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Persistence and transmission of natural type I feline coronavirus infection

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To examine the mode of natural transmission and persistence of feline coronavirus (FCoV), FCoV strains shed by domestic cats were investigated over periods of up to 7 years. An RT-PCR that amplified part of the 3′ end of the viral spike (S) gene was devised to distinguish FCoV types I and II. All but 1 of 28 strains of FCoV from 43 cats were type I. Nucleotide identities of the amplified 320 bp product from 49 type I FCoVs ranged from 79 to 100 %. The consensus partial S sequence of isolates recovered from persistently infected cats at time intervals spanning years was generally conserved. While most cats were infected with a single strain, a few may have been infected by more than one strain. Cats that were transiently infected and ceased shedding could be re-infected with either the same, or a different, strain. In most cases, whether a cat became persistently or transiently infected was independent of the virus strain. However, one strain was unusual in that it infected the majority of cats in the household simultaneously and was still being shed 18 months later. Factors that influence whether FCoV establishes lifelong infection in some cats and not others are determined mainly by the host response to infection.

INTRODUCTION

Feline coronavirus (FCoV) is an enveloped, positive-stranded RNA virus belonging to the family *Coronaviridae* within the order *Nidovirales* (den Boon *et al.*, 1991). Infection with FCoV is common in domestic cats. This was first recognized by the finding of a high prevalence of seropositive cats in the population, particularly in pure-bred cats in multi-cat households, among which transmission occurred readily (Herrewegh *et al.*, 1997; Kiss *et al.*, 1999; Pedersen, 1976). Until recently, defining the details of the epidemiology of FCoV was difficult because the virus could not be detected easily, being difficult to grow in cell culture. However, the application of RT-PCR has permitted the demonstration of FCoV RNA in animal tissues and excretions (Herrewegh *et al.*, 1995); thus, a picture of the way in which the virus occurs in the population has begun to emerge (Addie & Jarrett, 2001; Foley *et al.*, 1997; Herrewegh *et al.*, 1995). Of the cats that are exposed to the virus, 13 % become persistent, and probably lifelong, healthy carriers, excreting virus in the faeces (Addie & Jarrett, 2001). Most of the remaining cats become transiently infected, so that after a period of time, usually measured in months, they cease to excrete virus and subsequently revert to being seronegative. However, in this state they may become re-infected. A very few cats appear to be resistant to infection. It is presumed that the virus is maintained in the community by spread from persistently infected and transiently infected cats (Addie & Jarrett, 2001). What is not clear is the relative importance of these two sources of infection or the viral and host factors that promote the survival of the virus.

An unfortunate, inadvertent result of FCoV infection in a small proportion of cats is the development of the lethal, immune-mediated condition known as feline infectious peritonitis (FIP) (Poland *et al.*, 1996; Vennema *et al.*, 1998). In many parts of the world, FIP is considered to be the major, infectious cause of death in cats (Vennema *et al.*, 1998) and is of sufficient veterinary importance to create considerable demand for measures to control the infection, mainly through management practices (Addie & Jarrett, 1992; Kass & Dent, 1995). A more complete understanding of the epidemiology of the virus would permit more rational methods of control and may provide a model for the epidemiology of the severe acute respiratory syndrome virus. In particular, more information is required on the way in which the virus persists in the population.

Studies of other coronaviruses, particularly mouse hepatitis virus and infectious bronchitis virus of domestic poultry, have shown clearly that coronaviruses are prone to genetic variation (Cavanagh *et al.*, 1998), and one might imagine that variation would promote persistence through the generation of quasispecies from which escape mutants might emerge. The existence of FCoV quasispecies and variation has been shown already (Gunn-Moore *et al.*, 1998; Kiss *et al.*, 1999), which poses the question of whether variants arise in persistently infected cats that contribute

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both to the maintenance of persistence in these animals and to the re-infection of seronegative cats.

There are two types of FCoV: type I FCoV is wholly feline, whereas type II FCoVs have arisen by recombination events between type I FCoVs and canine coronavirus (CCoV), resulting in a FCoV genome consisting of the spike (S) gene and part of the adjacent genes from CCoV (Herrewegh *et al.*, 1998). The S protein is key: it is the S protein that attaches to the cellular receptor and it is antibodies to the S protein that mediate virus clearance (Gonon *et al.*, 1999). Type II FCoVs are easier to grow in cell culture and form most laboratory isolates but it is difficult to know how accurately laboratory infections with these strains reflect the real-life situation with type I viruses. The majority (90 %) of field isolates in Japan and the USA are type I (Vennema, 1999). Amongst cats with FIP in Japan, 30 % of FCoVs were type II (Hohdatsu *et al.*, 1992). Apart from the possibly greater chance of developing FIP with a type II infection, whether or not there is any correlation between FCoV type and outcome of infection in terms of becoming a carrier or being transiently infected has never been investigated previously. Until now, types I and II FCoV have been differentiated by monoclonal antibodies; in this paper, we describe an RT-PCR developed specifically to differentiate between type I and type II FCoVs.

To begin to answer these questions, we developed a method to identify FCoV isolates through amplification of a region of the S gene by RT-PCR and restriction endonuclease digestions of the product. This reaction distinguished FCoV of types I and II and indicated that the former was by far the more prevalent type in the UK. Phylogenetic analysis of viruses from a larger number of cats was performed to establish the range of variation in nucleotide sequence in our population. This study confirmed previous reports of considerable sequence variation between FCoV from different sources (Vennema *et al.*, 1998). A longitudinal study of cats in households in which the virus was endemic revealed that the amino acid sequence at the C terminus of the S gene of the virus in individual persistently infected cats appeared to be conserved over periods of up to 7 years. Finally, we investigated whether transiently infected cats that had recovered from infection could become re-infected with the same virus strain, excreted either from a persistently infected cat or from a transiently infected cat, or whether they could only be re-infected with a different virus strain. In this way, we expected to learn the relative importance of persistent and transient infections in FCoV survival.

METHODS

Samples from cats. The cats were part of a larger study: throughout the UK, 155 cats and 7 dogs from 29 households in which FCoV was naturally endemic were followed for periods of up to 7 years (Addie & Jarrett, 2001). Households were allocated letters of the alphabet and the cats within were given numbers (A1, A2 and A3, etc.). Faecal samples or rectal swabs were taken at regular intervals from the cats by veterinarians or by the owners for monitoring FCoV shedding by RT-PCR. Blood samples were submitted by the attending veterinary practitioner at intervals of 3–12 months and were tested for anti-FCoV antibodies.

In addition, we examined FCoV isolates from pleural and peritoneal effusions from eight naturally infected cats with FIP that had been submitted to our diagnostic laboratory (these were not from survey households).

RT-PCR. To monitor virus shedding, an RT-PCR that amplified the FCoV 7b gene (Herrewegh *et al.*, 1995) was performed on faecal samples or rectal swabs. In addition, a novel RT-PCR amplifying a partial S gene sequence (S RT-PCR) was developed to differentiate between FCoV types I and II (Fig. 1). Where there was sufficient material available, samples were also analysed using S RT-PCR. The target for the differentiating RT-PCR is located within the 3′ region from the S gene, which encodes the more conserved C-terminal part of the S protein and is believed to be responsible for the incorporation of the S protein into the FCoV particle (Godeke *et al.*, 2000). The region that was amplified is N-terminal to the proposed transmembrane domain and differences in the S genes of FCoV types I and II are apparent in this region (Motokawa *et al.*, 1995). A reverse sequence primer (Iubs) targeting a region that is conserved among FCoV and CCoV S genes was used to make cDNA, while the forward primers were directed specifically against the type I or II FCoV sequence, resulting in fragments of different sizes depending on the

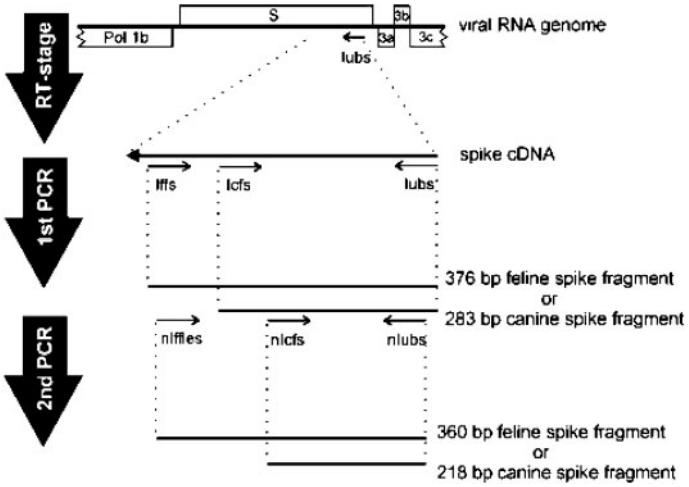

Fig. 1. RT-PCR to distinguish types I and II FCoV. For the RT stage with viral RNA as template, cDNA was transcribed from the S gene using the universal backward primer, Iubs. First-round PCR was performed with the Iubs primer and two specific forward primers designed exclusively to be specific for the FCoV (Iffs) or CCoV (Icfs) S sequences. Depending on the type of the isolate, this will yield a 376 bp fragment for type I or a smaller 283 bp fragment for type II FCoV. To increase sensitivity and specificity, a second PCR was performed with a nested universal backward primer (nIubs) and two nested specific forward primers (nIffles and nIcfs). This reaction resulted in a fragment of 360 bp for type I FCoV or 218 bp for type II FCoV.

Table 1. Nested PCR protocol to distinguish FCoV type I and II

	Primer	Sequence (5′→3′)	Nt position equivalent in sequence D30244() or X06170[ ]	PCR conditions
First-round PCR	Iffs	GTTTCAACCTAGAAAGCCTCAGAT	(3921-3944)	90 °C, 5 min
	Icfs	GCCTAGTATTATACCTGACTA	[3978-3998]	94 °C, 1 min ┐
	Iubs	CCACACATACCAAGGCC	(4280-4296)	50 °C, 1 min ┘ 35 cycles
			[4244-4260]	72 °C, 1 min
				72 °C, 7 min
Second-round PCR	nIffles	CCTAGAAAGCCTCAGATGAGTG	(3928-3949)	90 °C, 5 min
	nIcfs	CAGACCAAACTGGACTGTAC	[4041-4060]	94 °C, 1 min ┐
	nIubs	CCAAGGCCATTTTACATA	(4270-4287)	47 °C, 1 min ┘ 35 cycles
			[4234-4251]	72 °C, 1 min
				72 °C, 51 min

identity of the FCoV. To increase both the sensitivity and the specificity of the reaction, a nested PCR step was performed. The primers and temperature cycling programmes are described in Table 1.

Viral RNA was extracted from faecal samples using a modification of the guanidinium isothiocyanate silica protocol of Boom *et al.* (1990) and the RNA was eluted from the silica beads in 25 µl Tris/EDTA buffer. Primer annealing was conducted by the addition of 10 pmol Iubs primer to 9 µl RNA, heating to 100 °C for 5 min and cooling rapidly on wet ice. The RT mixture, which contained 1 × RT buffer (Gibco-BRL), 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL), 0·5 mM dNTPs (Perkin Elmer), 10 mM DTT and 30–40 U RNA guard/RNase inhibitor (Pharmacia Biochemicals) in a final volume of 20 µl, was added. This reaction mixture was centrifuged in a bench centrifuge and incubated at 37 °C for 1 h, adapted from Herrewegh *et al.* (1995).

A 10 µl sample of cDNA was added to the primary PCR mixture, which contained 1 × PCR buffer II (Perkin Elmer), 15 pmol Iubs, 20 pmol each specific forward primer (Iffs and Icfs), 2 mM $MgCl_2$, 0·8 mM dNTPs and 2·5 U *Taq* polymerase (Perkin Elmer) in a final volume of 100 µl. From this reaction, 10 µl was added to 90 µl of the secondary PCR mixture, which contained 1 × PCR buffer II, 20 pmol each primer (nIubs, nIffles and nIcfs), 1·5 mM $MgCl_2$, 0·8 mM dNTPs and 2·5 U *Taq* polymerase in a final volume of 100 µl. Samples of 10 µl of the secondary PCR were analysed on a 2 % agarose gel containing 0·5 µg ethidium bromide ml^{-1}.

Restriction enzyme analysis. The products of the differentiating RT-PCR were analysed by separate digestions with *Alu*I, *Sau*3AI (both from Gibco-BRL) and *Rsa*I (Quantum-Appligene). Digestions were performed with 10 µl PCR product, 12 µl H_2O, 1 × appropriate buffer and 5 units enzyme. Samples were analysed on 2 % agarose gels containing 0·5 µg ethidium bromide ml^{-1}.

Nucleotide sequencing and analysis. Direct sequencing of both strands of the RT-PCR products was performed by MWG-Biotech. At positions where more than one base was called in both forward and reverse reads, a variant position was recorded. This was presumed to be a consequence of a mixed target virus population, as no cloning step that might fix *Taq*-induced errors was performed. In several cats sampled sequentially, the emergence of a dominant nucleotide could be detected at positions that were variant in earlier samplings.

Sequence data were derived for 50 samples from cats in 14 households and from Primucell (Pfizer) vaccine strain FIPV-DF2 (Christianson *et al.*, 1989). Sequences were assigned the format AN/NNNN, where A is the household, N designates an individual cat and the numbers after the solidus refer to month and year of sampling (for example, J8/0196 was a sample collected from cat 8 in household J in January 1996). Sequences recovered from non-survey cats with FIP are prefaced by the letter F. Sequence alignment and unrooted phylogenetic trees were derived using the CLUSTAL_W program provided in MEGALIGN, version 5, using IUB weight matrix. Unrooted trees were derived through TREEVIEW, version 1.6.6 (Page, 1996). Pairwise sequence comparisons were performed using the Martinez–Needleman-Wunsch alignment facility within MEGALIGN. Where types I and II FCoVs were compared, phylogenetic trees were derived through analysis of the C-terminal 174 bp of a subset of type I amplicons and the full-length type II amplicons. Where only type I FCoV sequences were analysed, the entire 320 bp was compared. Type I sequences with nucleotide identities of over 97 % (Fig. 3) or over 99 % (Fig. 4) to specific samples were excluded from phylogenetic trees for purposes of clarity. Relatedness of these sequences to sequences incorporated in the phylogenetic trees is summarized as follows: G5/1095, (G1/0296 and G1/1100) + [G2/0500, G5/0600, G1/1095 and G5/0100], where the underlined sequence, G5/1095, is included in the tree, sequences in round brackets are >97 % identical to G5/1095 across the C-terminal 174 bp of amplicon and those in square brackets are >99 % identical to G5/1095 across the entire 320 nt amplicon. Other related sequences include: J8/0196, (J10/0897) + [J9/0301 and J10/0895]; J8/0197, [J7/1196]; J8/0201, [J4/0201, J5/0201, J6/0201 and J6/0300]; J10/0897, [J10/0998]; C10/0301, (C11/0301) + [C9/0301]; X1/0201, (H2/1100); P4/0900, (P2/0900) + [P4_0396]; and FQ1/1099, (Q8/1197). Published database sequences were also included as follows: type I strains were UCD1 (AB088222), Black (AB088223) and KU-2 (D32044); and type II strains were 79-1146 (X06170) and 79-1683 (X80799).

Serological testing. Titres of anti-FCoV antibody in cat plasma were determined by an indirect immunofluorescence antibody assay as described previously (Addie & Jarrett, 1992). Samples that failed to fluoresce at a dilution of 1 : 10 were deemed to be seronegative and given a titre of <10. Doubling dilutions were made up to 1 : 1280 and those samples which still produced strong fluorescence at 1 : 1280 were recorded as having an antibody titre of >1280.

RESULTS

Type I FCoV predominates in cats in the UK

A region of the S gene was amplified by RT-PCR. This region, located within the 3′ region of the S gene, was selected with the aim of differentiating type I and II strains and maximizing the number of FCoV isolates that could be amplified by a non-degenerate primer set.

Using this method, types I and II could be distinguished by the size of the PCR product (Fig. 1). The first round of the S RT-PCR produced fragments of the predicted 376 bp when used to amplify the UCD-1 and UCD-3 type I strains (Hoskins, 1997) of FCoV (kindly provided by D. Harbour, University of Bristol, UK). The nested step produced a product of 360 bp. From the type II Wellcome strain of FCoV, bands of 283 bp were produced by first-round PCR and 218 bp by second-round PCR (Fig. 2).

S RT-PCR proved to be less sensitive than the ORF 7b PCR and, consequently, amplicons were recovered from only a subset of the samples that proved positive by ORF 7b RT-PCR. It is possible that variant sequences in some isolates rendered primer binding ineffective in the S assay. S RT-PCR was applied successfully to 106 samples from 43 cats in 23 households throughout the UK. Further analysis of the product was carried out by restriction enzyme digestion and, in 51 samples, by nucleotide sequencing. Twenty-eight (97 %) strains from 43 cats were found to be type I and one was type II (several cats were infected with the same strain as each other, see below). Although these samples do not represent a random collection, this result suggests that there is a low prevalence of type II FCoV in cats in the UK, as in other areas of the world that have been studied (Hohdatsu *et al.*, 1992; Vennema, 1999). In three households, more than one strain of FCoV was found.

Comparative analysis of partial S sequences

To establish the origin of new strains within households, the range of sequence variation from cats across the UK was examined. Two households in which the virus was endemic over periods of up to 6 years were studied in detail. In these households, cats had been identified that were infected either persistently or transiently, as defined previously (Addie & Jarrett, 2001). Initial observations of whether a virus was type I or type II FCoV and the typing of isolates were made by restriction endonuclease digestion and verified by sequence analysis of a selection of samples. The 51 sequences were deposited in the EMBL database (accession nos AY159735–AY159785). A phylogenetic tree derived from comparison of a selection of these sequences and five reference type I (KU-2, Black and UCD1) and II (Wellcome and FIPV-DF2) S sequences is shown in Fig. 3. The range of nucleotide identities for all type I isolates (excluding reference strains) was 79–100 %. Sequence identity between 13 isolates each recovered from a distinct household was in the range of 82–95 %. Phylogenetic analysis of samples recovered from cats in the same household indicated a common origin of infection in some instances (sequence identities in the range of 95–100 %), but in other households, partial S sequences were as related to S genes of other households as they were to isolates circulating within their own household (sequence identities <95 %), implying that novel isolates had been introduced rather than emerging from within the household. Sequence identity between type I and II sequences was in the range of 60–67 %.

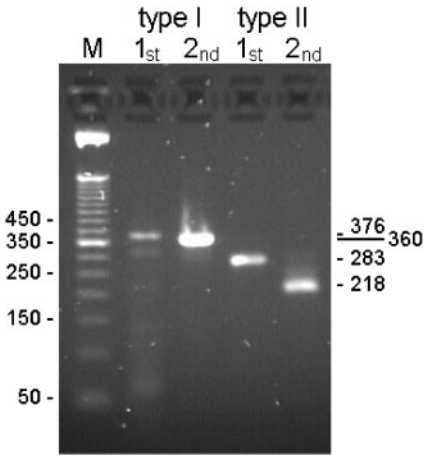

Fig. 2. PCR products showing the different band sizes distinguishing types I and II FCoV in both first-round (1st) and second-round (2nd) PCR.

FCoV in persistently infected cats is highly conserved

Persistently infected carrier cats that shed virus continuously in the faeces (Addie & Jarrett, 2001) were of particular interest since they might be expected to be reservoirs of the virus, as well as sources of virus variants. The 320 bp partial S sequences (exclusive of primer sequences) were obtained from five of these cats over periods of up to 6 years (Table 2). Sequence variation ranged from 0 changes in 17 months (100 % identity) (cat J3) to 9 nt in 5 years (97 % identity) (cat G1). For comparison, the sequences of three transiently infected cats that had recovered and become re-infected, on serological and molecular evidence, are included. In one of these, cat G5, there was very little change in sequence (>99 % sequence identity) and this cat may have been re-infected by cat G2 (100 % identity), a possible carrier of the type I strain detected initially in the household. However, in cats C5 and J7, which were infected at intervals of 12 and 13 months, respectively, there was over 8 % sequence change, which is suggestive of re-infection by distinct FCoV strains.

These data indicate that the variation in this region of the S sequence is not substantial in carrier cats.

Recovered transiently infected cats can be re-infected with the same or a different strain

We then investigated cats that had been transiently infected and subsequently re-infected with FCoV. We investigated whether the indigenous virus from the persistently infected cats in the same household or from another source had re-infected these cats. PCR products recovered from several of the transiently infected cats throughout the study were sequenced. From the results, presented in Tables 3 and 4, it appeared that both possibilities could occur.

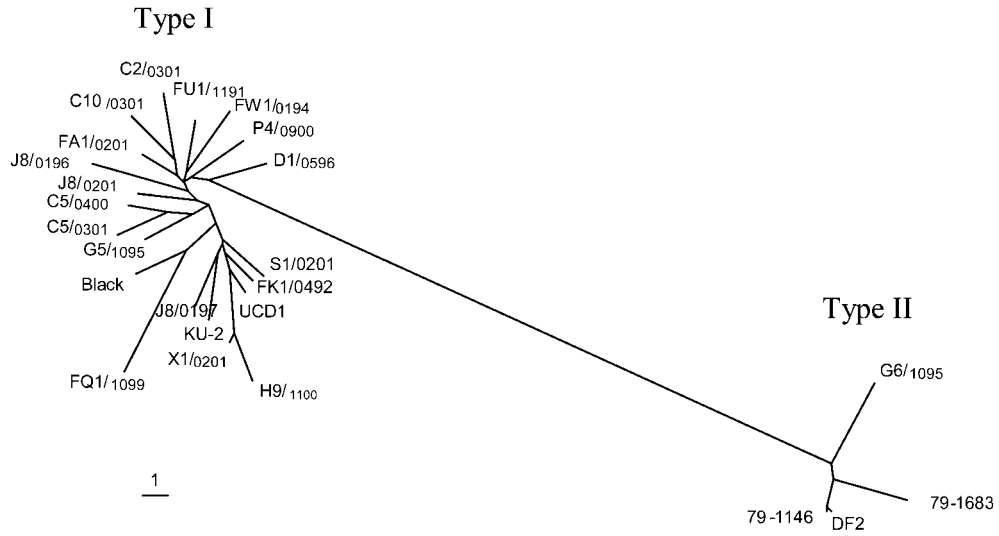

Fig. 3. Phylogenetic tree derived from analysis of coronavirus S sequences of types I and II field isolates, and types I (KU-2, Black and UCD1) and II (79-1146, 79-1683 and FIPV-DF2) database sequences showing the distance between the single type II isolate (G6/1095) sequenced and the type I FCoVs. Sequences were aligned across 174 bp (which comprises the entire type II amplicons and the C-terminal sequence of type I amplicons). The type I FCoVs are shown in greater detail in Fig. 4. Sets of type I sequences, which are over 97 % identical, are represented by a single strain in this tree and samples that might represent co-infections are not included (see Fig. 4). Households are designated by letters; individual cats are designated by letters and numbers (e.g. J10 is a cat from household J). Cats with FIP have the letter F. The month and year of samplings are also given (e.g. 0400 is April 2000).

These tables summarize a longitudinal study of two multi-cat households that contained carrier cats (G1 and J10) at the beginning of monitoring and in which some cats became carriers (J3, J4, J5 and J9). Both households contained pure-bred cats. Cats in household G were mainly neutered and were allowed to go outdoors, whereas cats in household J were breeding and show cats and were kept indoors. The tables show the anti-FCoV antibody titres and presence or absence of virus in the faeces, as determined by ORF 7b RT-PCR. Elimination of FCoV infection from a cat was assumed to have occurred when its antibody titre fell and virus excretion ceased. Tables 3 and 4 and Fig. 4 also show that individual cats can be re-infected by the same FCoV strain as before (>96 % sequence identity) or by a different strain (<92 % identity).

In household G, at the start of the survey, all but one cat were positive by ORF 7b RT-PCR (Table 4). By 14 months

Table 2. Sequence comparison between carrier and non-carrier cats

In contrast to the situation of the carrier cats, two distinct isolates harvested from two cats, C5 and J7, had identities of <92 %. In addition, a sample from cat G5 is included, showing strong similarity to that of G1, from which his infection appeared to originate.

Cat	FCoV status	Maximum interval of testing (months)	No. of differences (bp)	Similarity (%)	No. of amino acid changes
J10	Carrier	38	4	98·1	1
J9	Carrier	67	3	99·1	3
J3	Carrier	17	0	100	0
P4	Carrier	55	2	99·4*	2
G1	Carrier	62	9	96·9	7
G5	Re-infected same strain	57	2	99·1	1
C5	Re-infected different strain	12	29	90·3	11
J7	Re-infected strain *b*	13	39	87·8	14
J7	Re-infected strain c	50	26	91·9	10

*Compared across only 312 bp.

Table 3. FCoV strains in cats in household J over a 6 year period

Cattery J was monitored during this study for a period of 5 years and 10 months (initial letter code). The vertical axes shows the codes of the individual cats. The timescale is represented by the horizontal axis. Cats J3, J4 and J5 were born in January 1999, July 1998 and April 1998, respectively, while cat J10 died in April 1999. We screened for the presence (+) or absence (–) of FCoV using ORF 7b RT-PCR (Herrewegh *et al.*, 1995). Some of the positive samples were tested with the differentiating S RT-PCR and by sequence analysis (bold); all were FCoV type I. Three different sequences could be distinguished by restriction analysis and sequencing and are designated in the table as *a*, *b* or *c*.

	1995					1996											
Cat	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
J1	–	–	–		–	–	–	–	–	–	–	–	–	–	–	–	–
J2	–	–	–		–	–	–	–	–	–	–	–	–	–	–		
J3																	
J4																	
J5																	
J6	*a*	–	–		–	–	–	–	–	+	+	–	–		–	–	
J7	–	–	–		–	–	+	–	+	–	–		–		+	***b***	+
J8	–	–	–		+	***a***	+	–	+	–	–	+	–		+	+	+
J9	–	***a***	*a*		+	*a*	–	+	+	+	+	+	+	+	+	+	+
J10	***a***	+	+		+	+	+	+	+		*a*	+	+	+	+	+	+

	1996												1998											
Cat	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
J1	–	–	–			–		–		–				–	–		+	–	–		–		–	
J2	–		–		–	–		–	–	–	+	+	–	–	–		+							
J3																								
J4																							+	–
J5																		–	–		+	+	–	
J6	*b*	–	–	–	–	–		+	+	+	+	+	–	–	+	–	–		–				–	
J7	*b*	–	***b***	–	–			–	–	–	–	–		–	–	–	–	–		–	–			
J8	***b***		+	+	–	+		–	+	+	+	–	+		+	+	+			+	+	*a*	+	–
J9	–	–	–		+	+		+	+	+	+			+	+	+	+			+		+		
J10	+				+	+		***a***	+	+	+	+	+	+	+	+	+	+	+	+	***a***	+	+	

	1999												2000							2001	
Cat	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	O	F	M
J1		–							–		–			–	–				–		
J2			–						+		+			–		+					
J3		–	–	–			+		–	***c***	+			+	–	***c***				***c***	
J4	–		–	–			–	*c*		*c*	+	+	+	*c*	–	+	*c*	+		***c***	
J5		–	–	–			–		–		+	*c*	+	*c*	*c*	+	*c*	+		***c***	
J6	–	–	–					–	+	–	+	+	*c*	+	***c***			+	+	***c***	
J7			–	+	–		–	+		***c***	+		+	+	+	+	*c*	+		*c*	
J8	–		–	–			+	+	–	*c*	+	+		+	–			+	+	***c***	
J9				+		+		–	–		+		+	+				+			***a***
J10	*a*	+	+		Died																

later, several cats had apparently cleared the infection, as their antibody titres had fallen to <10 and viral sequences could not be amplified from their faeces. After a further 9 months, all of the cats, except G5, were virus positive by PCR and rising antibody titres confirmed that cats G3 and G6 had been re-infected. Analysis of the S amplicon of four cats at the 1995 sampling indicated that a type I FCoV was circulating in the majority of cats and cat G6 was infected with a type II FCoV strain. Sequence analysis of samples from cats G5 and G2 collected in 2000 indicated that the region of the S gene that was sequenced varied by fewer than 2 nt from the 1995 isolate. In contrast, the S gene of the confirmed carrier G1 had over nine differences. To summarize, in this household there was evidence for infection of cat(s) with types I and II FCoV. The majority of cats were re-infected with type I FCoV, which, on the basis of the S sequence, was likely to have originated from the same source.

In household J, cat J10 was a known carrier and cat J9 became a carrier. Sequences in this household grouped into three sets, designated *a*, *b* and *c*, in which same-set sequences were related by 97–100 % and sequences from different sets were related by 86–92 % (Table 3). Sequences were associated with infections in multiple cats in the household at different periods of the 7 year study: *a* sequences were recovered at the start of the study period, *b* sequences in 1996/1997 and *c* sequences at the end of the study period in 2001, consistent with circulation of new strains of FCoV introduced into the household. Most *a* and *b* infections were transient infections from which the cats recovered, although cat J10 was a carrier of sequence *a*. Cats infected with sequence *c* in 1999 were still shedding virus at the end of the study. Cats J6, J7 and J8 had several periods of virus shedding over this period. As can be seen from Table 3, cat J8 experienced three different FCoV infections in 1996 (sequence *a*), 1997 (sequence *b*) and 1999 (sequence *c*). That she was truly re-infected in 1999, rather than that her infecting strain had mutated, is confirmed by her antibody titre decreasing to 20 in May 1999 and rising again to 640 in July 1999.

Origin of two novel FCoV strains in household J

Sequence analysis indicated divergence between three sequences, *a*, *b* and *c*, associated with isolates recovered from household J: isolates *b* and *c* were as distant from isolates *a* as they were to sequences derived from isolates outside of this household (Fig. 4). This is suggestive of infection of cats in the household by three distinct strains of FCoV, although it does not preclude that recombination might have occurred between these coronaviruses in genome regions other than the S region sequenced herein.

To attempt to trace the origins of sequences *b* and *c*, the S amplicon of the FCoV from an in-contact cat, C9, from household C, who had mated cat J7 was sequenced. The sequence was found to be quite different to strains *b* (87 % identity) and *c* (90 % identity), so is unlikely to represent

Table 4. FCoV strains in cats in household G over a 6 year period

Monitoring of FCoV antibody titres (numbers) and virus shedding (+, virus shedding; –, negative result by ORF 7b RT-PCR). In addition, virus types as determined by sequencing are shown in bold. The single type II FCoV shed by G6 is indicated. The carrier cat, G1, shed virus at every test. This household appeared to eliminate FCoV infection by July 1996 but experienced re-infection by the next test in March 1997, when antibody titres increased and the number of cats shedding virus increased from one to six of seven cats. Months are indicated by their first letter.

	1995		1996			1997		1998		1999			2000		
Cat	**M**	**O**	**F**	**M**	**J**	**M**	**S**	**J**	**M**	**J**	**J/A**	**O**	**J/F**	**A/J**	**N**
G1	640	+	+	+	+	+	+	+		+	+			+	+
		>1280		1280		>1280									
G2		+	+	+	–	+	+	+						+	
		320		640											
G3		–	–	+	–	+	+	+	–		–	–	+	–	–
		<10		10		1280			1280						
G4		+	+	–	–	+	+	+			+				+
		<10		640											640
G5		+	+	–	–	–	–	–			+	+	+	+	+
		640		160											
G6		**Type II**	+	–	–	+	–	–			–				–
		<10		10		>1280								<10	
G7		+	–	–	–	+	–	–			–			–	–
		<10		<10											

their origin (Fig. 4). Cats in this household had been vaccinated with Primucell but this is a type II strain and identity with sequences *b* and *c* is under 65 %. Cat J3 had been to a cat show in June 1999, which may have been the source of the third strain found in this household.

Most cats are not superinfected with several strains of FCoV

Although evidence was obtained for the concurrent presence of multiple strains of FCoV in three households, most cats were infected with only a single FCoV strain. However, three samples (C12, P2 and FZ1) had multiple variant positions within the sequences to an extent that this may reflect co-infection of these cats with two distinct strains. This is the first indication that co-infection or superinfection may occur with FCoV.

DISCUSSION

Viruses that establish persistent infections cause many of the most important diseases of animals and man. The value of persistence for the survival of viruses is clear from the variety of mechanisms that many quite different types of viruses have evolved to achieve this interaction with their hosts. Most species that are infected by coronaviruses are short-lived either naturally (mouse) or because they are bred for food (pigs and chickens). We have shown lifelong infection by coronavirus in a companion animal, the cat, whose lifespan can be up to 20 years. In addition, long-term shedding of CCoV has been observed in puppies [A. Pratelli (Department of Animal Health and Well-Being, Faculty of Veterinary Medicine, Bari, Italy), personal communication]. No persistent infection of humans by coronaviruses has been demonstrated so far but our results indicate that such a carrier state may exist and should be looked for.

We have shown for the first time that FCoV of types I and II can be distinguished using molecular biological methods. In addition, the PCR products can be digested using a combination of enzymes to produce a characteristic fingerprint for each FCoV strain. This method provides a useful tool for examining transmission patterns in natural infections in the field. Only 1 of 43 (2 %) cats tested was infected with type II FCoV, which is in agreement with figures from other countries in which type I is the endemic strain (Hohdatsu *et al.*, 1992; Vennema, 1999). Thus, in a Japanese study, 10 % of healthy FCoV-infected cats and 31 % of infected cats with FIP were infected with a type II FCoV (Hohdatsu *et al.*, 1992). Since type II FCoV arises by recombination of type I FCoV and CCoV (Herrewegh *et al.*, 1998), it might have been expected that cats in our households that contained dogs would have had a greater prevalence of type II. However, only five households had the seven dogs that were studied, which may have been too few to expect concurrent CCoV infection and in which to find recombinant virus. The one household that contained a cat with a type II virus did not have a dog. Whether type II viruses are transmitted naturally among cats is not known.

Carrier cats were defined as cats that shed virus continually, as opposed to intermittently, probably for life (Addie & Jarrett, 2001). Although the region of the S gene chosen showed up to 20 % variability across isolates, the amount of

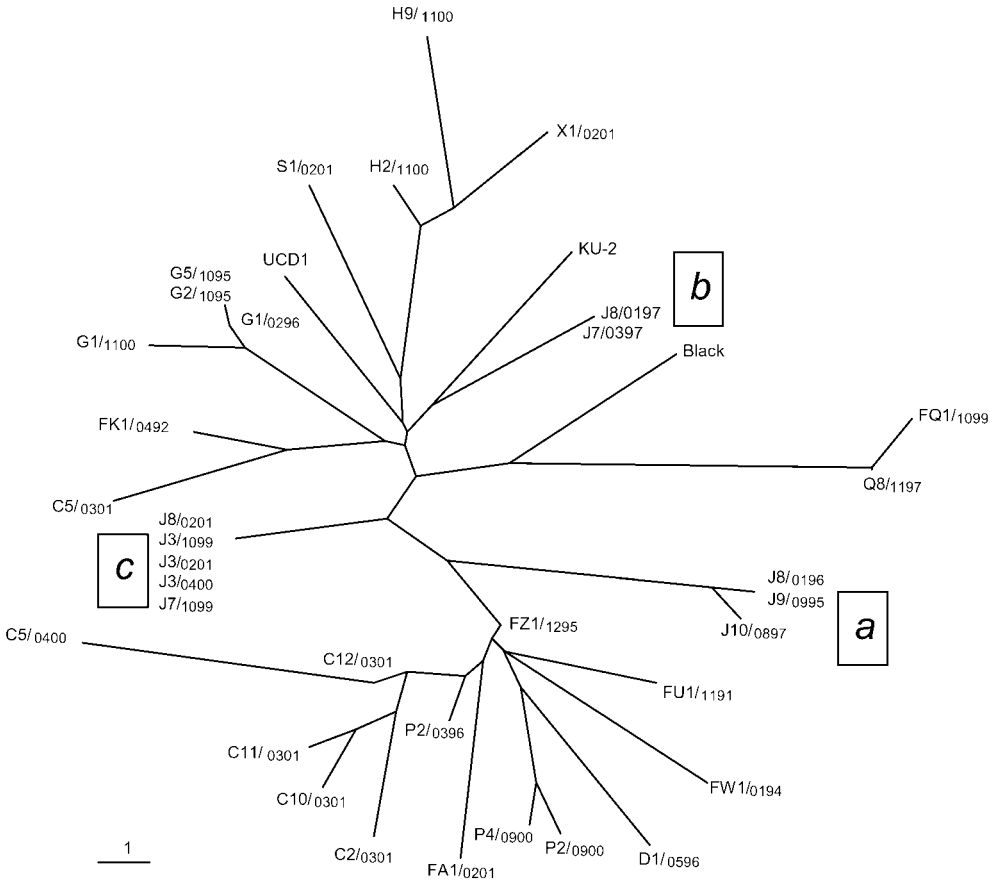

Fig. 4. Phylogenetic analysis of type I isolates based on the entire 320 bp amplicon. All type I sequences are represented except those with identities between 99 and 100 % to represented sequences (see Methods for details of sequences omitted). Households are designated by letters; individual cats are designated by letters and numbers (e.g. J10 is a cat from household J). Cats with FIP have the letter F. The month and year of samplings are also given (e.g. 0400 is April 2000). Sequences *a*, *b* and *c* from household J are illustrated in boxes, showing that they are more distant from each other than from isolates from non-J households.

variation within lifelong carrier cats over periods of up to 5·5 years was minimal, suggesting that carrier status may not be maintained by quasispecies variation within the individual animal. Virus clearance has been correlated with humoral (Gonon *et al.*, 1999) and cell-mediated immune responses to the S glycoprotein (de Groot-Mijnes *et al.*, 2002). However, it is likely that the region of the S gene that we chose to examine was not the region involved in immune clearance of the virus and, therefore, might not be subject to selection pressure. Further sequencing of the whole S gene of these isolates might provide additional information in this regard. Our data demonstrated clearly that virus persistence in the carrier cats was due to the maintenance of the same virus over time and was not due to re-infection by different FCoV strains.

Most cats that become infected with FCoV mount an immune response, eliminate the virus and may then become re-infected (Addie & Jarrett, 2001; Foley *et al.*, 1997). We have shown that cats can become re-infected not only with different strains but also with the same strain of FCoV. In household G, re-infection of cat G5 was by a strain only 2 nt different from the strain with which he had been infected previously. In household J, over a 3 year period, only one mutation occurred in the sequenced region of the virus that was excreted by the carrier cat, J10. In contrast, there were 31 changes between this sequence (isolate *a*) and isolates circulating in the household, suggesting that a new virus had been introduced into the household. A possible candidate for introducing the virus was from another survey household, in which matings had occurred between two pairs of cats. However, the new virus in household J did not match any of the sequences recovered from the in-contact household (C).

Interestingly, no cat that became re-infected with FCoV developed FIP, in contrast to laboratory infections, where

second infections commonly lead to more rapid and fulminant development of FIP (Vennema *et al.*, 1990), a phenomenon known as antibody-dependent enhancement.

The factors that cause a cat to become a carrier or to be transiently infected are unknown. It has been proposed that a deletion or mutation must occur in FCoV for it to cause FIP in the host (Poland *et al.*, 1996; Vennema *et al.*, 1998), so it seemed reasonable to expect that carrier status of the host be a consequence of genetic change in the virus. In household J, cats became re-infected with a type of FCoV that appeared to be more persistent than the previous indigenous virus, since it continued to be shed by a high proportion of cats for at least 18 months, including cats that had eliminated previous strains within a year. However, it was notable that the novel strain did not superinfect the existing persistently infected cat. With the exception of the cats mentioned above, most transiently infected cats did not become re-infected while they were shedding virus or were seropositive. However, after recovery from shedding and the loss of antibodies, these cats could become re-infected with the same or a different virus. More research is required to define the roles of both virus variation and host immune response that allow persistence of this virus. One possible explanation for the persistence of FCoV in healthy carrier cats is that they maintain a cell-mediated response indefinitely, with a focus of infection acting as a perpetual antigenic stimulus.

It has been proposed that cats infected with FCoV do not become superinfected, that is, infected with more than one strain of FCoV (Herrewegh *et al.*, 1997). We have conflicting evidence on that subject. Thus, in one household, cat J9 did not become infected with the more infectious strain *c* virus, while in two other households, two other cats, C12 and P2, appeared to be infected with two virus strains.

In three cats (H2, H9 and Hub1; data not shown) from two households there was a deletion of 6 nt (resulting in the loss of 2 aa). These cats were from different breeds and had never had contact but were from neighbouring counties. This finding supports the conclusion of Vennema *et al.* (1998): there are many geographical differences in FCoV strains. Had these cats had FIP or been carriers, it might have been tempting to conclude that this deletion was responsible for the phenotypic change, whereas it was merely a geographical difference. This finding illustrates the dangers of drawing conclusions about virus virulence from changes in limited regions of the FCoV genome of only a few isolates.

In conclusion, we present a simple molecular biological method for examination of natural strains of FCoV. This method was used to show that most FCoVs infecting cats in the UK are type I. Carrier cats shed the same strain of FCoV continually. Most cats eliminate FCoV infection but are susceptible to re-infection with the same or a different FCoV strain. Whether a cat becomes a carrier or simply transiently infected appears to be a property of the cat rather than a property of the virus strain.

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