individual cats as complex viral populations. While the presence of variation within cell-culture maintained virus argues against FCoV heterogeneity being driven solely by pressure from the immune system, it remains likely that changes in selection pressure will have an effect on viral variation.

It was hypothesised that quasispecies may vary according to whether or not the cat has FIP, the age of the cat, the general health of the cat, and the particular household within which the cat has lived, i.e. the particular FCoVs to which the cat has been exposed. From the current study the exact relevance of these factors remains unclear. Little difference was found in the quasispecies patterns according to age, while the majority of cats with FIP demonstrated rather limited viral variation. The diversity of exposure appeared to correlate with increased viral heterogeneity, since kittens from mixed-source households showed the most marked variability, possibly representing co-infection with a number of different FCoVs.

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