Studies on feline calicivirus with particular reference to persistence.

by

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CONTENTS.

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ABBREVIATIONS.

cpe	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DIP	Defective interfering particles
DNA	Deoxyribonucleic acid
dn	Non-synonymous mutations per non-synonymous site
dNTP	Deoxynucleotide triphosphate
ds	Synonymous mutations per synonymous site
EBHS(V)	European brown hare syndrome (virus)
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
FCV	Feline calicivirus
FIV	Feline immunodeficiency virus
FPV	Feline picornavirus
HCMV	Human cytomegalovirus
HEV	Hepatitis E virus
HuCV	Human calicivirus
HIV	Human immunodeficiency virus
HV	Hypervariable
HVR	Hypervariable region
mAb	Monoclonal antibody
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility
NK	Natural killer
NV	Norwalk virus
ORF	Open reading frame
PCR	Polymerase chain reaction
RHD(V)	Rabbit haemorrhagic disease (virus)
RNA	Ribonucleic acid
SMSV	San Miguel sea lion virus
SRSV	Small round structured virus
TAP	Transporter associated with antigen processing
URTD	Upper respiratory tract disease
VES(V)	Vesicular exanthema of swine (virus)
VN	Virus neutralisation
VPg	Genome-linked viral protein
wt	Wild-type

ABSTRACT.

The molecular evolution of feline calicivirus (FCV) was studied in cell culture and in persistently infected cats. Sequence analysis of the 5' hypervariable region of the FCV capsid (5'HVR; located at the 5'end of variable region E), a region known to contain linear neutralising B cell epitopes, showed FCV existed as a quasispecies which evolved at the nucleotide and amino acid level during persistent infection. Quasispecies heterogeneity tended to decrease during the course of persistence. Sequential isolates from a cat showed marked antigenic variation during the course of persistent infection.

Sequential passage of FCV in cell culture was also associated with sequence evolution of the 5'HVR. However, these isolates showed no change in antigenicity suggesting that individual substitutions observed in viruses from cats, but not in viruses from cell culture, may be responsible for changes in antigenicity. Alternatively, the observed antigenic changes may be associated with mutations elsewhere in the genome.

In order to identify regions of the FCV capsid protein containing linear B-cell epitopes, two approaches were used. Firstly, an expression library containing random, short (100-300bp) fragments of an FCV capsid gene was constructed. This library was screened using polyclonal antisera from a cat that had been challenged experimentally with FCV to identify immunoreactive clones containing B-cell epitopes. Initial screening identified five clones that reacted positively to feline antisera in immunoblots. FCV derived sequence from these clones all mapped to the 5'HVR suggesting this region contains the immunodominant linear epitopes of the capsid.

The second approach used to identify B-cell epitopes was to map more accurately the epitope of a neutralising monoclonal antibody (IG9) which had already been shown to lie in a 37 amino acid region of the 5'HVR (Milton *et al.* (1992), Journal of General Virology 73, 2435-2439). Replication of plaque purified IG9-sensitive parent virus in sub-neutralising concentrations of IG9 led to the generation of a neutralisation resistant escape mutant. Sequence analysis of this mutant and the parent virus revealed a single non-synonymous nucleotide substitution within the 5'HVR suggesting this residue is critical to the correct formation of the IG9 epitope.

A method to type FCVs based on sequence analysis of the 5'HVR was established. Most isolates appeared relatively homogenous. However, some isolates, both from vaccines and vaccine failures, appeared to contain more than one FCV. Comparison of 5'HVR sequences from different isolates showed that most isolates were either 0-5.3% different (related isolates) or 20.7-42.7% different (unrelated isolates). The majority of the related isolates shared an epidemiological link, implying they represented isolates that originated from a common source. Comparisons of sequences obtained from vaccine failures and vaccine virus fell into two similar categories; those with closely related sequences (0.0-5.3%) implying a role for the vaccine in disease and those with divergent sequences (21.3-38.7%) implying field virus caused the disease.
These results were compared with those obtained by using a serotyping method based on virus neutralisation (VN) which exploits differences in antigenicity between most FCVs gene (Dawson *et al.* (1993), Veterinary Record 132, 346-350). VN and sequence analysis gave the same typing result in 65-73% of individual cases. Based on these results and the difficulty of interpreting VNs, we suggest that molecular based sequence analysis may be more suitable to the epidemiological investigation of FCV related disease particularly in the case of vaccine reactions.

INTRODUCTION.

1 The *Caliciviridae.*

The *Caliciviridae* are a large, diverse and expanding family of viruses. Initially, due to several shared characteristics, early members were incorrectly grouped in the *Picornaviridae* (Fenner, 1976). However, important differences between the two families have established the *Caliciviridae* as a relatively new virus family (Melnick, 1974; Cubitt *et al.*, 1995) distinct from the *Picornaviridae*. Despite this classification, the *Caliciviridae* still contain many similarities to the *Picornaviridae* and this is reflected in their shared classification in RNA virus superfamily II (Strauss & Strauss, 1988).

2 Members of the *Caliciviridae*.

2.1 Vesicular exanthema of swine virus (VESV).

The first reported outbreak of vesicular exanthema of swine (VES) was in California in 1932 (described in Sawyer, 1976; Smith & Akers, 1976). The importance of VES largely resided in its similarity to foot and mouth disease, both inducing vesicles which later break down to form erosions and ulcers (House & House, 1992). VES was largely confined to the United States of America and at its height affected 42 states due to transport of infected individuals and infected pig meat. Following the introduction of radical control measures, including slaughter of affected pigs and heat treatment of pig feed, the disease was declared eradicated from the United States in 1959 (USDA, 1959). The isolation of a virus from aborting sea lions on the island of San Miguel (San Miguel sea lion virus; SMSV) off the coast of California with similar physicochemical characteristics to VESV, and the demonstration that this new virus was capable of inducing a disease in pigs similar to VES (Smith *et al.*, 1973), led to the suggestion that VESV may have spread from sea lions or similar species to domestic pigs (Breese & Dardiri, 1977; Sawyer *et al.*, 1978), probably in contaminated feed. This was supported by serological evidence of SMSV infection

in marine and terrestrial mammals (Prato *et al.*, 1974). More recently, the isolation of a serotype of SMSV from ocean fish which is also capable of inducing VES-like disease in experimental pigs (Smith *et al.*, 1980) has suggested the possibility that fish may represent a potential reservoir of SMSV spreading to pigs either directly or via marine mammals.

2.2 San Miguel sea lion virus.

San Miguel sea lion virus (SMSV) was first isolated from a rectal swab of an aborting Californian sea lion from the island of San Miguel off the coast of California (Smith *et al.*, 1973; Studdert, 1978). However, a causal relationship between SMSV and abortion has not been demonstrated (Smith *et al.*, 1973). Subsequently, the isolation of serologically related but distinct viruses from Stellar sea lions (Skilling *et al.*, 1987), fish, elephant seals and a sea lion trematode (Smith *et al.*, 1980), and northern fur seals (Sawyer *et al.*, 1978; Smith *et al.*, 1981), has brought the number of SMSV serotypes to 17.

The importance of SMSV rests in its ability to induce VES-like disease in pigs (see above) and its close relation with VESV which has recently been confirmed by phylogenetic analysis based on non-structural genes (Neill *et al.*, 1995).

2.3 Rabbit haemorrhagic disease virus

An acute, highly infectious, and usually fatal disease of rabbits characterised by hepatic necrosis and haemorrhage, was first reported in China in 1984 (rabbit haemorrhagic disease; RHD) (Liu *et al.*, 1984). Following early difficulties in classification (Gregg & House, 1989), the new virus, termed rabbit haemorrhagic disease virus (RHDV), was characterised as a calicivirus (Ohlinger *et al.*, 1990; Parra & Prieto, 1990). The distribution of the disease has subsequently become largely world-wide, first appearing in the United Kingdom in 1992 (Fuller *et al.*, 1993). In 1995, RHDV was introduced into Australia (Tribe, 1995; Cooke, 1997) following its inadvertent release from an experimental facility on a off-shore island, where field trials were underway to evaluate the use of RHDV as a biological control agent of

large rabbit populations (Robinson & Westbury, 1997). In 1997, following a decision earlier that year not to use RHDV as a biological control agent in New Zealand (O'Hara, 1997), the first cases of RHD were also reported in New Zealand (Ministry of Agriculture, 1997).

Retrospective analysis of rabbit sera collected between 1975 and 1985, well before RHD became widespread in Europe, demonstrated the presence of anti-RHDV antibodies (Rodák *et al.*, 1990) suggesting the existence of a non-pathogenic virus antigenically related to RHDV. Recently, such a virus has been isolated and characterised (Capucci *et al.*, 1996). Their is some confusion regarding the nomenclature of the caliciviruses of rabbits as the term rabbit calicivirus is being used both in Australia and New Zealand to describe virulent RHDV, and by others as the name for the newly characterised avirulent virus related to RHDV (Capucci *et al.*, 1996). In order to avoid any confusion, the author will refer to all caliciviruses of rabbits as RHDV, and where the distinction is necessary, will describe them as virulent or avirulent.

An comprehensive review article on RHD and RHDV has recently been published (Chasey, 1997).

2.4 European brown hare syndrome virus.

A disease of European brown hares termed European brown hare syndrome (EBHS) was first described in the 1980s and has spread throughout most of Europe including the U.K. (Chasey & Duff, 1990). Despite similarities between EBHS and RHD (Henriksen *et al.*, 1989; Moussa *et al.*, 1992) and between EBHS virus (EBHSV) and RHDV (Chasey *et al.*, 1992; Wirblich *et al.*, 1994), the viruses remain host-specific, unable to induce disease out of their natural host (Chasey *et al.*, 1992). Most recently, antigenic analysis has suggested that the two viruses represent two serotypes of the same serogroup (Laurent *et al.*, 1997). However, phylogenetic analysis based upon capsid gene sequence places the two viruses in distinct groups (Nowotny *et al.*, 1997).

2.5 Human caliciviruses.

Acute, self-limiting, non-bacterial gastroenteritis was a long recognised condition of people often referred to as winter vomiting disease due to its frequent seasonal incidence. An infectious aetiology was first described for an outbreak in Norwalk, Ohio by recreating the disease in healthy 'volunteer' prisoners challenged orally with bacterial-free stool filtrates from diseased individuals (Dolin *et al.*, 1971). Subsequently, a virus was detected in an infectious stool filtrate from the same outbreak by immune electron microscopy and termed Norwalk virus (NV) (Kapikian *et al.*, 1972). Virus particles with similar morphology have since been isolated from numerous outbreaks of acute, non-bacterial gastroenteritis and named according to the geographical location of isolation (Kogasaka *et al.*, 1981). All these viruses are termed 'small round structured viruses' (SRSVs) as they possess an amorphous surface structure lacking obvious geometric symmetry (Caul & Appleton, 1982). The term SRSV is used synonymously with Norwalk-like viruses.

The first report of caliciviruses in humans showing the typical "star of David" morphology (classic HuCVs) was in 1976 in stool samples from children in Glasgow (Madeley & Cosgrove, 1976). Subsequently, similar viruses have been isolated from outbreaks of acute, non-bacterial gastroenteritis in children and elderly patients throughout the world (reviewed by Caul, 1996b).

As well as being distinct morphologically, the SRSVs and classic HuCVs divide upon the epidemiological characteristics of the diseases they are associated with. The former are primarily associated with acute, non-bacterial gastroenteritis of children and adults whereas classic HuCVs are rarely implicated in disease of adults. In SRSV associated disease, high secondary infection attack rates contrast to the relatively low level of secondary infection in the classic HuCVs. The immunological response to the two virus groups also differs with SRSV immunity being relatively short lived allowing re-infection whereas classic HuCV immunity tends to last longer.

Sequence analysis has allowed the human caliciviruses to be divided into three genogroups (reviewed by Carter & Cubitt, 1995; Green *et al.*, 1997). Genogroup I contains SRSVs alone and includes NV. Genogroup II contains SRSVs and classic HuCVs (Green *et al.*, 1994; Dingle *et al.*, 1995). Genogroup III currently only

contains classic HuCVs (Lambden *et al.*, 1994; Matson *et al.*, 1995). Antigenic analysis has allowed the SRSVs to be divided into at least four serogroups (Lambden *et al.*, 1993). However, there is only a poor correlation between genogroups and serogroups (Carter & Cubitt, 1995).

A review by Caul has recently been published and covers the clinical, diagnostic and epidemiological factors of SRSV and classic HuCV infection (Caul, 1996a, b).

2.6 Hepatitis E virus

Outbreaks of enterically transmitted, non-A, non-B hepatitis, suggested a role for a novel viral agent in epidemics of human hepatitis (Khuroo, 1980). Subsequently, due to important differences with other known viral aetiologies of hepatitis (hepatitis A, B, C and D), the virus was tentatively classified as a calicivirus (Bradley & Balayan, 1988) and given the name hepatitis E virus (HEV) (Reyes *et al.*, 1990). Whilst the classification of HEV within the *caliciviridae* is currently accepted (Cubitt *et al.*, 1995; Berke *et al.*, 1997) it is still somewhat controversial due to important differences in genome organisation (Cubitt *et al.*, 1995) and codon usage (Berke *et al.*, 1997) with the other members of the family. Such differences have led to the speculation that HEV may be related to another, as yet uncharacterised virus family (Berke *et al.*, 1997).

2.7 Feline calicivirus.

During a project initially undertaken to attempt the isolation of the viral agent responsible for feline panleucopenia, Fastier reported the isolation of an unrelated virus capable of inducing intense cytopathic effect (cpe) in cell culture which he called kidney cell degenerating virus (KCD) (Fastier, 1957). Subsequently, further reports were made of viruses from cats capable of inducing similar cpe (Crandell & Madin, 1960; Crandell *et al.*, 1960). These viruses were tentatively grouped together on the basis of low serological cross-reactivity and similar cpe (Crandell & York, 1966). However, a clear association between the new group of viruses and a disease was still not apparent; indeed Fastier had suggested that the KCD isolate was a "filterable

agent in search of a disease" or an "orphan virus" (Fastier, 1957). Early attempts to classify these viruses initially suggested they were feline picornaviruses (FPVs) (Burki, 1965). However, this was later disputed due to important differences between the physicochemical (Studdert *et al.*, 1970) and morphological (Zwillenberg, 1966; Almeida, 1968) characteristics of the new viruses and the known picornaviruses. However, reports in the literature continued to refer to the new viruses as FPVs (Spradbrow *et al.*, 1970; Love & Baker, 1972; Hoover & Kahn, 1973; Povey *et al.*, 1973). It was not until 1974 that on the basis of capsid structure, VESV and FPV were suggested to be members of a new virus family, the *Caliciviridae* (Burroughs & Brown, 1974) and FPV became feline calicivirus (FCV).

Unlike SMSV and the human caliciviruses (see above), FCVs are considered to constitute a single serotype (Kalunda *et al.*, 1975; Burki *et al.*, 1976) although the majority of isolates can be distinguished using conventional cross-neutralisation tests (Povey, 1974; Knowles *et al.*, 1990).

FCV is discussed in more detail in section 4 below.

2.8 Other caliciviruses.

Candidate caliciviruses have now been isolated from many species (Table 1). However, in many cases, their classification is on the basis of limited evidence and therefore may best be termed calicivirus-like.

Virus	Reference
Canine calicivirus	Evermann *et al.*, 1981, 1985; Schaffer *et al.*, 1985; Crandell, 1988; Sangabriel *et al.*, 1996, 1997.
Bovine enteric calicivirus	Smith, *et al.*, 1983a; Bridger *et al.*, 1984; Bridger & Dastjerdi, 1997; Dastjerdi *et al.*, 1997.
Mink calicivirus	Long *et al.*, 1980; Evermann *et al.*, 1983.
Primate calicivirus	Smith, *et al.*, 1983b, 1985a, b.
Insect calicivirus	Hillman *et al.*, 1982.
Reptile calicivirus	Smith, *et al.*, 1986.
Amphibian calicivirus	Smith, *et al.*, 1986.
Avian caliciviruses	Gough & Spackman, 1981; Wyeth *et al.*, 1981; Gough *et al.*, 1992, 1997; Sironi, 1994.

Table 1; Calicivirus-like particles isolated from other species. In many cases, classification is purely on the basis of morphology.

3 Properties of the *Caliciviridae*.

Early attempts to classify caliciviruses led to their initial classification in the *Picornaviridae* (Melnick, 1974; Fenner, 1976). Subsequently, analysis of physico-chemical properties, morphology and capsid structure, identified the *Caliciviridae* as a distinct family (Schaffer, 1979). More recently, features of genome transcription and organisation have come to be the hallmarks of the *Caliciviridae*. However, the morphology of the viral capsid is still one of the major determinants that initially classifies a virus as a calicivirus.

3.1 Capsid structure.

Early work on SMSV, VESV and FCV demonstrated that the capsid contained only a single major capsid protein (Bachrach & Hess, 1973; Burroughs & Brown, 1974; Burroughs *et al.*, 1978). This distinguished them from the known picornaviruses (Minor *et al.*, 1995) and was instrumental in the establishment of the caliciviruses as a distinct viral family. More recently, a second minor capsid protein has been identified in the second open reading frame (ORF2) of RHDV (Wirblich *et al.*, 1996). However, since virus particles can be assembled *in vitro* from just the major capsid protein (Nagesha *et al.*, 1995; Green *et al.*, 1997; Laurent *et al.*, 1997), it has been suggested that this minor structural protein may facilitate the interaction of viral RNA and capsid protein thereby facilitating encapsidation (Wirblich *et al.*, 1996).

3.2 Morphology.

Calicivirus virions range in diameter from 35-40nm making them considerably smaller than the picornaviruses. However, it was the distinct appearance of the capsid surface which strongly suggested the caliciviruses were a separate family (Burki, 1965; Zwillenberg, 1966). Using negative staining electron microscopy (EM) the surface of the virus appeared to be covered in cup-like depressions and it was from this feature that name of the virus originated (calyx being latin for cup or chalice).

More recently, the 3-dimensional structure of the capsid has been determined by electron cryomicroscopy for both a calicivirus of classic morphology (Prasad & Matson, 1994) and an SRSV (Prasad *et al.*, 1994). In both cases, the capsid was shown to be made up of 90 arch-like capsomeres, each a dimer of the capsid protein, arranged in a T=3 icosahedral symmetry. This confirmed earlier work based on the molecular weight of the capsid protein (Burroughs *et al.*, 1978). In the case of the classic calicivirus morphology, the capsomeres were arranged around 32 large surface hollows at the icosahedral 5- and 3-fold axes which may correspond to the cup-like depressions seen under the EM (Prasad & Matson, 1994).

3.3 Genome structure.

Sequence analysis of caliciviruses has shown the whole genome is between 7.2 and 7.7kb. It has positive sense (Oglesby *et al.*, 1971) ribonucleic acid (RNA) genome (Bachrach & Hess, 1973) which, as in the picornaviruses, is covalently linked to a small, 10 to 15kDa protein termed VPg (genome-linked viral protein) at the 5' end (Burroughs & Brown, 1978; Schaffer *et al.*, 1980; Wimmer, 1982; Meyers *et al.*, 1991a; Herbert *et al.*, 1997)(Figure 1). Removal of VPg by protease digestion renders calicivirus RNA uninfectious (Burroughs & Brown, 1978) and it has recently been demonstrated that for FCV, *in vitro* transcribed genomic RNA which lacks a VPg is only infectious following the addition of a mammalian cap analogue (Sosnovtsev & Green, 1995).

The genome is polyadenylated at the 3'-end (Ehresmann & Schaffer, 1977; Black *et al.*, 1978)(Figure 1). The 3' non-coding region of the caliciviruses is predicted to contain RNA stem-loop structures (Seal *et al.*, 1994) which in other viruses, are believed to be important for RNA replication (Weiner & Maizels, 1987).

3.4 Genome organisation.

A distinguishing feature of the calicivirus genome organisation is that the non-structural proteins are located at the 5' end of the genome (Neill, 1990) and precede

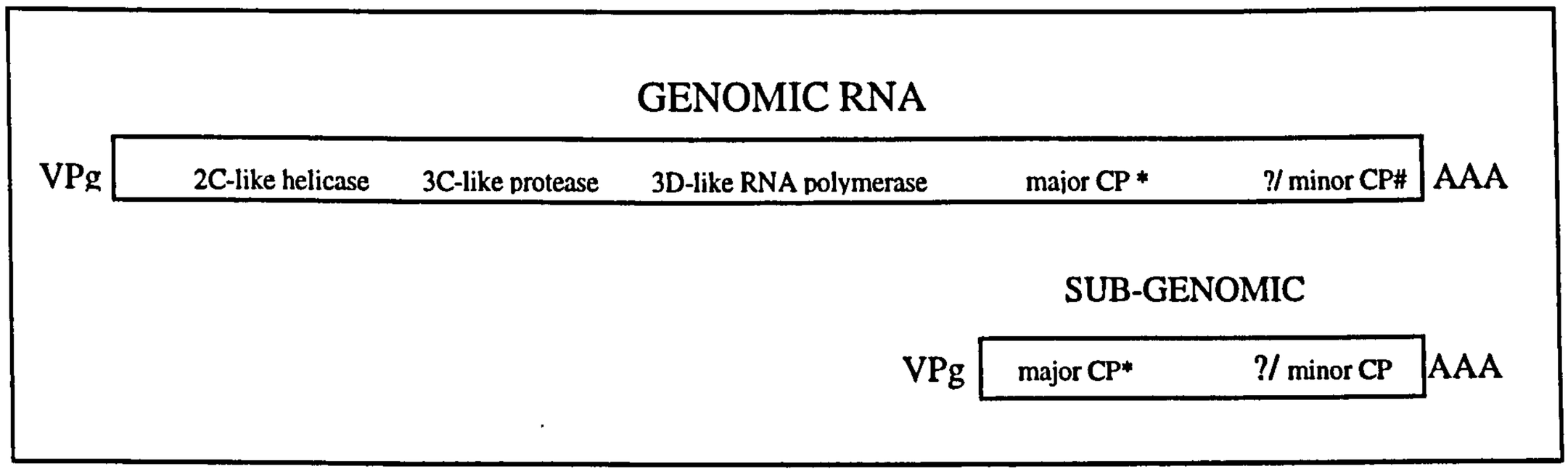


Figure 1; Generic putative genomic RNA structure and gene organisation of the caliciviruses. Both genomic and sub-genomic RNAs are polyadenylated at the 3' end and linked to protein (VPg) at the 5' end. The non-structural proteins, identified on the basis of homology with the non-structural proteins of picornavirus (2C-like helicase, 3C-like protease and 3D-like RNA-dependent RNA polymerase), are encoded towards the 5' end of the genome. *CP; capsid protein. # ?/minorCP; this small ORF has recently been identified as a minor structural protein in RHDV (Wirblich *et al.*, 1996).

the major structural protein (Figure 1). This contrasts to the *Picornaviridae*, where this order is reversed (Minor *et al.*, 1995).

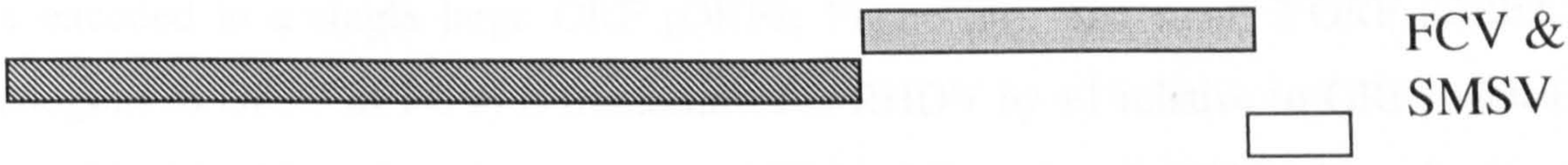
Entire genomic sequences are available for FCV (Neill, 1990; Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Oshikamo *et al.*, 1994; Sosnovtsev & Green, 1995; Glenn, 1997), RHDV (Meyers *et al.*, 1991b; Rasschaert *et al.*, 1994; Gould *et al.*, 1997), EBHSV (Le Gall *et al.*, 1996), SRSV (Jiang *et al.*, 1993; Lambden *et al.*, 1993, 1995; Dingle *et al.*, 1995; Hardy & Estes, 1996), classic HuCV (Liu *et al.*, 1995, 1997) and HEV (Tam *et al.*, 1991). Analysis of such sequences demonstrates a basic conserved genome organisation for the *Caliciviridae*. The 5' end of the genome encodes the non-structural proteins in a single large open reading frame (ORF) and includes by homology with the picornaviruses, a 2C-like helicase, a 3C-like cysteine protease and a 3D-like RNA-dependent RNA polymerase (Figure 1). Downstream of this region is the gene for the major capsid protein. This is followed at the 3'end of the genome by a small ORF which has recently been shown in RHDV to encode a minor structural protein (Wirblich *et al.*, 1996).

Within this overall conserved genome organisation, the *Caliciviridae* may be divided into those in which the ORF encoding the non-structural proteins is separate from that encoding the major capsid protein (FCV, SMSV, SRSV and HEV; Figure 2a) and those in which the two ORFs are fused in-frame creating a single large ORF (RHDV, EBHSV and classic HuCV; Figure 2b).

In FCV, the non-structural ORF (ORF1) and major capsid ORF (ORF2) are separated by two nucleotides and the start codon of the small 3' ORF overlaps the termination codon of ORF2 by four nucleotides (Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Glenn, 1997). The genome organisation of SMSV is similar to FCV except there are five nucleotides between the stop codon of ORF1 and the start codon of ORF2 (Neill, 1992).

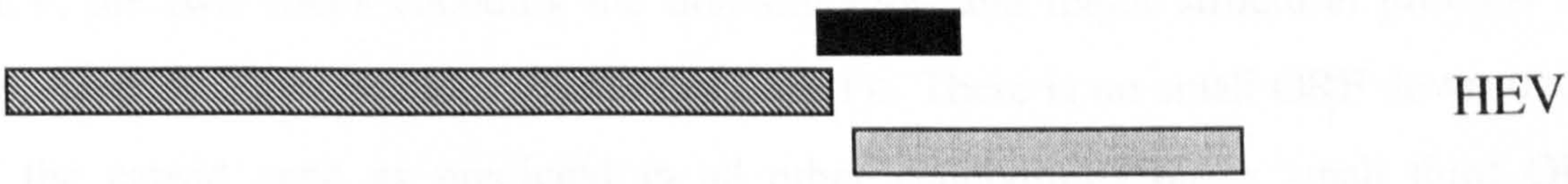
The SRSVs again have a similar genome organisation to FCV in having three distinct ORFs (Figure 2a). However, ORF1 and ORF2, rather than being separated, overlap* one another by 17 (Jiang *et al.*, 1993), 17 (Lambden *et al.*, 1993) or 20 (Dingle *et al.*, 1995) nucleotides for Norwalk, Southampton and Lordsdale viruses respectively, ORF2 always being frameshifted +1 relative to ORF1. In each SRSV, the stop codon

2a; Basic Genome Type 1; major capsid and non-structural proteins are encoded in separate ORFs.



ORF1 and ORF2 are separated by 2 (FCV) and 5(SMSV) nucleotides. The start codon of ORF3 overlaps the termination codon of ORF2 by 4 nucleotides in both FCV and SMSV.

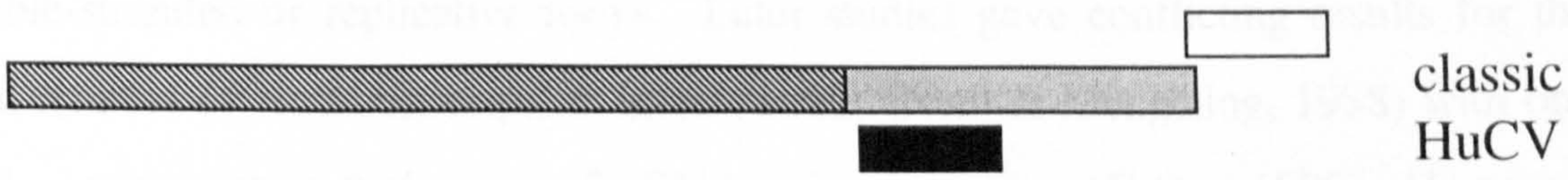
ORF2 overlaps ORF1 always maintaining +1 frameshift. The start codon of ORF3 overlaps the termination codon of ORF2 by 1 nucleotide.



Stop codon of ORF1 and start codon of ORF2 are separated by 37 nucleotides. A third small ORF overlaps ORF1 and ORF2.

2b; Basic Genome Type 2; major capsid and non-structural proteins are encoded in a single ORF.

ORF1 contains both the non-structural and major capsid proteins. ORF2 overlapps ORF1 by 20 nucleotides.



The start codon of the 3'ORF overlaps the stop codon of ORF1 by a single nucleotide. A third potential ORF overlaps the capsid gene region of ORF1.

Figure 2; Genome organisation amongst the caliciviruses (see text). The different regions of the genome are indicated by diagonal striped boxes (non-structural proteins), grey boxes (major capsid protein), open boxes (putative minor structural protein) and black boxes (putative ORFs).

of ORF2 and initiation codon of ORF3 overlap by a single nucleotide.

For RHDV, EBHSV and classic HuCV, the non-structural proteins and major capsid are encoded in a single large ORF (ORF1; Figure 2b). The small 3'ORF (ORF2; analogous to ORF3 of FCV) is frameshifted in RHDV by +1 relative to ORF1 which it overlaps* by 20 nucleotides in virulent RHDV (Meyers *et al.*, 1991b; Rasschaert *et al.*, 1994; Gould *et al.*, 1997) and 8 nucleotides in the avirulent RHDV (Capucci *et al.*, 1996) and EBHSV (Le Gall *et al.*, 1996). In the classic HuCV, ORF2 is frameshifted by -1 relative to ORF1, the ORF2 start codon overlapping the stop codon of ORF1 by a single nucleotide (Liu *et al.*, 1995).

Further variations on these two basic genome arrangements have been predicted. In HEV, the two ORFs encoding the non-structural and major structural proteins are separated by 37 nucleotides (Tam *et al.*, 1991). There is no small ORF downstream of the capsid gene as predicted in all other caliciviruses but a small third ORF encoding an immunoreactive epitope partially overlaping the first and second is predicted in the -1 frame relative to ORF1 (Tam *et al.*, 1991)(Figure 2a). In the classic HuCVs, a third ORF potentially encoding for a small basic protein overlaping the capsid region of ORF1 is predicted on the basis of sequence analysis (Liu *et al.*, 1995)(Figure 2b).

3.5 RNA.

Early studies on intracellular RNA suggested the presence of both a large (presumed full-length genome) and a small but abundant sub-genomic RNA (Ehresmann & Schaffer, 1977, 1979; Black *et al.*, 1978). Both were present in single-stranded and double-stranded or replicative forms. Later studies gave conflicting results for the total number of viral transcripts in infected cells (Neill & Mengeling, 1988) with one study demonstrating 8 virus specific RNAs of various sizes (Carter, 1990). However, recently only two intracellular RNA species have been demonstrated for FCV in cell culture corresponding to the genome and a 3' co-terminal sub-genomic mRNA (Figure 1) (Meyers *et al.*, 1991a; Herbert *et al.*, 1996). The start of the sub-genomic mRNA has been mapped in FCV strain F9 (Herbert *et al.*, 1996) and CFI/68 (Neill *et*

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* For consistency, the ORF1/ ORF2 overlap has been calculated by including the ORF1 stop codon.

al., 1991) and corresponds to nucleotides 5296-5297 of the F9 genomic RNA (Carter *et al.*, 1992a).

Like the full-length genome, the sub-genomic RNA is covalently linked at its 5' end to a VPg (Figure 1) (Meyers *et al.*, 1991a; Herbert *et al.*, 1997) and in the case of RHDV, may also be packaged into viral capsids (Meyers *et al.*, 1991a). The major capsid protein is believed to be translated primarily from this sub-genomic mRNA (Neill *et al.*, 1991; Herbert *et al.*, 1996) along with in FCV, a small protein encoded down-stream of the capsid gene in ORF3 (Herbert *et al.*, 1996). This ORF is analogous to ORF2 of RHDV, which has recently been shown to encode a minor structural protein (Wirblich *et al.*, 1996).

Analysis of entire calicivirus genomes has demonstrated the presence of a conserved sequence at the 5' end of both the genomic and sub-genomic RNAs (Meyers *et al.*, 1991a; Carter *et al.*, 1992a; Lambden *et al.*, 1995; Hardy & Estes, 1996). It has been suggested that this may be involved in the regulation of replication, translation or packaging.

3.6 Proteins encoded by the calicivirus genome.

Proteins encoded by the calicivirus genome, both known and potential, fall predominantly into one of two categories; non-structural and structural.

3.6.1 Non-structural proteins.

The non-structural proteins of the Caliciviridae are encoded in a large ORF at the 5' end of the genome. In the case of RHDV, EBHSV and the classic HuCVs (basic genome type 2; figure 2b) this ORF is fused in frame with the gene for the major structural protein (see above). Following the production of a large polyprotein from ORF1, co- and post-translational cleavage by viral-encoded proteases is required to release functional proteins. Such a strategy is common to many positive-stranded RNA viruses and some double-stranded RNA viruses, allows the activation of subsets of proteins with different biological functions from the same precursor molecule (Spall *et al.*, 1997). The various functions of the non-structural proteins are still poorly

characterised and have been largely assigned on the basis of conserved amino acid domains shared with viral proteins from the *Picornaviridae* (Neill, 1990). In common with a large number of other viruses the order of the genes in ORF1 is conserved, a 2C-like helicase being towards the N terminus, followed by a 3C-like protease and a 3D-like RNA dependent RNA polymerase (Argos *et al.*, 1984; Strauss & Strauss, 1988; Neill, 1990).

3.6.1.1 2C-like helicase.

The presence of a homologue of the 2C helicase of the picornaviruses has been demonstrated in most caliciviruses for which sequence from ORF1 is available (Neill, 1990; Meyers *et al.*, 1991b; Liu *et al.*, 1995; Neill *et al.*, 1995). The 2C protein of picornaviruses is believed to be involved in RNA synthesis. Mutants in 2C are deficient in RNA synthesis (Li & Baltimore, 1988; Mirzayan & Wimmer, 1992) and mutants which escape the selective inhibition of RNA synthesis by guanidine map to 2C (Pincus *et al.*, 1986; Baltera & Tershak, 1989). The 2C protein of picornavirus is a putative helicase (Mirzayan *et al.*, 1994; Paul *et al.*, 1994). There is also evidence for ATPase and GTPase activity in poliovirus 2C (Rodriguez & Carrasco, 1993). The presence of a motif, thought to be responsible for nucleotide binding has been identified within the putative 2C-like helicase of the caliciviruses (Neill, 1990; Meyers *et al.*, 1991b; Dingle *et al.*, 1995).

3.6.1.2 3C-like protease.

Like other potential proteins in ORF1 of the caliciviruses, the 3C-like protease was initially described only on the basis of a relatively low-level of sequence homology with the 3C cysteine proteases of the picornaviruses (Neill, 1990; Meyers *et al.*, 1991b; Boniotti *et al.*, 1994; Dingle *et al.*, 1995). However, more recently, the function of the 3C-like protease has been analysed in RHDV by expression in *Escherichia coli* (*E.coli*) and *in vitro* translation in rabbit reticulocyte cell-free lysate. *In vitro* translation of RNA corresponding to the 3C, 3D and capsid regions of ORF1 of RHDV led to the release of mature capsid (Boniotti *et al.*, 1994). Site-directed

mutagenesis using 3C protease expressed in *E.coli* largely confirmed the requirement of certain conserved residues that form the so-called catalytic triad in other 3C proteases (Boniotti *et al.*, 1994).

Analysis of cleavage products generated in *E.coli* identified the NH_2-terminal 3C-protease, COOH-terminal 3C-protease and NH_2-terminal capsid cleavage sites as glutamate-glycine, glutamate-threonine and glutamate-glycine respectively (Figure 3) (Wirblich *et al.*, 1995). Subsequently, site-directed mutagenesis was used to determine the specificity of the protease cleavage recognition site. The P1 position of the cleavage site had a requirement for glutamate, glutamine or aspartate; P1' preferred glycine, serine or alanine; P2 substitutions had little effect on cleavage efficiency (Wirblich *et al.*, 1995). A later study identified four gene products released from the ORF1 polyprotein during processing corresponding to an 80 kDa protein which includes the 2C helicase, a 43 kDa protein, a 73 kDa protein representing fused 3C-like protease and 3D RNA polymerase and the capsid (60 kDa)(Martín Alonso *et al.*, 1996). N-terminal sequence analysis confirmed earlier work on RHDV 3C-protease that for the 43 and 73 kDa fragments, cleavage occurred between glutamate and glycine peptide bonds (Martín Alonso *et al.*, 1996). The genetic map of polyprotein processing has been extended to include seven probable cleavage sites which are used *in vitro* to different extents in the order NH_2-p16-p23-2Chelicase(p37)-p30-VPg-3Cprotease-3Dpolymerase-capsid-COOH (Figure 3) (Wirblich *et al.*, 1996).

The only human calicivirus for which information on ORF1 polyprotein processing and 3C protease activity is available is an SRSV (Liu *et al.*, 1996). Three cleavage products were identified corresponding to an N-terminal 48kDa protein, the 41 kDa 2C helicase and a 113 kDa protein representing a fusion of 3C protease and 3D RNA polymerase. The cleavage sites which release the 2C-like helicase were identified as glutamine-glycine (Figure 3) (Liu *et al.*, 1996).

Recently, the cleavage sites that release the VPg and 3C protease have been reported for FCV and as in RHDV, there is a preference for glutamate at P1 (Figure 3) (Clarke & Lambden, 1997).

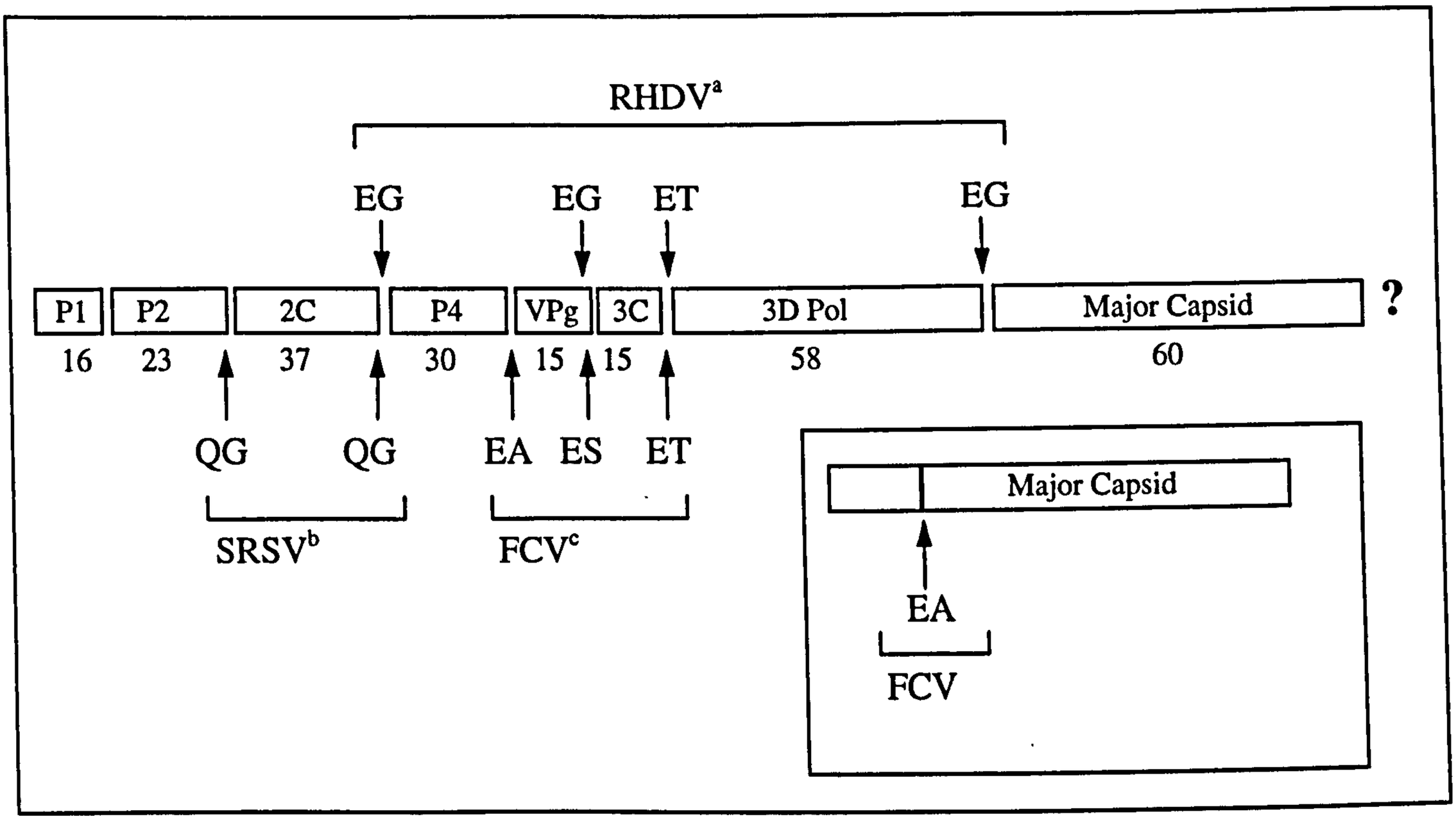


Figure 3; Summary of polyprotein cleavage in the caliciviruses (adapted from Clarke & Lambden, 1997). a; RHDV. b; Southampton SRSV. c; FCV. ?; the mechanism of production of the 3'ORF gene product is not known. Inset; cleavage site of the major capsid protein precursor in FCV (Carter *et al.*, 1992b). Amino acid abbreviations: A; alanine. E; glutamate. G; glycine. Q; glutamine. S; serine. T; threonine.

3.6.1.3. 3D RNA dependent RNA polymerase.

Sequence analysis has identified domains within the putative 3D polymerase of the caliciviruses including GLPSG, YGDD and FLKR (Neill, 1990; Meyers *et al.*, 1991b; Green, S.M. *et al.*, 1994; Oshikamo *et al.*, 1994; Liu *et al.*, 1995; Neill *et al.*, 1995) which are conserved in RNA polymerases of other plant, animal and bacterial viruses (Kamer & Argos, 1984). However, despite this, function has not been attributed to this region of the genome.

3.6.2 Major structural protein

Caliciviruses possess a single major capsid protein which in FCV and VESV has been shown to be derived from a precursor protein by proteolytic cleavage (Fretz & Schaffer, 1978; Carter, 1989; Carter *et al.*, 1992b) probably utilising a viral protease (Shin *et al.*, 1993). In the case of RHDV, the mature capsid is produced *de novo*, predominantly from the subgenomic mRNA (Parra *et al.*, 1993) although, as mentioned above, the capsid is also released from the ORF1 polyprotein following proteolytic cleavage (Boniotti *et al.*, 1994; Wirblich *et al.*, 1995, 1996; Martín Alonso *et al.*, 1996).

Comparison of sequences from the capsid protein genes of different isolates of FCV and of other animal caliciviruses has allowed the capsid to be divided on the basis of sequence conservation into six regions, A-F (Neill, 1992; Seal *et al.*, 1993)(Figure 4). In the following discussion, each region is defined relative to FCV isolate F9 (Carter *et al.*, 1992a; Seal *et al.*, 1993)

3.6.2.1 Region A (amino acids 1-124).

The definition of the extent of region A of the capsid gene is currently unclear. It was initially described as that region which showed little homology between SMSV and FCV (Neill, 1992). This contrasted to region B where SMSV, FCV and RHDV are relatively similar. However, the same author stated that the border between region A and B was poorly defined and therefore only approximate. Subsequently, in the first report of multiple FCV capsid gene sequence comparisons, the poorly defined border

previously described between region A and region B was rigorously applied (Seal *et al.*, 1993). In that work, region A was defined as amino acids 1-120 of the capsid precursor corresponding to "the NH_2-terminal portion of the capsid precursor which is theoretically cleaved to produce the functional capsid protein". The same author stated that cleavage occurred at a conserved hexapeptide sequence FRLEAD in agreement with previous work (Carter *et al.*, 1992b) and placing the actual precursor cleavage site between amino acids 124 and 125. This site conforms to the reported cleavage requirements of the FCV 3C protease in the ORF1 polyprotein (see above and Clarke & Lambden, 1997). Therefore, the author will use the term region A in FCV to describe amino acids 1-124 of the capsid precursor which are theoretically cleaved to generate the mature capsid. Since the mechanism of precursor cleavage is not currently reported for FCV and the site is only reported for a single strain (Carter *et al.*, 1992b), further work to clarify the exact site of cleavage is required. The fate of the region A post-cleavage has not been determined.

The extent of variability within region A is somewhat controversial. It was initially described as that region which showed little homology between SMSV and FCV (Neill, 1992). Recently it has been identified as hypervariable (Geissler *et al.*, 1997). Using a similarity plot based on 18 FCV capsid gene sequences, it appears that region A is best defined in FCV as of intermediate variability (Glenn, 1997).

3.6.2.2 Region B (amino acids 125-396).

This region of the capsid is conserved both within FCV and between members of the *Caliciviridae*. There is also considerable homology with picornavirus structural protein VP3 (Tohya *et al.*, 1991) suggesting possible similarities in structure and function (Tohya *et al.*, 1991; Neill, 1992). In picornaviruses, this region is believed to fold into a β-barrel structure (Hogle *et al.*, 1985; Acharya *et al.*, 1989; Arnold & Rossman, 1990). A putative ATP/GTP binding site motif and a myristylation site are predicted to occur in region B (Seal *et al.*, 1993).

3.6.2.3 Region C (amino acids 397-401).

This region constitutes a small island of considerable variability both within the FCVs and between other animal caliciviruses.

3.6.2.4 Regions D and F (amino acids 402-425 and 524-668 respectively).

Regions of sequence conservation within the FCVs and other caliciviruses.

3.6.2.5 Region E (amino acids 426-523).

In FCV, region E has been further divided into a relatively conserved central sequence separating two hypervariable (HV) regions (Seal *et al.*, 1993; Seal, 1994; Glenn, 1997). The 5' HV region (corresponding to nucleotides 6589-6693 of the F9 strain of FCV (Carter *et al.*, 1992a) is strongly implicated as a target for antibody-mediated virus neutralisation as it contains epitopes for neutralising monoclonal antibodies (Milton *et al.*, 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997) and synthetic peptides which include this region induce the formation of neutralising polyclonal antisera (Guiver *et al.*, 1992). More recently, studies using an infectious clone of FCV strain Urbana showed that sequence corresponding to region E when cloned from FCV isolate NADC into the urbana infectious clone conveyed neutralisation characteristics of NADC upon the recombinant virus (Neill *et al.*, 1997). Epitopes mapped to the capsid protein are reviewed in figure 5.

3.6.2.6 Capsid variability in other caliciviruses.

RHDV demonstrates a high degree of sequence conservation between individual isolates of different geographical origin. In one study, only 30 amino acid differences existed between two isolates, and these clustered towards the N-terminus of ORF1 and the middle of the major capsid (Gould *et al.*, 1997).

For the human caliciviruses, variability between isolates has led to the division of the capsid into three regions. Region 1 and 3 are relatively conserved and equivalent to FCV regions B and F respectively. Region 2 is more variable, and equivalent to regions C-E of FCV (Lew *et al.*, 1994).

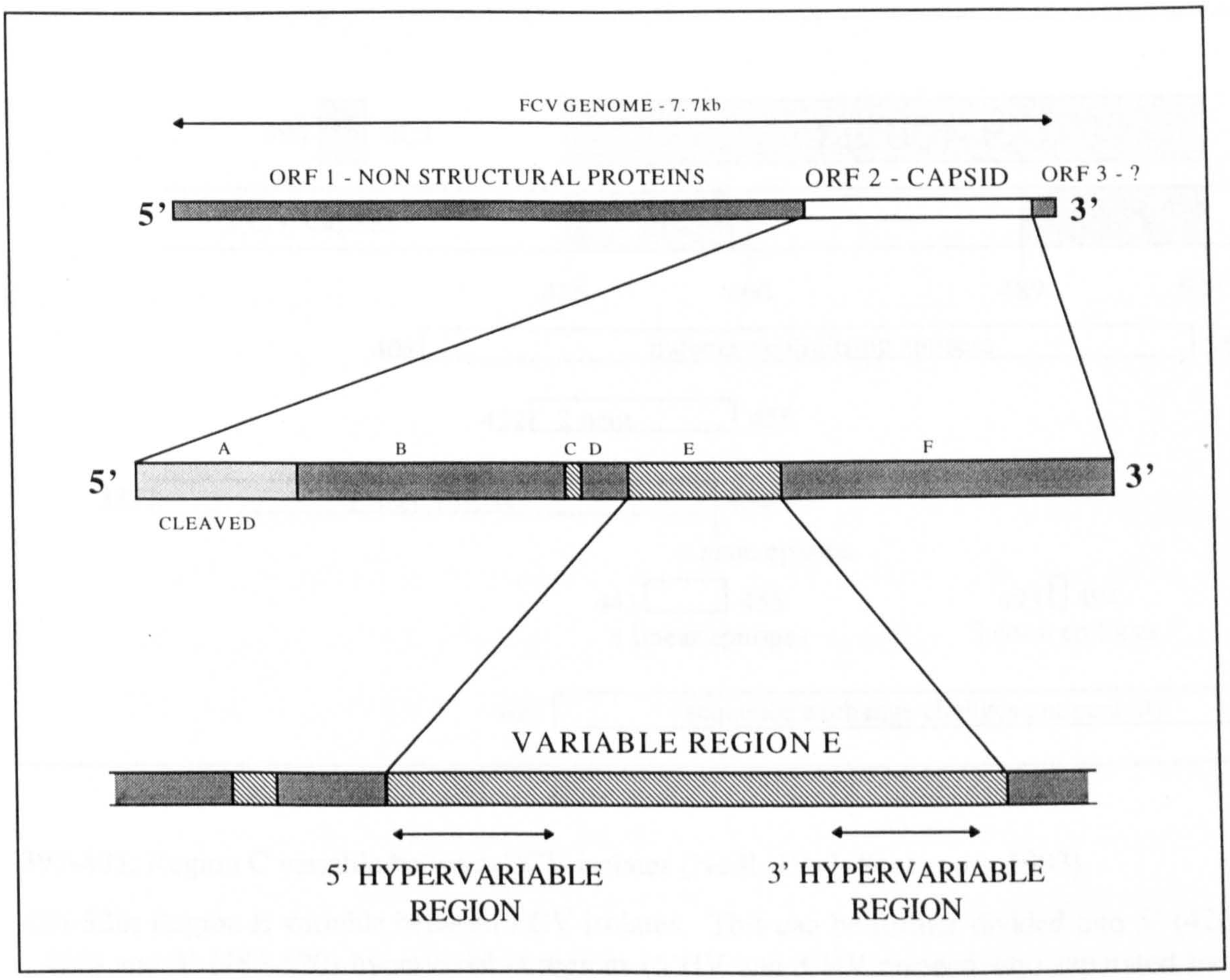
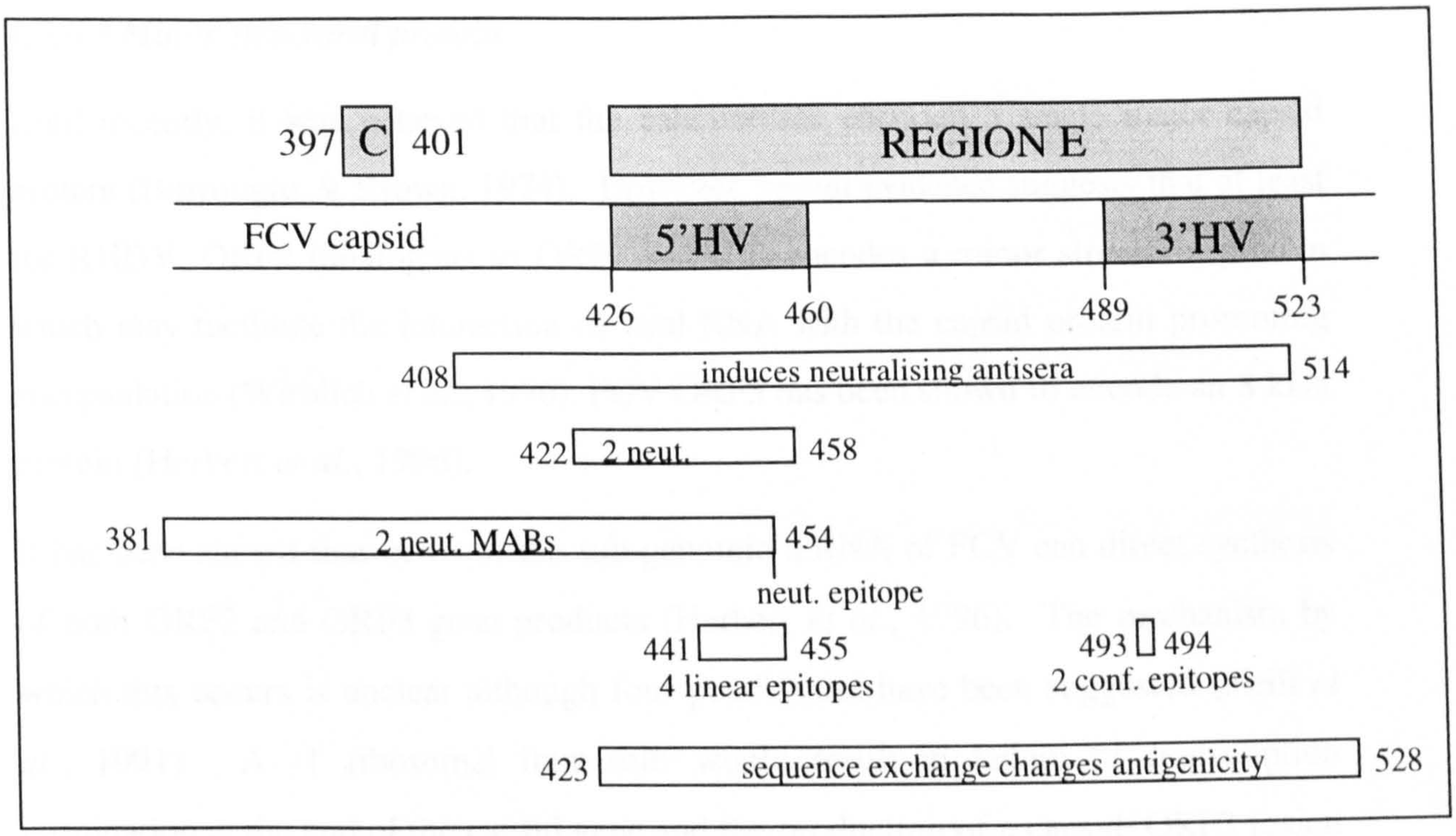


Figure 4; Capsid variability of FCV. ORF2 has been divided into regions A-F on the basis of sequence variability between different FCV isolates. Regions A,B,D and F are relatively conserved whilst regions C and E are variable. Region E has been further divided into a 5' and 3' hypervariable region (Neill, 1992; Seal *et al.*, 1993; Seal, 1994; Glenn, 1997).



397-401; Region C variable between FCV isolates (Neill, 1992; Seal *et al.*, 1993).

426-520; Region E variable between FCV isolates. This can be further divided into 5' (426-460) and 3' (489-520) hypervariable regions (5'HV and 3'HV respectively) separated by a conserved region (461-488) regions (Seal *et al.*, 1993; Seal, 1994; Glenn, 1997).

408-514; a recombinant peptide corresponding to this region of FCV F9 induced the formation of neutralising polyclonal antisera in rabbits, and cats vaccinated with F9 produced a polyclonal antisera that reacted to this peptide in immunoblots (Guiver *et al.*, 1992).

422-458; a recombinant peptide corresponding to this region contained the epitopes for 2 neutralising mouse monoclonal antibodies (mAbs) by immunoblot (Milton *et al.*, 1992).

381-454; recombinant capsid with region 381-454 deleted loses epitopes for 2 mAbs and the region around 454 is associated with two further epitopes (Shin *et al.*, 1993) (see also Tohya *et al.*, 1997).

441-445; sequence analysis of neutralising mAb escape mutants of FCV F4 localise 4 linear epitopes to this region (Tohya *et al.*, 1997).

493-494; sequence analysis of neutralising mAb escape mutants of FCV F4 localise 2 conformational epitopes to this region (Tohya *et al.*, 1997).

423-528; sequence corresponding to this approximate region exchanged from FCV NADC to Urbana conveyed neutralisation characteristics of NADC upon the NADC/ Urbana recombinant (Neill *et al.*, 1997).

Figure 5; Representation of known epitopes within the FCV major capsid protein. All numbers relate to FCV F9 (Carter *et al.*, 1992b), are for amino acids, and are inclusive.

1.3.6.3 Minor structural protein

Until recently, it was believed that the caliciviruses encoded a single major capsid protein (Burroughs & Brown, 1974). However, recent evidence suggests that at least for RHDV, ORF2 (analogous to ORF3 in FCV) encodes a minor structural protein which may facilitate the interaction of viral RNA with the capsid protein promoting encapsidation (Wirblich *et al.*, 1996). FCV ORF3 has been shown to encode an 8 kDa protein (Herbert *et al.*, 1996).

It has been shown that *in vitro*, the sub-genomic mRNA of FCV can direct synthesis of both ORF2 and ORF3 gene products (Herbert *et al.*, 1996). The mechanism by which this occurs is unclear although four possibilities have been suggested (Neill *et al.*, 1991). A -1 ribosomal frameshift would result in failure of transcription termination at the end of the capsid gene and the production of a capsid/ ORF3 fusion protein. Similar mechanisms are well characterised in retroviruses and coronaviruses (reviewed by Brierley, 1995) where infrequent frameshift may allow the relatively low level production of the down-stream ORF relative to the upstream ORF. Frameshift signals have been described which include a homopolymeric 'slippery' sequence of nucleotides and a downstream region of RNA secondary structure and these have been predicted to occur in FCV (Neill *et al.*, 1991; Neill, 1992). However, such RNA secondary structure is not predicted in the SRSVs (Jiang *et al.*, 1993) and the presence of the predicted ORF2/3 fusion protein has not been demonstrated.

A second potential mechanism for ORF3 translation is 'leaky scanning'. The scanning model for translation states that the 40S ribosomal subunit binds at the 5'-end of a mRNA and then proceeds towards the 3'-end, initiating translation at the first start codon in a favourable context (Kozak, 1978). Leaky scanning has been suggested as a mechanism whereby upstream start codons are ignored allowing translation to initiate further downstream (Kozak, 1987, 1989). However, this is thought unlikely to occur in the caliciviruses due to the poor context of the ORF3 start codon relative to upstream AUGs in more favourable contexts.

Thirdly, it has been suggested that following termination, the close context of the ORF2 stop codon and the ORF3 start codon may lead to re-initiation and translation of ORF3 as has been demonstrated in other bicistronic mRNAs. Finally, it has been

suggested that, using a similar mechanism described classically for the picornaviruses (Jackson *et al.*, 1990) but also for other viruses (Tsukiyama-Kohara *et al.*, 1992)and cellular mRNAs (Le & Maizel, 1997), ribosome complexes may initiate translation at the ORF3 start codon internally. These mechanisms have a requirement for an internal ribosome entry site (IRES) (Chen & Sarnow, 1995) associated with complex stem-loop RNA secondary structure. Such structures have not been predicted in the caliciviruses.

A review of the molecular biology of the caliciviruses has recently been published (Clarke & Lambden, 1997).

4 Feline calicivirus.

FCV is a major cause of acute oral and upper-respiratory tract disease in the domestic cat (reviewed by Gaskell & Dawson, 1994) which causes a persistent infection in some recovered cats. Despite widespread vaccination, FCV is still present at a high prevalence in domestic cat populations.

4.1. Clinical disease.

FCV was first fortuitously isolated from the spleens of cats in an attempt to isolate the virus responsible for feline panleucopenia (Fastier, 1957). Initially, no association was made between the new virus and disease leading them to be called orphan viruses (Fastier, 1957). Subsequently, it has been demonstrated that along with feline herpesvirus, FCV is one of the major causes of 'cat flu' (reviewed by Gaskell & Dawson, 1994).

Typically FCV is associated with a relatively mild syndrome characterised by mild ocular and nasal discharge, vesicle and ulcer formation in the oral cavity, particularly on the dorsal margin of the tongue, and pyrexia. Cats may also become anorexic, and develop sneezing and conjunctivitis (Kahn & Gillespie, 1971; Povey & Hale, 1974; Hoover & Kahn, 1975; Wardley & Povey, 1977b; Ormerod *et al.*, 1979; Knowles *et al.*, 1991; reviewed by Gaskell and Dawson, 1994).

However, FCV can induce a wide spectrum of clinical disease from inapparent infections (Fastier, 1957; Povey & Hale, 1974) to fatal pneumonia in kittens (Kahn & Gillespie, 1971; Povey & Hale, 1974). Early experiments using aerosolised virus to challenge naive kittens may have artefactually induced more severe forms of disease (Kahn & Gillespie, 1971; Ormerod *et al.*, 1979). Indeed, it has been demonstrated that fomite transmission and not aerosol transmission of virus is most likely responsible for the infection of naive cats (Wardley & Povey, 1977a).

Some isolates of FCV induce an acute febrile lameness syndrome which is reproducible experimentally. Lameness post-FCV infection was reported as an occasional clinical sign in early experiments (Crandell & Madin, 1960; Studdert *et al.*, 1970). However, it was more than 10 years before a clear association between some

strains of FCV infection and lameness was suggested (Pedersen *et al.*, 1983; Dawson *et al.*, 1994). Subsequently, lameness has been associated with certain live attenuated vaccines (Church, 1989; Dawson *et al.*, 1993a, b).

Some studies report a strong association between FCV isolation and chronic stomatitis in the field (Thompson *et al.*, 1984; Knowles *et al.*, 1989, Harbour *et al.*, 1991). However, attempts to reproduce this condition experimentally have failed (Knowles *et al.*, 1991) and it is likely that other factors, particularly dual infection with feline immunodeficiency are involved (Knowles *et al.*, 1989; Tenorio *et al.*, 1991).

FCV has been isolated from cats with many other patterns of disease including sudden death in kittens (Love & Baker, 1972), ulceration of the skin (Cooper & Sabine, 1972; Love & Zuber, 1987), jaundice and abortion (Ellis, 1981) and feline urological syndrome (Rich & Fabricant, 1969; Fabricant & Rich, 1971). However, as FCV is able to induce an inapparent persistent infection (see below, section 4.3), it is possible that some of these disease associations represent fortuitous co-infection with FCV.

4.2 Vaccination.

Vaccination of the domestic cat population against FCV is widespread (Gaskell & Dawson, 1994). Vaccines traditionaly incorporated live, attenuated FCV strain F9 or similar, F9-like isolates, as part of multivalent, FCV-feline herpesvirus-feline panleucopenia vaccines. More recently, some vaccines have incorporated isolates distinct from F9 (Dr. R.M.Gaskell, personal communication). Whilst generally effective at preventing clinical signs post-FCV infection, these vaccines do have several potential draw-backs. Firstly, both FCV and FHV associated disease can occur if vaccine virus is administered oronasally as may occur at the time of vaccination if an aerosol is produced or if cats lick the injection site (Povey, 1977). Secondly, there is some evidence to suggest that even following correct administration, vaccine virus may be shed from the oropharynx (Bennett *et al.*, 1989; Pedersen & Hawkins, 1995). Thirdly, as current vaccines are only based on a single FCV isolate, there is the possibility that vaccine induced immunity, although widely cross-protective (Kalunda *et al.*, 1975; Knowles *et al.*, 1990), will not protect against

infection with all field isolates (Kalunda *et al.*, 1975). Finally, whilst preventing the majority of disease, vaccine-induced immunity does not appear to prevent infection and vaccinated cats may become persistently infected (Gaskell *et al.*, 1982). Indeed, in one study, pre-existing vaccine-induced immunity prolonged virus shedding following subsequent FCV challenge (Dawson *et al.*, 1991).

It is perhaps therefore not surprising that vaccine failures are not uncommon and FCV-associated disease post-vaccination is well characterised (Church, 1989; Dawson *et al.*, 1993a). These vaccine failures have previously been described according to the time post-vaccination that clinical signs are first seen as vaccine reactions (vaccination to disease interval of 21 days or less) and vaccine breakdowns (vaccination-to-disease interval of greater than 21 days), the majority of reported cases being reactions (Dawson *et al.*, 1993a). There is some serological evidence to suggest that FCV derived from one vaccine (currently no longer marketed - personal communication R.M.Gaskell) was associated with vaccine reactions (Dawson *et al.*, 1993a). This is discussed further in section 4.4 under typing of FCVs.

4.3 FCV persistent infection.

Following recovery from clinical disease many cats continue to shed FCV from the oropharynx and act as a reservoir of infection to naive animals (Povey *et al.*, 1973). The duration of this period is variable but in some experimental systems, approximately half of infected cats still shed virus 75 days post-infection (Wardley & Povey, 1977b). Individual animals have been shown to shed virus for up to two years (Povey *et al.*, 1973; Wardley, 1976). Such persistently infected carrier animals are believed to be widespread in the cat population due to a high FCV isolation rate from apparently healthy cats in the field (Wardley *et al.*, 1974; Coutts *et al.*, 1994). Levels of shedding are approximately 25% in cat-show populations (Coutts *et al.*, 1994) and were as high as 41% in some colonies prior to vaccination (Wardley *et al.*, 1974).

Individual carrier animals have been described as high, medium or low-level shedders according to the titre of FCV recoverable from the oropharynx (Wardley, 1976). Shedding is believed to stop abruptly rather than following a gradual decline in the amount of virus shed (Wardley, 1974) although this has only been studied in a small

number of cats. Some experimental animals cease shedding detectable virus for short periods during the carrier state (Wardley, 1976; Dr. S.Dawson, personal communication). It is believed this represents virus shedding reducing to below experimentally detectable levels rather than a period of true 'latency' followed by recrudescence. The level of FCV shedding appears to be independent of stressors like exogenous corticosteroids and environmental temperature fluctuations (Wardley, 1976). As well as the oropharynx, FCV is occasionally isolated from faeces of carrier animals although the significance of this unclear (Wardley, 1976).

The site of FCV persistence within carrier cats is still uncertain although several studies have shown the tonsil is the tissue from which virus is most readily isolated in carrier cats (Povey *et al.*, 1973; Povey & Hale, 1974; Wardley, 1974). Using immunohistochemistry, viral proteins have been demonstrated in the superficial tonsilar epithelium and the stratum germinativum of the adjacent fossa mucosa (Dick *et al.*, 1989). However, tonsillectomy of carrier cats failed to terminate viral shedding (Wardley, 1974) and virus can be isolated from other non-tonsillar tissues (Povey *et al.*, 1973) implying the tonsil is not necessary for persistence.

The mechanism by which FCV persists within carrier cats is unclear. The reisolation of antigenic variants from persistently infected cats has led to the suggestion that antigenic variation may allow FCV to escape from a humoral immune response (Wardley, 1974; Johnson, 1992; Pedersen & Hawkins, 1995). Since the site of virus persistence in carrier cats is still uncertain, the possibility of viral replication in immune-privileged sites should not be discounted.

4.4 Typing of FCV.

Unlike the human caliciviruses, attempts to group FCVs antigenically or genotypically using serological or sequence based techniques respectively have failed. Therefore, FCV isolates are considered to be a diverse but closely and equally related group of viruses constituting a single serotype and genotype. However, differences between FCVs mean most isolates can be distinguished by serology and sequence analysis. This variability has formed the basis of typing-methods using conventional cross-neutralisation tests (Povey, 1974; Knowles *et al.*, 1990; Dawson *et al.*, 1993a, b;

Lauritzen *et al.*, 1997), monoclonal antibody reactivity (McArdle *et al.*, 1996; Geissler *et al.*, 1997) and sequence analysis (Geissler *et al.*, 1997, Glenn, 1997). These techniques however have largely failed to produce an association between the FCV isolate and either the disease it was associated with, the location it was isolated from or the year of its isolation. The only exceptions to this have been reported using cross-neutralisation tests. Using such techniques, chronic stomatitis isolates were shown to be relatively distinct from those associated with acute oral and respiratory disease (Dawson *et al.*, 1993a, b), and isolates grouped geographically and by time of isolation showed different patterns of serological cross-reactivity (Lauritzen *et al.*, 1997).

The antigenic differences between most FCVs have been exploited to facilitate the epidemiological investigation of FCV-associated disease. Serum neutralisation tests implicated one particular vaccine-derived FCV in some vaccine reactions although the majority of cases appeared to be caused by antigenically distinct strains of field virus (Dawson *et al.*, 1993a). However, serologically-based methods can be difficult to interpret as some FCV strains are not distinguishable serologically (Kalunda *et al.*, 1975; Dawson *et al.*, 1993b) and the neutralisation patterns of some strains of FCV may change on replication *in vitro* or *in vivo* (Pedersen & Hawkins, 1995). There have been no reports of the use of sequence analysis in the epidemiological investigation of FCV-associated disease.

5 Mechanisms of viral persistence.

Viruses have adopted several strategies to allow their persistence within the host and these include down-regulation of viral protein expression, evasion of the host immune response, down-regulation of the host immune response (either specifically or generally) and immunotolerance. There is also some evidence to suggest that in cell culture, the host evolves to favour the establishment of viral persistence. There follows a brief review of the major mechanisms of viral persistence. Potential mechanisms for FCV persistence will be discussed under relevant sections.

5.1 Down-regulation of viral protein expression.

The immune response relies almost exclusively on the detection of foreign, non-self proteins and peptides for its target selection. Viruses utilise several methods that allow down-regulation of viral protein expression either generally or of specific immunodominant proteins. These may facilitate the persistence of viruses undetected by the host.

5.1.1 Proviral integration.

One way to switch off viral protein expression but still guarantee viral genome partition during host cell division is to integrate the genome into that of the host as a provirus. Proviral integration is almost exclusive to the retroviruses and has been reviewed (Coffin, 1990). Briefly, viral-encoded, reverse transcriptase converts viral RNA to double-stranded DNA. This is stably integrated into the host genome, maintaining viral gene order. Subsequent provirus replication and transcription is entirely dependent on host mechanisms.

5.1.2 Latency.

One of the best examples of restricted viral gene expression is the classic latent infection of neurones by alphaherpesviruses including herpes simplex virus (Fawl & Roizman, 1994) and feline herpesvirus (Gaskell *et al.*, 1985), another important

respiratory pathogen of cats (Gaskell & Dawson, 1994). In latently infected cells, infectious virus is not recoverable. The viral genome persists in a non-integrated, circular or linear and concatenated form. Viral gene expression is down-regulated to the transcription of a limited number of latency associated transcripts (LATs) and no viral protein is detectable. Periodically, usually associated with host stress, latent virus can be reactivated. The function of LATs is uncertain and not essential to viral latency or reactivation under experimental conditions (reviewed by Rock, 1993; Fawl & Roizman, 1994).

5.1.3 Defective interfering particles.

Defective interfering particles (DIPs) constitute mutants of wild-type (wt) virus from which they are derived, that due to some functional mutation are incapable of replicating unless complimented by parental wt virus. By parasitising wt virus proteins for their own replication, DIPs can specifically down-regulate replication of wt virus (reviewed by Perrault, 1981; Holland, 1990). Such auto-interference may lead to cyclical variation of virus titre in cell culture (Henry *et al.*, 1979; Kirkwood & Bangham, 1994) and animals (Cave *et al.*, 1985).

DIPs have been demonstrated for the majority of RNA virus groups (Perrault, 1981) but the evidence for their existence in caliciviruses in general, and FCV in particular, is limited. Repeated, high multiplicity passage of SMSV in cell culture (conditions usually associated with the accumulation of DIPs) did not alter SMSV RNA expression (Schaffer, 1979). Similar passage of FCV was associated with the accumulation of virus particles with a reduced buoyant density when compared to wt virus (Jensen & Coates, 1976), and these viruses were able to inhibit replication of wt virus.

There is considerable evidence supporting a role for DIPs in facilitating persistent infections of cell cultures (reviewed by Holland *et al.*, 1980). However, evidence for their existence *in vivo* is largely circumstantial based on sequence analysis (Martell *et al.*, 1992; Adami *et al.*, 1995) and any potential role for DIPs in persistence *in vivo* has largely been extrapolated from their properties of specific down-regulation of wt virus in cell culture.

5.1.4 Antibody induced antigenic modulation.

Antibody induced antigenic modulation refers to the down-regulation of viral antigen expression by specific antibody and is classically described for measles virus (reviewed by Fujinami & Oldstone, 1984; Schneider-Schaulies *et al.*, 1994). In cell culture, measles virus antibodies lead to reduced cell-surface and intra-cellular viral protein expression (Fujinami & Oldstone, 1979, 1980) and reduced viral gene transcription (Schneider-Schaulies *et al.*, 1992). A role for antibody in the pathogenesis of measles virus persistence has been suggested both on experimental (Wear & Rapp, 1971; Rammohan *et al.*, 1981) and retrospective clinical evidence (Rammohan *et al.*, 1982).

5.1.5 Downregulation of specific immunodominant protein expression.

In adenovirus type 5, an E3 region gene product has been shown to specifically reduce expression of the E1a immunodominant antigen by a post-transcriptional mechanism (Zhang *et al.*, 1991).

5.2 Evasion of the host immune response.

Many persisting pathogens, not just viruses, have adopted a strategy to evade the host immune response by frequently changing their immunodominant epitopes including bacteria (Saunders, 1989; Seifert, 1996) and other parasites (Dessaint & Capron, 1993; Lanzer *et al.*, 1997) most notably *Plasmodium species*, the cause of malaria (Reeder & Brown, 1996).

For the larger pathogens with greater coding capacity, this antigenic variation is often achieved by possessing multiple variants of genes that encode the immunodominant proteins and these are switched between silent loci (where the gene is not expressed) and active transcription sites (Borst & Rudenko, 1994; Borst *et al.*, 1995; Seifert, 1996; Barry, 1997; Deitsch *et al.*, 1997; Zhang *et al.*, 1997).

Most examples of viral antigenic variation are associated with RNA viruses where small genome sizes limit coding capacity. However, this small genome size means fidelity in replication is not so critical. Indeed, there is considerable evidence suggesting RNA viruses replicate their genome using error prone, low fidelity

polymerases (Steinhauer & Holland, 1987; Preston *et al.*, 1988; Holland *et al.*, 1992; Drake, 1993). It has been estimated for an average sized RNA virus of approximately 10kb, that each time the genome is replicated, approximately one mistake is made in copying the genome (Holland *et al.*, 1992). The implication of this is that replicating RNA viruses will exist as diverse, yet closely related populations of viruses containing many slightly different sequences (quasispecies) (Holland *et al.*, 1992; Domingo *et al.*, 1996, 1997). Error-prone viral replication also explains why most (if not all) RNA virus quasispecies contain a large number of non-functional viral genomes constituting defective interfering particles (see above).

The quasispecies structure of RNA virus populations allows them to adapt rapidly to changes in their environment by the selection of viruses with higher 'fitness' for the new environment from the existing population. This has important clinical implications in the emergence of novel infectious diseases, and the generation of escape mutants that are resistant to antiviral chemotherapy and the developing host immune response (Domingo *et al.*, 1997). Many viruses that cause persistent infections generate mutants during the course of infection with altered immunoreactivity, either in T or B cell epitopes (Table 2). There is considerable evidence to suggest that these mutants may evade the host immune response and be critical to the establishment and maintenance of persistent infections.

In the case of T cell epitopes, these escape mutant progeny may do more than just escape neutralisation. In Influenza A virus (Dong *et al.*, 1996), hepatitis B virus (Bertoletti *et al.*, 1994) and human immunodeficiency virus (Klenerman *et al.*, 1995; McAdam *et al.*, 1995; Meier *et al.*, 1995), it has been demonstrated that mutated peptide epitopes may also promote survival of their wild type parent virus by antagonising cytotoxic T cells specific for wild type peptide.

Although error prone RNA polymerase activity may explain most antigenic variation in RNA viruses, a novel host-encoded enzyme may also facilitate viral genome mutation. Modification of double stranded RNA by the host-encoded enzyme double-stranded RNA adenosine-deaminase results in the conversion of adenosine to inosine with an associated biased hypermutation of adenine to guanine (Bass & Weintraub, 1988; Wagner *et al.*, 1989; Nishikura, 1992). Such biased hypermutation is

implicated in host RNA sequence evolution (Sommer *et al.*, 1991), the mechanism of interferon activity (Patterson *et al.*, 1995) and antisense RNA technology (Nishikura, 1992). Sequence analysis has also identified regions of biased hypermutation in persisting viruses in particular measles virus (Schneider-Schaulies *et al.*, 1994)and retroviruses (Hajjar & Linial, 1995). It has been suggested that this may promote the generation of viral variants resistant to the host immune response.

More recently nutrition has been suggested as another mechanism promoting viral evolution (Domingo, 1997). It has been demonstrated that replication of a coxsackievirus in vitamin E or selenium deficient mice is associated with an increase in viral mutation and a dramatic change from avirulent to virulent phenotype (Beck *et al.*, 1995; Beck, 1997). Several mechanisms for this observation have been suggested including direct oxidative damage of RNA. The interaction of diet and dietary deficiencies on RNA virus mutation, evolution and pathogenesis was the subject of a recent symposium (Levander, 1997).

Although antigenic variation offers a simple and plausible explanation for many persistent infections, the true story may be more complicated as demonstrated by visna virus, a retrovirus that induces a persistent infection of sheep. In some sheep infected with visna virus, no antigenic variants emerge during infection (Thormar *et al.*, 1983) and in other cases, antigenic variants emerge but persist in the presence of neutralising antibodies (Narayan *et al.*, 1978; Thormar *et al.*, 1983; Kennedy-Stoskopf & Narayan, 1986). Recent work has suggested that antibodies induced by visna virus (Kennedy-Stoskopf & Narayan, 1986) are of low affinity and probably not relevant to the occurrence of antigenic variants or the establishment of persistence (Kennedy-Stoskopf & Narayan, 1986). In caprine arthritis and encephalitis virus, a related virus of goats, sialic acid residues on the virus surface are responsible for inhibiting the neutralisation activity of antibodies (Huso *et al.*, 1988).

5.3 Down-regulation of the host immune response.

Some viruses have adopted intricate strategies that down-regulate specific components of the host immune response in particular major histocompatibility (MHC) -dependent antigen presentation, natural killer cells, apoptosis, cytokine

Virus	Epitope type*	References
Human Immunodeficiency Virus	B	Reitz *et al.*, 1988; Nara *et al.*, 1990; Wolfs *et al.*, 1991
	class I	Phillips *et al.*, 1991; McAdam *et al.*, 1995
Equine infectious anaemia virus	B	Montelaro *et al.*, 1984; Salinovich *et al.*, 1986
Visna virus	B	Scott *et al.*, 1979; Lutley *et al.*, 1983
Hepatitis A virus	B	Lemon *et al.*, 1991
Hepatitis B virus	class I	Bertoletti *et al.*, 1994; Tai *et al.*, 1997
	class II	Hosono *et al.*, 1995
	B	Waters *et al.*, 1992
Hepatitis C Virus	B	Weiner *et al.*, 1992; Yamaguchi *et al.*, 1994
Foot and mouth disease virus	B	Gebauer *et al.*, 1988; Mateu *et al.*, 1990; Salt, 1993
Feline calicivirus	B	Johnson, 1992

Table 2; Some viruses for which antigenic variation has been suggested to play a role in persistent infection. *epitope type; B = B-cell epitope, class I = Major histocompatibility (MHC) class-I restricted T cell epitope, class II = MHC class-II restricted T cell epitope.

function and antibody function. Other viruses induce a generalised immunosuppression favouring persistence of themselves and other pathogens.

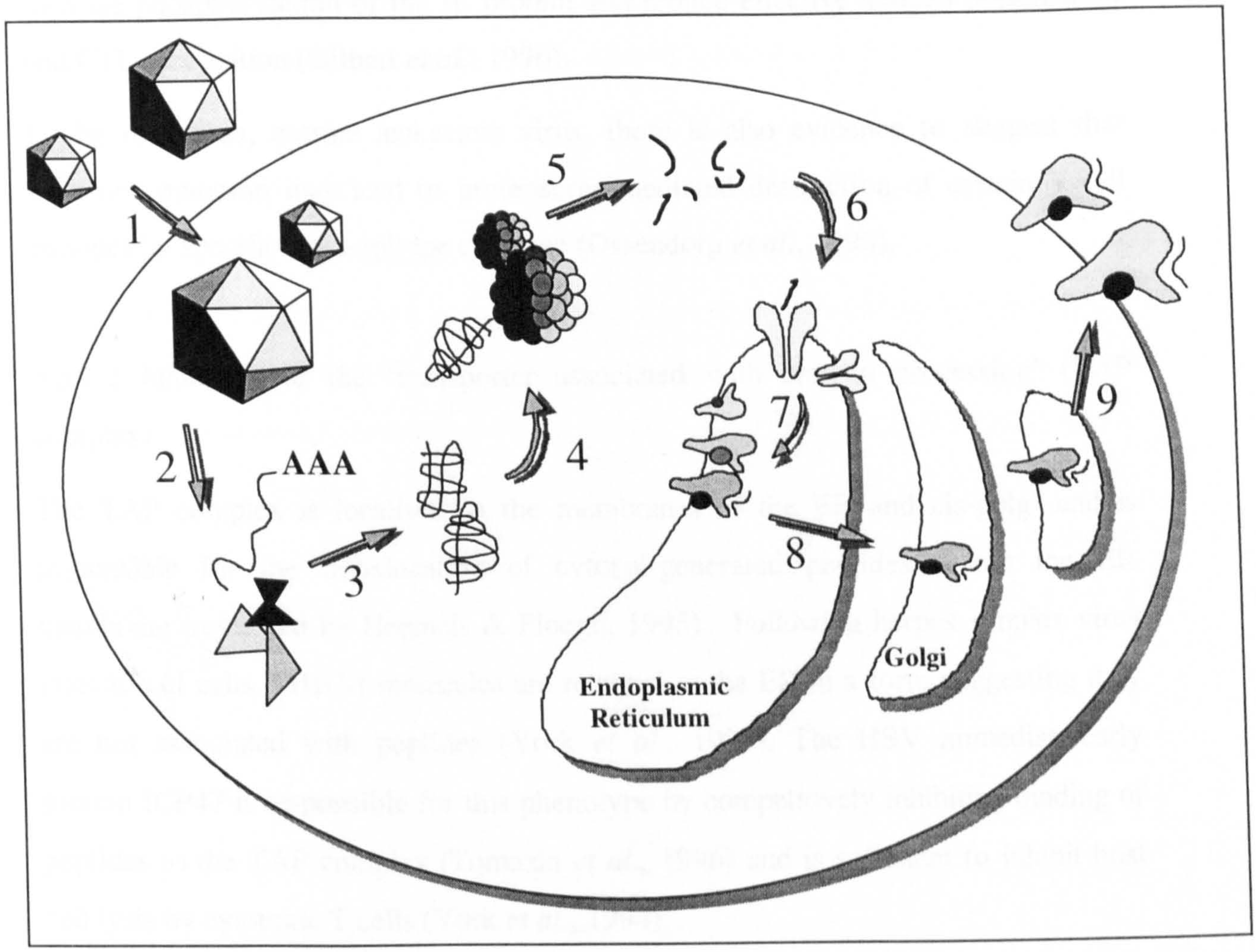
5.3.1 Down-regulation of MHC dependent antigen presentation.

The detection by the immune response of infected cells due to the presence of virally derived peptides on their surface represents an intricate pathway of protein cleavage, peptide transport, peptide-protein interactions, intracellular protein complex trafficking and cell-to-cell interactions (Figure 6 and reviewed by Heemels & Ploegh, 1995; York & Rock, 1996; Koopmann *et al.*, 1997). The end result is death of the infected cell. To overcome this, viruses have developed mechanisms that interfere with many of the steps involved in this process which are discussed below and shown in table 3 (reviewed by Hill, 1996; Hengel & Koszinowski, 1997; Oldstone, 1997).

5.3.1.1 Reduced antigen processing

The Epstein-Barr virus (EBV)-encoded nuclear antigen (EBNA1) is expressed in latently EBV-infected B lymphocytes that persist for life in healthy virus carriers (Tierney *et al.*, 1994). However, EBNA1-specific cytotoxic T lymphocyte (CTL) responses have not been demonstrated (Khanna *et al.*, 1992). Sequence analysis has shown that EBNA1 contains an internal glycine-alanine repeat (Hennessy & Kieff, 1983; Baer *et al.*, 1984). This repeat motif encodes a *cis*-acting inhibitor of antigen processing and therefore MHC-I restricted epitope expression (Levitskaya *et al.*, 1995; reviewed by de Campos-Lima *et al.*, 1996).

Products of early phase gene expression from human cytomegalovirus (HCMV) inhibit the transporter associated with antigen processing complex and target MHC molecules in the endoplasmic reticulum (ER) for degradation, thereby efficiently down-regulating CTL recognition (see below). However, an immediate-early (IE) essential transcription factor is produced prior to this blockade. Despite this apparent mismatch in MHC blockade and viral protein production, few IE specific CTLs are found in HCMV infected individuals and these lyse HCMV infected target cells



1; Virus entry.

2; Uncoating and preparation of viral mRNA.

3; Translation.

4; Proteins targeted to proteosome.

5; Proteosome degradation of intracellular proteins.

6; Peptides trafficked across the endoplasmic reticulum (ER) membrane by the transporter associated with antigen processing complex (Heemels & Ploegh, 1995).

7; Peptides associate with MHC-β2 microglobulin complex.

8; Mature MHC class-I complex leaves the ER and trafficked through the golgi.

9; Mature MHC class-I complex expressed on cell-surface for T cell receptor interaction.

Figure 6; MHC class-I antigen presentation pathway (reviewed by Heemels & Ploegh, 1995; York & Rock, 1996; Koopmann *et al.*, 1997).

poorly (Gilbert *et al.*, 1993). An HCMV-encoded protein, pp65, has been shown to increase phosphorylation of the IE protein and reduce effective antigen presentation and CTL recognition (Gilbert *et al.*, 1996).

In the retrovirus, murine leukaemia virus, there is also evidence to suggest that sequence mutation may lead to proteosome-mediated destruction of certain T cell epitopes by specific intra-epitope cleavage (Ossendorp *et al.*, 1996).

5.3.1.2 Inhibition of the 'transporter associated with antigen processing' (TAP complex).

The TAP complex is localised to the membranes of the ER and cis-golgi and is responsible for the translocation of cytosol-generated peptides across the ER membrane (reviewed by Heemels & Ploegh, 1995). Following herpes simplex virus infection of cells, MHC-I molecules are retained in the ER in a form suggesting they are not associated with peptides (York *et al.*, 1994). The HSV immediate-early protein ICP47 is responsible for this phenotype by competitively inhibiting binding of peptides to the TAP complex (Tomazin *et al.*, 1996) and is sufficient to inhibit host cell lysis by cytotoxic T cells (York *et al.*, 1994).

The HCMV gene product US6 has recently also been demonstrated to inhibit TAP function by an as yet, poorly defined but distinct mechanism to ICP47 (Ahn *et al.*, 1997; Hengel *et al.*, 1997; Lehner *et al.*, 1997).

There is also evidence to suggest that in cell lines transformed by the highly oncogenic adenovirus 12, levels of messenger RNA encoding the proteins of the TAP complex are reduced resulting in reduced cell-surface expression of MHC-I molecules (Rotem-Yehudar *et al.*, 1994).

5.3.1.3 Down-regulation of surface MHC production.

The recognition of infected cells by cytotoxic T cells depends on the interaction between T cell receptor, MHC molecule and antigenic peptide. Some viruses are associated with persistent infection in cell types that do not normally express surface MHC molecules the classic example being the latent infection of herpes simplex virus

in neurones (Oldstone, 1991). Many viruses however replicate in MHC expressing cells but encode proteins that specifically either down-regulate MHC production, increase MHC degradation or reduce MHC translocation to the cell surface.

Reduced MHC production.

The highly oncogenic human adenovirus 12 (Ad-12) has been shown to be associated with down regulation of MHC-I heavy chains (Schrier *et al.*, 1983). It is postulated that this leads to failure of CTL recognition allowing proliferation of transformed cells and tumour formation (Bernards *et al.*, 1983). Down regulation of MHC-I heavy chains has been mapped to the immediate early region 1A of Ad-12 (Ad12E1a) (Schouten *et al.*, 1995). In cells transformed with Ad12E1a, the processing of important MHC-I pro-transcription factor precursors is reduced leading to reduced MHC-I transcription (Schouten *et al.*, 1995).

Increased MHC degradation

Following HCMV infection there is a decline in MHC-I complexes associated with an accelerated degradation of class I heavy chains (Beersma *et al.*, 1993). This phenotype maps to two separate loci (Jones *et al.*, 1995), US2 (Wiertz *et al.*, 1996b; Jones & Sun, 1997) and US11 (Wiertz *et al.*, 1996a). In both cases, MHC-I complexes are targeted for degradation in the cytosol probably by the proteosome. In the case of US2, translocation from the ER to the cytosol is believed to be mediated by the sec61 complex (Rapoport *et al.*, 1996) in what appears to be a reversal of the mechanism by which sec61 translocates nascent proteins synthesised in the cytosol into the ER (Wiertz *et al.*, 1996b). It has been suggested that this apparent duplication of gene function evolved in response to MHC polymorphism as both US2 and US11 have different MHC-type specificities (Machold *et al.*, 1997).

There is some evidence to suggest that the HIV protein Nef may also increase the degradation of MHC-I complexes by targeting them to the endolysosomal compartment (Scheppler *et al.*, 1989; Schwartz *et al.*, 1996; Mangasarian *et al.*, 1997).

Reduced MHC transport

One of the first examples of viruses inhibiting the MHC pathway was the E3/19K protein of adenoviruses. E3/19K associates with MHC class I molecules and prevents their maturation through the golgi (Andersson *et al.*, 1985; Burgert & Kvist, 1985). The domains of E3/19K responsible for MHCI retention in the ER and for interaction with MHCI have been mapped to the extreme carboxy-terminal cytoplasmic tail (Nilsson *et al.*, 1989; Cox *et al.*, 1991) and the amino-terminal ER lumenal domain (Hermiston *et al.*, 1993) respectively.

The US3 gene product of HCMV (Ahn, 1996; Jones *et al.*, 1996) and gp40, the m152 gene product of murine cytomegalovirus (MCMV) (Del Val *et al.*, 1992; Ziegler *et al.*, 1997) have both also been shown to associate with mature MHC-class I complexes leading to their transport arrest.

5.3.1.4 Natural Killer cell inhibition.

Many of the immuno-modulatory mechanisms discussed above have as a final pathway, reduced or abolished cell surface expression of MHC. However, natural killer (NK) cells are able to recognise and destroy host cells that do not express class I MHC molecules (Brutkiewicz & Welsh, 1995). Therefore, viruses that have 'protected' their host cell against CTL lysis by down-regulation of class I MHC expression risk eventually losing it from NK cell attack. The UL18 gene product of HCMV (Beck & Barrell, 1988)and its homologue in murine CMV both share considerable structural and functional similarities to MHC class I heavy chains and have been shown to protect MHC-depleted cells from NK attack both *in vitro* (Reyburn *et al.*, 1997) and *in vivo* (Farrell *et al.*, 1997) respectively.

Despite MHC class I ER retention associated with MCMV early gene expression (see above), it has recently been shown that some mature class I complexes do leave the ER in association with an abundant MCMV-encoded glycoprotein, gp34 (Kleijnen *et al.*, 1997). It has been speculated that surface expression of gp34 class I MHC complexes acts to decoy NK cells.

Virus	Gene Product	Function	Reference
HCMV	US2	increased MHC class-I degradation	Wiertz *et al.*, 1996b
	US3	reduced MHC class-I transport	Ahn, 1996; Jones *et al.*, 1996
	US6	inhibition of TAP	Ahn *et al.*, 1997; Lehner *et al.*, 1997
	US11	increased MHC class-I degradation	Wiertz *et al.*, 1996a
	pp65	reduced antigen processing	Gilbert *et al.*, 1996
	UL18	NK cell decoy	Reyburn *et al.*, 1997
MCMV	gp40	reduced MHC class-I transport	Del Val *et al.*, 1992; Ziegler *et al.*, 1997
	gp34	NK cell decoy	Kleijnen *et al.*, 1997
	m144	NK cell decoy	Farrell *et al.*, 1997
HSV	ICP47	Inhibition of TAP	York *et al.*, 1994; Tomazin *et al.*, 1996
Adeno-viruses	E3/19K	reduced MHC class-I transport	Andersson *et al.*, 1985; Burgert & Kvist, 1985
	Ad12E1a	reduced MHC class-I production	Schouten *et al.*, 1995
	Ad12	reduced TAP mRNA	Rotem-Yehudar *et al.*, 1994
Epstein-Barr virus	EBNA-1	reduced antigen processing	de Campos-Lima *et al.*, 1996

Table 3; Viruses associated with persistent infections that down-regulate the MHC class-I antigen presentation pathway.

5.3.2 Down-regulation of apoptosis.

Apoptosis refers to a process of controlled and programmed self-destruction of individual cells in response to a variety of stimuli including redundancy, oncogenic transformation, radiation damage and viral infection. Many viruses encode proteins that manipulate this pathway either to suppress it or to induce it and these have been the subject of recent reviews (Cuff & Ruby, 1996; Spriggs, 1996; Teodoro & Branton, 1997). Whilst it is believed that many of these mechanisms are involved in early viral replication allowing progeny virus to be produced in acute infection prior to cell destruction, some viruses are believed to interact with apoptotic pathways to facilitate persistent infection. The latently expressed membrane protein (LMP1) of Epstein-Barr virus has been shown to protect cells from apoptosis and is believed to be important in maintenance of persistence (Henderson *et al.*, 1991; Kawanish, 1997). In adenoviruses, two viral transcripts E1B-19K and E1B-55K inactivate bak and p53 respectively, both of which are important cellular proteins in the apoptosis pathway (Hayder & Müllbacher, 1996).

5.3.3 Down-regulation of cytokines.

Cytokines are important secreted messenger molecules that co-ordinate the immune response to infection. Many viruses encode proteins that interfere with cytokine function (Spriggs, 1996). Indeed many of these molecules are homologous to cytokines and cytokine receptors and are believed to act as decoys, down-regulating cytokine function by competitive inhibition. Such cytokine inhibitors are classically described for poxviruses (Alcamí & Smith, 1995; Smith *et al.*, 1997) and Epstein-Barr virus (Moore *et al.*, 1990). However, their function is believed to be primarily associated with down-regulation of inflammation in response to acute tissue damage and their role in persistence is uncertain.

5.3.4 Down-regulation of antibody function.

Antibodies consist of a variable functional domain which is responsible for binding specific antigens and a conserved domain (Fc) which interacts with other components of the immune response. Herpes simplex virus type 1 (Frank & Friedman, 1989;

Dubin *et al.*, 1991) and MCMV (Thäle *et al.*, 1994) are known to encode surface expressed proteins which bind to the Fc portion of antibodies and are capable of inhibiting antibody induced killing of viruses and virally infected cells.

5.3.5 Generalised immunosuppresion.

In the later stages of human immunodeficiency virus infection there is a decline in the numbers of cytotoxic T cells and a rise in viral titre, ultimately leading to acquired immunodeficiency syndrome. The immunodeficiency is primarily associated with loss of the viruses main cellular target, CD4 positive cytotoxic T cells. Since HIV will probably have already been persistent for several years it is uncertain that this immunosuppresion is necessary for HIV persistence. However, this stage of disease may facilitate persistent infection with other pathogens not normally associated with persistent infection (Rocha *et al.*, 1991; Angel *et al.*, 1996).

5.4 Immunotolerance.

Immunotolerance refers to specific failure of the immune response to recognise non-self antigens as foreign. With bovine viral diarrhoea virus and Border disease virus of sheep, *in utero* infection at particular stages of gestation leads to immunotolerance (McClurkin *et al.*, 1984; Duffell & Harkness, 1985). In such persistently infected immunotolerant animals, virus is detected in peripheral blood mononuclear cells but the animals remain seronegative (Lopez *et al.*, 1993; Woldehiwet & Hussin, 1994a,b).

5.5 Host cell evolution and viral persistence.

It is in the interest of a host to develop a less pathogenic relationship with its pathogens and it could be argued that a pathogen would persist better in an individual host if it was less virulent. As such, low virulence in a particular host-pathogen system may reflect long-term co-evolution of host and pathogen towards a less virulent state. Whilst this phenomenon may be difficult to demonstrate in whole animal-pathogen systems, there is some evidence that it may occur in cell culture. During the establishment of persistently infected cell lines with foot and mouth disease

virus (Martín Hernández *et al.*, 1994) or mouse hepatitis virus (Chen & Baric, 1996), cells became more resistant to the virus.

Aims of this thesis.

FCV is an important pathogen of cats which, despite widespread vaccination, remains at a high prevalence in the cat population. The cause of this high prevalence is not known but may be contributed to by persistent infection of individual cats, the continued use of live vaccines and the circulation of FCVs within the cat population in animals with either naturally or artificially acquired pre-existing immunity.

To understand this high prevalence it will be necessary to determine the origins of FCVs circulating in the cat population. Current methods of FCV typing rely on serological differences between FCV isolates. However, such methods may be difficult to interpret. In this thesis, a sequence-based method for typing FCV was established. The method was used to investigate the role of vaccine-derived virus in FCV-associated disease in the cat population. The results of sequence-based typing were to compared with those based on antigenic differences between isolates.

The mechanism by which FCV persists in individual cats is not known but may involve antigenic variation of the major capsid protein. In order to further investigate FCV persistence, the quasispecies evolution of a proposed immunodominant region of the FCV major capsid protein was analysed in persistently infected cats. Since the immunodominant regions of the FCV capsid have not been determined in detail, we have also constructed an expression library to facilitate more thorough B-cell epitope mapping within the capsid gene.

MANUSCRIPT 1

Quasispecies evolution of a hypervariable region of the feline calicivirus capsid gene in cell culture and persistently infected cats†.

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ABSTRACT.

Feline calicivirus (FCV) is a respiratory pathogen of cats capable of causing persistent infections. This study examined the evolution of a hypervariable region of the FCV capsid gene both during 90 passages in cell culture and during replication in persistently infected cats. This region of the capsid protein is known to contain neutralisation epitopes and may be a target for immune evasion during viral persistence in the host. Sequence analysis showed that FCV exists as a quasispecies which evolved both in cell culture and in persistently infected cats. Changes involved both loss of sequence present in the infecting isolate and a gain of both synonymous and non-synonymous nucleotide substitutions to generate sequences not detected within earlier isolates. Overall, these changes led to a reduction in population heterogeneity over time. Where virus populations were highly homogenous allowing a consensus sequence to be determined, evolution rates for the consensus sequence ranged from 0.10-1.07 substitutions per nucleotide per year. Marked changes in virus neutralisation profiles were seen in isolates obtained sequentially from a persistently infected cat. This was not the case with cell culture passaged virus, suggesting that the individual amino acid changes found only in virus from persistently infected cats may significantly alter the antigenic profile of FCV, and may be the result of immune selection.

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INTRODUCTION.

Feline calicivirus (FCV) belongs to the family *Caliciviridae* (Cubitt *et al.*, 1995) and is a major cause of acute, oral and upper respiratory tract disease in domestic cats (Gaskell & Dawson, 1994). Unlike calicivirus infections in other species, there is a well-characterised carrier state where many individuals recovered from clinical disease continue to shed FCV in the oro-pharynx (Povey *et al.*, 1973; Wardley & Povey, 1977b). Duration of shedding is variable but experimentally, approximately 50% of infected cats cease shedding virus by 75 days post-infection (Wardley & Povey, 1977b). Individual cats have been shown to shed virus for up to 2 years (Povey *et al.*, 1973; Wardley, 1976). Such persistently infected animals are believed to be widespread (Harbour *et al.*, 1991; Coutts *et al.*, 1994) and are important in the maintenance of FCV and clinical disease within the domestic cat population.

The mechanisms by which FCV persists within an individual cat are unclear. However, changes in the neutralisation profiles of serially isolated FCVs from persistently infected cats (Wardley, 1974; Johnson, 1992; Pedersen & Hawkins, 1995) have led to the suggestion that, as for other RNA viruses (Clements *et al.*, 1982; Salinovich *et al.*, 1986; Phillips *et al.*, 1991; Weiner *et al.*, 1992; van Doorn *et al.*, 1995), antigenic variation may play a role in the establishment and maintenance of persistent FCV infections.

FCV has a single stranded, positive sense RNA genome approximately 7.7kb in length which encodes the non-structural proteins at the 5' end and the single major capsid protein towards the 3' end (Carter *et al.*, 1992a; Cubitt *et al.*, 1995). Comparison of sequences from the capsid protein genes of different isolates of FCV and of other caliciviruses has allowed the capsid to be divided on the basis of sequence conservation into six regions (Neill, 1992; Seal *et al.*, 1993). Regions A, B, D and F are conserved between isolates whilst regions C and E are variable. Region E has been further divided into a relatively conserved central sequence separating two hypervariable (HV) regions (Seal *et al.*, 1993; Seal, 1994). The 5' HV region (corresponding to nucleotides 6589-6693 of the F9 strain of FCV (Carter *et al.*, 1992a) is strongly implicated as a target for antibody-mediated virus neutralisation as it contains epitopes for neutralising monoclonal antibodies (Milton

et al., 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997), and synthetic peptides which include this region induce the formation of neutralising polyclonal antisera (Milton *et al.*, 1992).

In this study, we have examined the evolution of nucleotide sequence from the 5' HV region under the differing selective pressures of passage in cell culture and persistence in experimentally infected cats.

METHODS.

Virus.

FCV LS015 was originally isolated from a persistently infected domestic cat (Knowles, 1988) and experimentally, induces oral ulceration, mild ocular and nasal discharge, and pyrexia (Knowles, 1988; Dawson *et al.*, 1991).

Experimental animals.

Twelve week old specific-pathogen-free cats were obtained from a registered commercial supplier and housed under barrier conditions. Serum samples were collected and shown to be negative for FCV neutralising antibodies. Cats were infected with LS015 between 21 and 22 weeks of age.

Virus evolution in cell culture.

In vitro sequence evolution of the 5' HV region of strain LS015 was examined by serial transfer of undiluted cell culture harvest from 5th to 95th passage in Fef cells (Intervet).

Virus evolution in experimentally infected cats.

In vivo sequence evolution was examined in cat Oscar inoculated intranasally with 10^5 50% tissue culture infective dose ($TCID_{50}$) 5th passage LS015. Oropharyngeal (OP) swabs were taken at regular intervals into virus transport medium (VTM) and stored at -80°C until ready for virus culture (Wardley *et al.*, 1974) or RNA isolation.

Serum samples for virus neutralisation were obtained prior to infection, 17 days post-infection (p.i.) and 9 weeks p.i.

Longer-term sequence evolution in cats was studied using virus isolates from a previous study (Dawson *et al.*, 1991) avoiding unnecessary use of experimental animals. Briefly, three kittens (Sam, Charlie and Sally) were infected oronasally with $10^{7.6}$ $TCID_{50}$ of a 4th passage LS015 and OP swabs taken as above. Charlie had been vaccinated subcutaneously twice, first four weeks and then one week previously with inactivated LS015. Sally had been infected nine weeks previously with feline immunodeficiency virus (FIV) strain E77 (Dawson *et al.*, 1991).

Virus titrations.

Virus titrations were performed on 5th passage LS015 and all isolates from Oscar as previously described (Wardley, 1976) and titres expressed as 50% end points (Reed and Muench, 1938).

Virus neutralisation tests.

Virus neutralisation (VN) tests were performed on 5th and 95th passage virus from cell culture and isolates taken from Oscar 16, 21 and 39 days p.i. All isolates for use in VN tests were passaged once in Fe cells (ICN Biomedicals Ltd). The antisera used was obtained from cat Oscar (see above). VN tests were performed in duplicate, essentially according to Dawson *et al.*, 1993b. Titres were expressed as the mean of the reciprocal of the 50% end point.

RNA isolation and cDNA synthesis.

Total RNA was isolated from 100µl of either freeze-thawed cell lysate (for 5th and 95th passage LS015) or directly from VTM (for *in vivo* studies using LS015) according to the manufacturer's instructions (RNA Isolator; Genosys) and dissolved in 33µl of RNase free water (Sigma). cDNA was prepared by oligo dT primer extension using all of the RNA prepared above (Ready-To-Go, Pharmacia). Negative controls consisting of VTM, mock-infected Fe cells or OP swabs from uninfected cats were processed simultaneously.

Amplification of cDNA by nested polymerase chain reaction.

The nested polymerase chain reaction (PCR) was performed to amplify a 235 nucleotide region of the capsid gene equivalent to residues 6533-6767 of FCV strain F9 (Carter *et al.*, 1992a). Amplification was performed using *Pfu* DNA polymerase (Stratagene) and oligonucleotide primers (Table 1, Kings College, London) in 50µl reaction volumes. Thermal cycling conditions for both rounds of nested PCR consisted of DNA denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, primer annealing at 40°C for 1 minute and primer extension at 72°C for 3 minutes. A final extension was performed at 72°C for 5 minutes. First round amplification was carried out using 5µl of cDNA with 1.5µM of each of primers 1 and 2 and 100µM of each deoxynucleotide triphosphate (dNTP) (Advanced Biotechnologies Ltd). Second round nested amplification was performed in quadruplicate using 1µl of either neat or one-in-ten diluted first round PCR reaction as template with 240nM of each of primers 4 and 5 and 200µM of each dNTP. Pooled PCR products were purified using the Wizard PCR preps DNA purification system (Promega).

Primer	Sequence (5'-3')	binding site*
1	CCCTTTGTGTTCCAAGCAAATCG	6406-6428
2	CCTCTCCGATACCAGTGTATCC	6934-6913
4	TTGCAACTGATTATATTGTTCCTGG	6533-6557
5	GCAGTGTTGGATATTTTCTTGTCACC	6767-6742

Table 1; Primers used for PCR and sequencing. * Binding sites relate to FCV strain F9 (Carter *et al.*, 1992a).

Sequencing.

Direct sequencing of PCR products to produce a consensus sequence for each isolate was performed using the *fmol* and omnigene DNA sequencing systems (Promega). In order to determine sequence variation within an isolate, multiple clones of PCR products were also sequenced. Briefly, purified PCR products were ligated either directly into pCR-script SK(+) (Stratagene) or, following the addition of a deoxyadenosine tail using *Taq* (Advanced Biotechnologies) into pCR (T.A. cloning system; Invitrogen). Products of the ligation reaction were transformed into competent cells (One Shot cells (Invitrogen) or Ligator (R&D systems)). Plasmids were isolated according to the manufacturer's instructions (Wizard minipreps, Promega) and inserts sequenced essentially according to Sanger *et al.*, 1977. Primers 4 and 5 (Table 1) were used throughout for sequencing. All presented sequence, except where indicated, was generated from, and identical in, both strands of DNA.

Sequence analysis software.

Sequence comparisons were performed using version 8 of the Wisconsin package (1994), Genetics Computer Group (Deveraux *et al.*, 1984). Phylogenetic analysis was performed using the Phylip package (Felsenstein, 1989). Where clear consensus sequences were available for consecutive isolates from the same cat, 60 nucleotide, sliding window analyses of synonymous substitutions per synonymous site (ds) and non-synonymous substitutions per non-synonymous site (dn) were performed using WINA C++ version 0.3 (Endo *et al.*, 1996).

RESULTS.

Virus evolution in cell culture.

Sequences of clones derived from LS015 5th passage showed the virus existed as a diverse population of related sequences (quasispecies) (Figure 1*a*). By the 95th cell culture passage, the quasispecies apparently evolved at both the nucleotide (Figure 1*a*) and amino acid (Figures. 2*a* and 3*a*) levels. This was due both to loss of sequence variability, largely resulting from loss of sequence found in 5 clones of the

```
1                                                   50
..........  ..........  ......g..t  .t........  .c........  2†; 95th
..........  ..........  ......g..t  .t........  .c........  pcr 95
..........  ..........  ......g..t  .t........  .c........  4; 95th
..........  ..........  ......g..t  .t........  .c........  1; 95th
..........  ..........  ......g..t  .t........  .c........  1; 95th
c.........  ..........  ......g..t  .t........  .c........  1; 95th
..........  ..........  ......g..t  .t........  .c........  1; 95th
..........  ..........  ......g..t  ..........  .c........  2; 5th
..........  ..........  ......g..t  ..........  .c........  1; 5th
..........  ..........  ......g..t  ..........  ..........  1; 5th
..........  ..........  ......g..t  ..........  .c........  1; 5th
..........  ..........  ..........  ..........  ..........  1; 5th
tggccagata  ccacaatccc  atcagaactc  acccctgcag  gtaattacgc  4; 5th
^                                   ^

51                                                  100
t.....c..a  ...g......  .........g  ..........  .a........  2; 95th
t.....c..a  ...g......  .........g  ..........  .a........  pcr 95
t.....c..a  ...g......  .........g  ..........  .a........  4; 95th
t.....c..a  ...g......  .........g  ..........  ta........  1; 95th
t.....cc..  ...g......  .........g  ..........  .a........  1; 95th
t.....c...  ..........  ..........  ..........  .a........  1; 95th
t.....c...  ..........  ..........  ..........  .a........  1; 95th
t.....c...  ....g.....  ..........  ..........  .a........  2; 5th
t.....c...  ....g.....  ........gt  ..........  .a........  1; 5th
t.....c...  ....g.....  ........gt  ..........  .a........  1; 5th
t.....c...  ....g.....  ........--  ----------  ----------  1; 5th
..........  ..........  ..........  ..........  ..........  1; 5th
cataactagt  ggaaatggga  atgatattaa  aacagctgca  gcatatgatt  4; 5th
      ^ ^     ^^                  ^^              ^^

101                                                 150
..........  .....t....  ..........  .t........  .....t...  2; 95th
..........  .....k....  ..........  .t........  .....t...  pcr 95
..........  ..........  ..........  .t........  .....t...  4; 95th
..........  .....t....  ..........  .t........  .....t...  1; 95th
..........  ..........  ..........  .t........  .....t...  1; 95th
..........  ......g...  ..........  .t........  .....t...  1; 95th
..........  ......g...  ..........  .t........  .....t...  1; 95th
..........  ..........  ..........  .t........  .....t...  2; 5th
..........  ..........  ..........  .t........  .....t...  1; 5th
..........  ..........  ..........  ..........  ..........  1; 5th
----------  ----------  ----------  ---------.  ..........  1; 5th
..........  ..........  ..........  .t........  ..........  1; 5th
cagctgatgt  gatcagaaac  aacaccaatt  tcagaagtat  gtacatctgt  4; 5th
                  ^
```

Figure 1a; Nucleotide sequence alignments of the 5'HV region for 5th and 95th cell culture passaged virus. Sequence prefixed by 'pcr' indicates consensus sequence. All other sequences are from clones, † representing the number of clones with that sequence. - represents deletions. ^ indicates a non-synonymous nucleotide substitution. ? is sequence unattainable. t at nucleotide 91 in a single 95th passage clone generates an in-frame stop codon. Ambiguity codes for consensus sequences: r = a or g, y = t or c, k = t or g, m = a or c and w= t or a. Nucleotide 1 is equivalent to nucleotide 6571 of FCV strain F9 (Carter *et al.*, 1992a). The 5' HV (Seal *et al.*, 1993) is between nucleotides 19-123 and is in bold.

```
1                                                  50
                                                      1†; Oscar-39
.......... .......... .......... .......... .......... 1; Oscar-39
.......... .......... .......... .......... ..g....... 1; Oscar-39
.......... .......... .......... .......... ..g....... 8; Oscar-39
.......... .......... .......... .......... ..g....... 1; Oscar-21
.......... .......... .......... .......... .......... 2; Oscar-21
.......... .......... .......... .......... ..g....... 4; Oscar-21
.......... .......t.. .......... .......... ..g....... 1; Oscar-21
.......... .......... .......... .......... ..g....... 1; Oscar-21
.......... .......... .......... .......... ..g....... 1; Oscar-21
.......... .......... .......... .......... ..g....... pcr Oscar-21
.......... .......... ......r..y .......... .yr....... pcr Oscar-8
.......... .......... ......r..t .......... .y........ pcr Oscar-2
.......... .......... ......g..t .......... .c........ 3; Oscar-2
.......... .......... .......... .......... .c........ 1; Oscar-2
.......... .......... .......... .......... .......... 1; Oscar-2
tggccagata ccacaatccc atcagaactc acccctgcag gtaattacgc 5; Oscar-2
                                                    ^

51                                                 100
.......... .......... ....t...g. .......... .......... 1; Oscar-39
.......... a......... ....t...g. .......... .......... 1; Oscar-39
.......... .......... ....t...g. .......... .......... 8; Oscar-39
.......... .......... ....t..... .......... .......... 1; Oscar-21
.......... .......... ....t...g. .......... .......... 2; Oscar-21
.......... .......... ....t...g. .......... .......... 4; Oscar-21
.......... .......... ....t...g. .......... .......... 1; Oscar-21
.......... .......... ....t.---. .......... .......... 1; Oscar-21
.......... .......... ....t..c.. .......... ...------- 1; Oscar-21
.......... .......... ....t...r. .......... .......... pcr Oscar-21
y.....y... ....r..... ....t...rw .......... .m........ pcr Oscar-8
y.....y... ....r..... ....y...rw .......... .......... pcr Oscar-2
t.....c... ....g..... ....t...gt .......... .a........ 3; Oscar-2
t.....c... --..g..... ....t...gt .......... .a........ 1; Oscar-2
.......... .......... ....t..... .......... .......... 1; Oscar-2
cataactagt ggaaatggga atgacattaa aacagctgca gcatatgatt 5; Oscar-2
           ^   ^           ^^           ^

101                                                150
.......... .......... .......... .......... .......... 1; Oscar-39
.......... .......... .......... .......... ....g..... 1; Oscar-39
.......... .......... .......... .......... ....g..... 8; Oscar-39
.......... .......... .......... .......... .......... 1; Oscar-21
.......... .......... .......... .......... .......... 2; Oscar-21
.......... .......... .......... .......... .......... 4; Oscar-21
.......... .......... .......... .......... .......... 1; Oscar-21
.......... .......... .......... .......... .......... 1; Oscar-21
--------- --------- --------- ------...  .......... 1; Oscar-21
.......... .......... .......... .......... .......... pcr Oscar-21
.......... .......... .......... .y........ ......t... pcr Oscar-8
.......... .......... .......... .y........ .......... pcr Oscar-2
.......... .......... .......... .t........ ......t... 3; Oscar-2
.......... .......... .......... .......... .......... 1; Oscar-2
.......... .......... .......... .......... .......... 1; Oscar-2
cagctgatgt gatcagaaac aacaccaatt tcagaagtat gtacatctgt 5; Oscar-2
                                                    ^
```

Figure 1b; Nucleotide sequence alignments of the 5'HV region for isolates from Oscar, 2, 8, 21 and 39 days p.i. with 5th passage LS015. Sequence prefixed by 'pcr' indicates consensus sequence. All other sequences are from clones, † representing the number of clones with that sequence. - represents deletions. ^ indicates a non-synonymous nucleotide substitution. ? is sequence unattainable. Ambiguity codes for consensus sequences: r = a or g, y = t or c, k = t or g, m = a or c and w= t or a. Nucleotide 1 is equivalent to nucleotide 6571 of FCV strain F9 (Carter *et al.*, 1992a). The 5' HV (Seal *et al.*, 1993) is between nucleotides 19-123 and is in bold.

```
1                                                    50
........c. .......... .......... .......... ..g..... pcr Charlie-55
........c. .......... .......... .......... ..g..... pcr Sam/Charlie-25
.......... .......... .........c .......... .......... pcr Sally-106
.......... .......... .......... .......... .......... pcr Sally-25
tggccagata ccacaatccc atcagagctt acccctgcag gcaattacgc pcr Sam/Charlie/Sally-1
                                                     ^

51                                                   100
......t... ...g...... ........ac ......c... .c........ pcr Charlie-55
......t... ...g...... ........ac ......c... .c........ pcr Sam/Charlie-25
.......... ....a..... .........t .......... .......... pcr Sally-106
.......... .......... ........a. .......... .......... pcr Sally-25
tataaccagt ggaagtggga atgatattga aacagctgca gaatatgatt pcr Sam/Charlie/Sally-1
              ^^                ^^                ^

101                                                  150
.......c.. .......... g......... .c........ .....??... pcr Charlie-55
.......c.. .......... .......... .c........ ......c... pcr Sam/Charlie-25
.......... .......... .......... .......... .....??... pcr Sally-106
.......... .......... .......... .......... .......... pcr Sally-25
cagctgatgt gatcagaaac aacaccaatt ttagaagtat gtacatttgt pcr Sam/Charlie/Sally-1
                     ^
```

Figure 1c; Nucleotide sequence alignments of the 5'HV region for isolates from Sam, Charlie and Sally obtained between 1 and 106 days p.i. Sequence prefixed by 'pcr' indicates consensus sequence. ^ indicates a non-synonymous nucleotide substitution. ? is sequence unattainable. Ambiguity codes for consensus sequences: r = a or g, y = t or c, k = t or g, m = a or c and w= t or a. Nucleotide 1 is equivalent to nucleotide 6571 of FCV strain F9 (Carter *et al.*, 1992a). The 5' HV (Seal *et al.*, 1993) is between nucleotides 19-123 and is in bold.

(a)

```
1                                                  50
.......... I........R .D....R... *.......I. .......... 1†; 95th
.......... I........R .D....R... E.......I. .......... 2; 95th
.......... I........R .D....R... E......... .......... 5; 95th
.......... I......... .......... E......... .......... 1; 95th
R......... I......... .......... E......... .......... 1; 95th
.......... .......... .S........ E......... .......... 2; 5th
.......... .......... .S....V... E......... .......... 2; 5th
WPDTTIPSEL TPAGNYAITS GNGNDIKTAA AYDSADVIRN NTNFRSMYIC 5; 5th
```

(b)

```
1                                                  50
.......... ....D..... R.....E... .......... ........V. 1; Oscar-39
.......... ....D..... ......E... .......... ........V. 8; Oscar-39
.......... .......... ......E... .......... .......... 1; Oscar-39
.......... .......... ......E... .......... .......... 2; Oscar-21
.......... ....D..... ......E... .......... .......... 5; Oscar-21
.......... ....D..... .....-.... .......... .......... 1; Oscar-21
.......... ....D..... .......... .......... .......... 1; Oscar-21
.......... .......... .S....V... E......... .......... 2; 5th & 3 Oscar-2
.......... .......... .S........ E......... .......... 2; 5th
WPDTTIPSEL TPAGNYAITS GNGNDIKTAA AYDSADVIRN NTNFRSMYIC 5; 5th & 6 Oscar-2
```

(c)

```
1                                                  50
.......... ....D..... .G....T... .......... D......... pcr Charlie-55
.......... ....D..... .G....T... .......... .......... pcr Sam/Charlie-25
.......... .......... ......V... E......... .......... pcr Sally-106
.......... .......... .S........ E......... .......... pcr Sally-25
.......... .......... .S....E... E......... .......... pcr Sam/Charlie/Sally-1
WPDTTIPSEL TPAGNYAITS GNGNDIKTAA AYDSADVIRN NTNFRSMYIC Consensus
```

Figure 2; Alignments of deduced amino acid sequences of the 5'HV region for a) 5th and 95th cell culture passaged virus. b) isolates from Oscar, 2, 8, 21 and 39 days p.i. with 5th passage LS015. c) isolates from Sam, Charlie and Sally obtained between 1 and 106 days p.i.. Sequence prefixed by 'pcr' indicates consensus sequence. All other sequences are from clones, † representing the number of clones with that sequence. - represents deletions. ? is sequence unattainable. In Figure 2a, * at codon 31 in a 95th passage clone represents a stop codon. Underlined sequence in Figure 2c corresponds to those regions for which dn exceeds ds. The 5' HV (Seal *et al.*, 1993) is between amino acids 7-41 and is in bold.

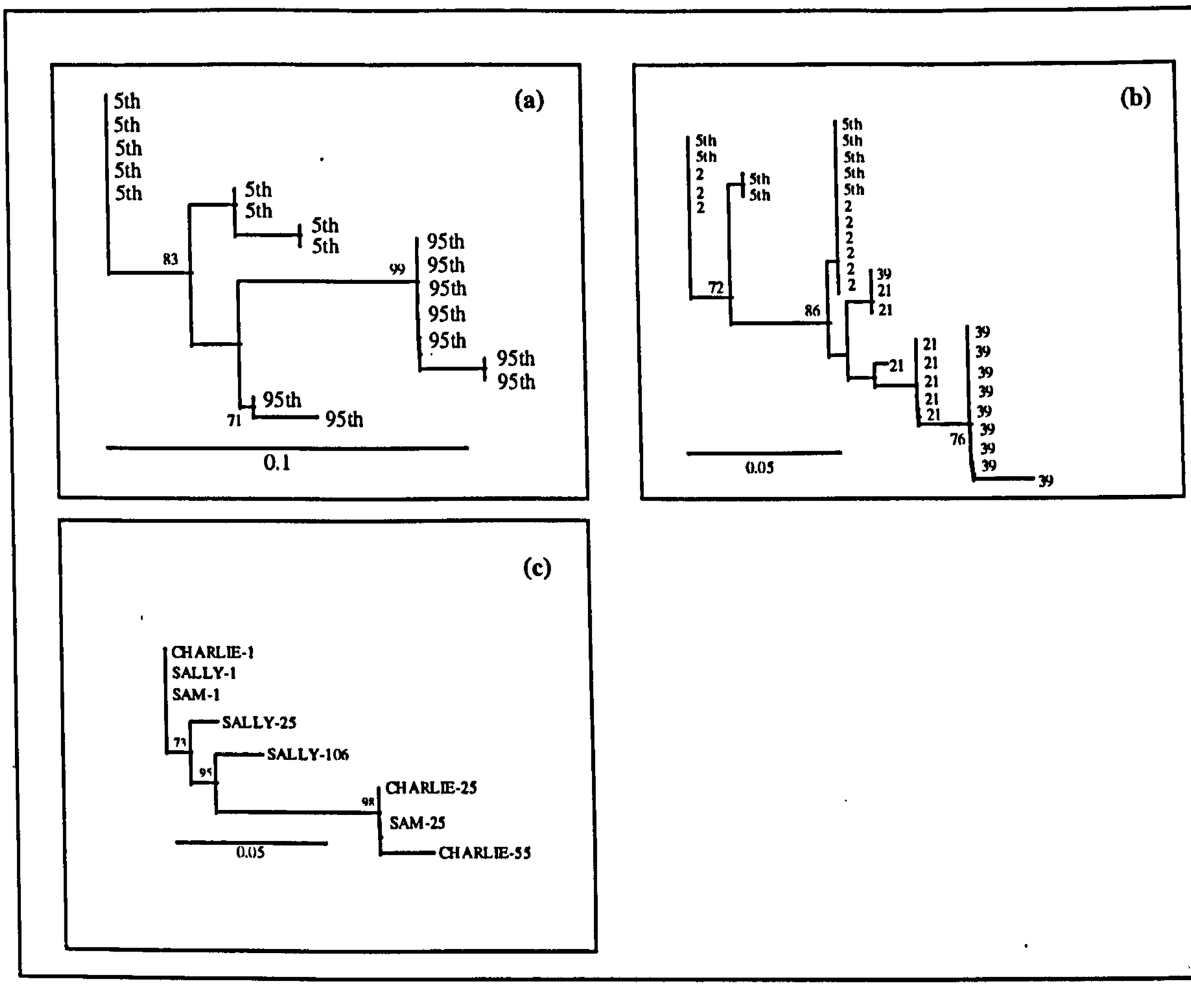


Figure 3; Phylogenetic analysis of full length 5' HV deduced amino-acid sequences (SEQBOOT, PROTDIST (Dayhoff-PAM), and NEIGHBOR (Neighbor-joining) (Felsenstein, 1989)). a) Cloned sequences derived from 5th and 95th cell culture passaged virus. b) Cloned sequences derived for virus isolated from Oscar, 2, 21 and 39 days p.i. with 5th passage LS015. c) Consensus sequences for isolates from Sam, Charlie and Sally obtained between 1 and 106 days p.i. Numbers at major nodes are the bootstrap values >70 out of 100 replications. The scale is equivalent to amino acid distances.

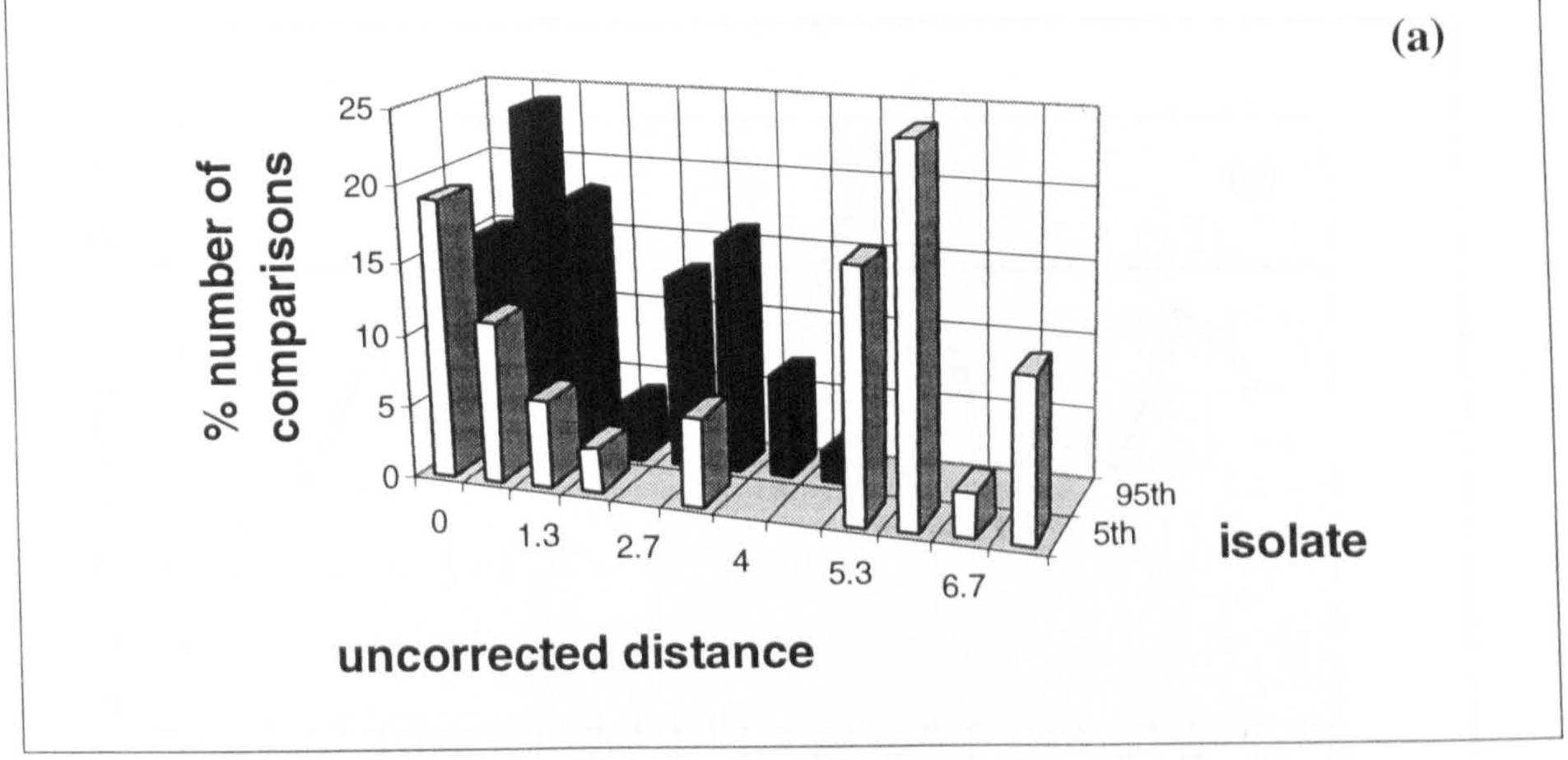
5th passage virus, and the generation of apparently novel sequences in the 95th passage virus (Figure 1*a*). These changes included (i) loss of nucleotide sequence variability at 10 sites, 3 of which were non-synonymous leading to loss of amino acid variability, and (ii) a gain at 7 sites, 6 of which generated new amino acids. Overall, these changes led to a reduction in quasispecies variability, as assessed by uncorrected nucleotide distance distributions within individual populations (Figure 4a). The presence of defective viral genomes was suggested by a 61 nucleotide deletion and an in-frame stop codon in single clones derived from the 5th and 95th pass respectively (Figures 1a and 2a).

Virus evolution in experimentally infected cats.

Following inoculation with FCV LS015, cat Oscar developed signs of typical FCV disease with mild pyrexia (Figure 5a) and oral ulceration. Virus was isolated from the oropharynx up to 39 days p.i. (Figure 5b).

Sequence comparison of the two day p.i. virus (Figure 1b) with the infecting isolate (5th passage LS015, Figure 1a) showed they were very similar, the two day p.i. virus containing one synonymous nucleotide substitution in 5 clones (T to C at nucleotide 75) and a 2 nucleotide deletion. Subsequent evolution occurred, as in cell culture, by a combination of sequence loss and the generation of novel nucleotide (Figure 1b) and amino acid (Figures. 2b and 3b) sequences. By 39 days p.i., these changes resulted in loss of sequence variability at 12 sites, 4 of which led to non-synonymous changes, and a gain at three non-synonymous sites. This was reflected by a marked reduction in the distribution of nucleotide distances between cloned sequences within successive isolates (Figure 4*b*). There were also 2 deletions of 3 and 44 nucleotides in individual clones derived from the 21 day p.i. isolate.

VN tests showed that virus isolated from Oscar evolved antigenically over the course of infection (Table 2). Viruses isolated 17 and 21 days p.i. were markedly (>4-fold) more sensitive to neutralisation than the infecting isolate (5th passage LS015) with antisera obtained 9 weeks p.i. Subsequently, the virus isolated 39 days p.i. appeared to become slightly less sensitive again to neutralisation with the same antisera. In contrast, virus passaged in cell culture showed no significant change in



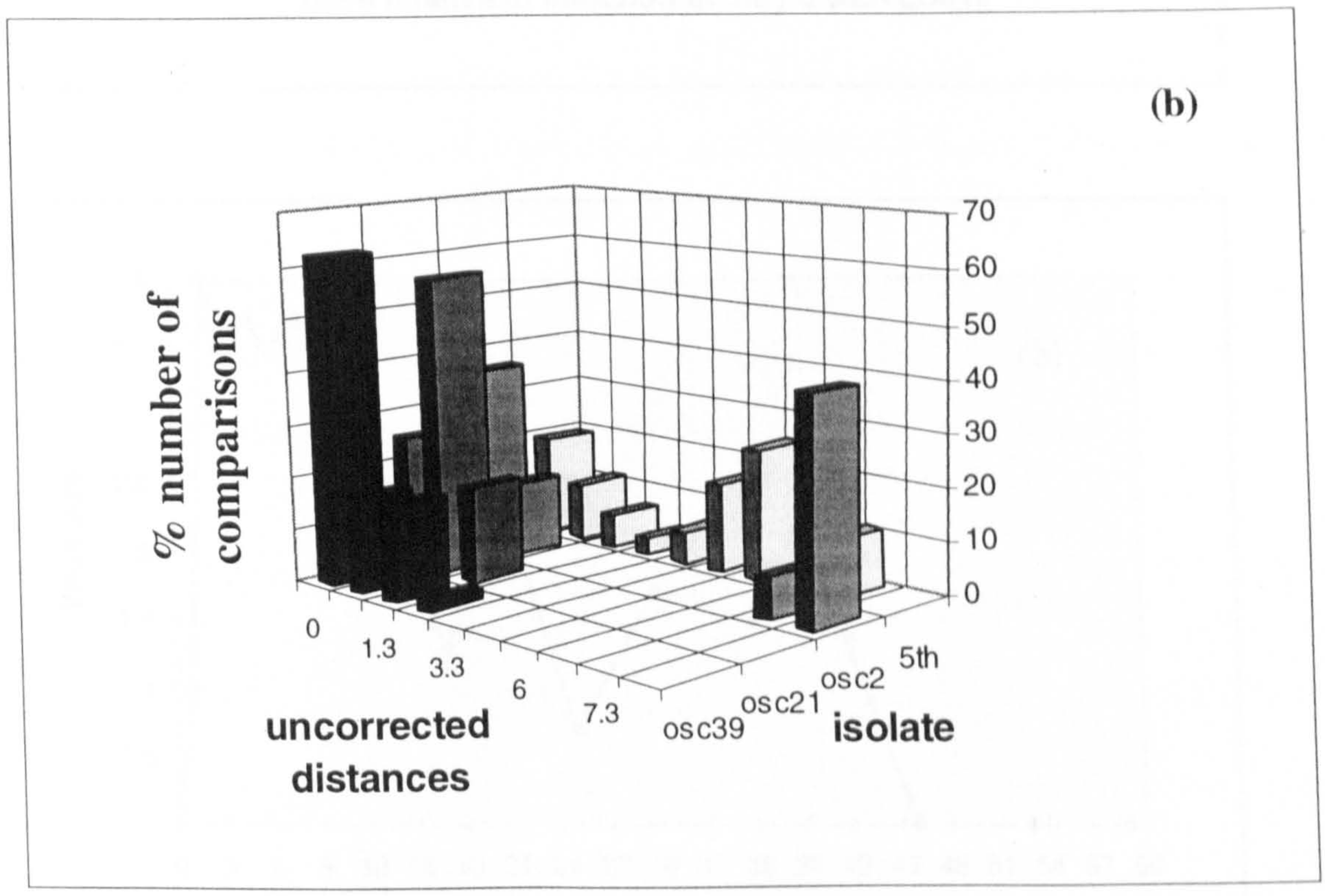
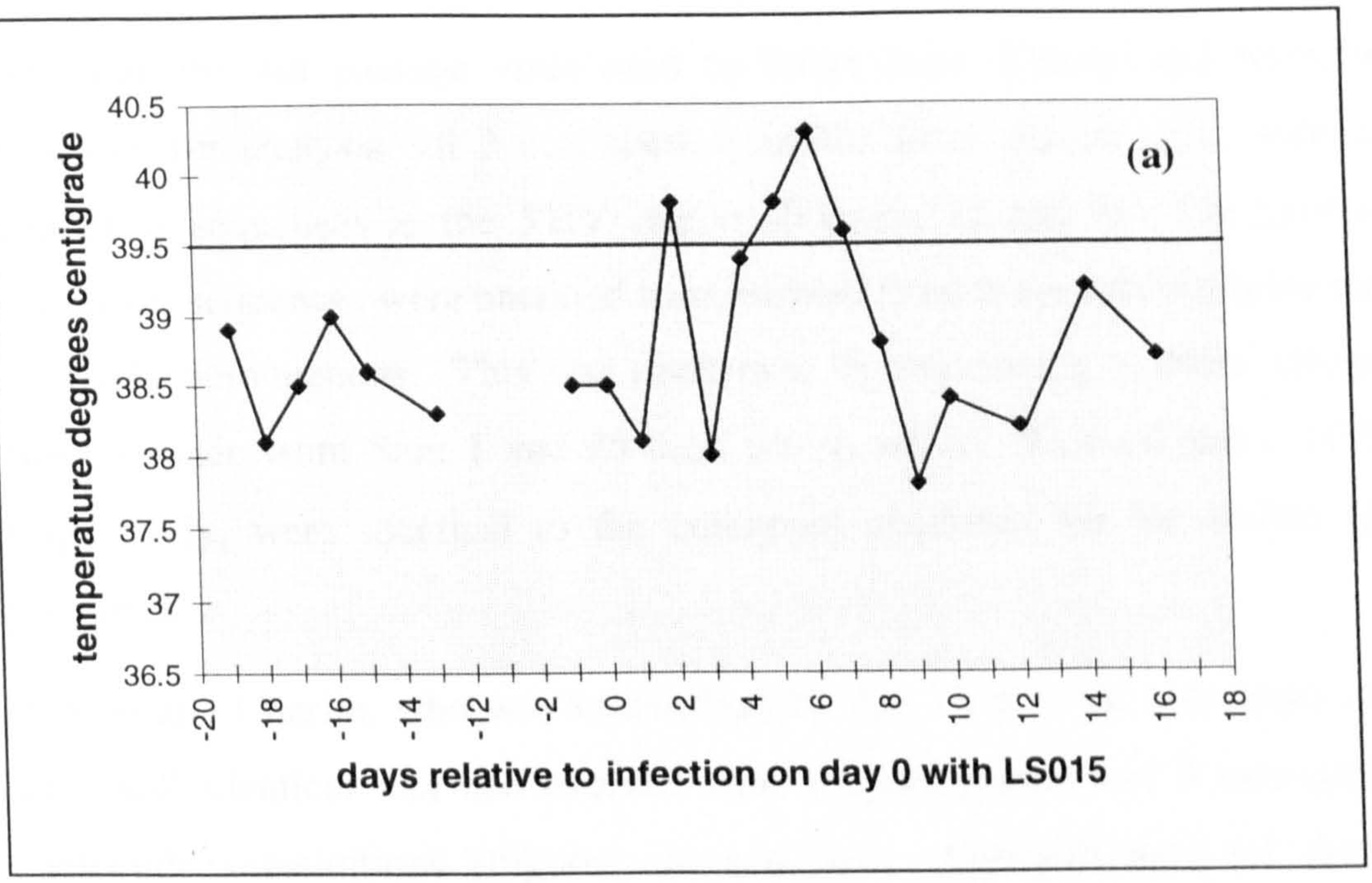


Figure 4; Uncorrected distance distributions (DISTANCE) (Deveraux *et al.*, 1984). Distance distributions for each isolate for which multiple cloned sequence data were available were produced from the uncorrected distance values for each cloned sequence relative to all other sequences in that isolate. The percentage of the total number of comparisons made within an isolate with a particular distance value is on the y-axis. The uncorrected distance is on the x-axis. a) 5th and 95th cell culture passaged virus. b) virus isolated from persistently infected cat Oscar, 2, 21 and 39 days p.i. with 5th pass LS015.



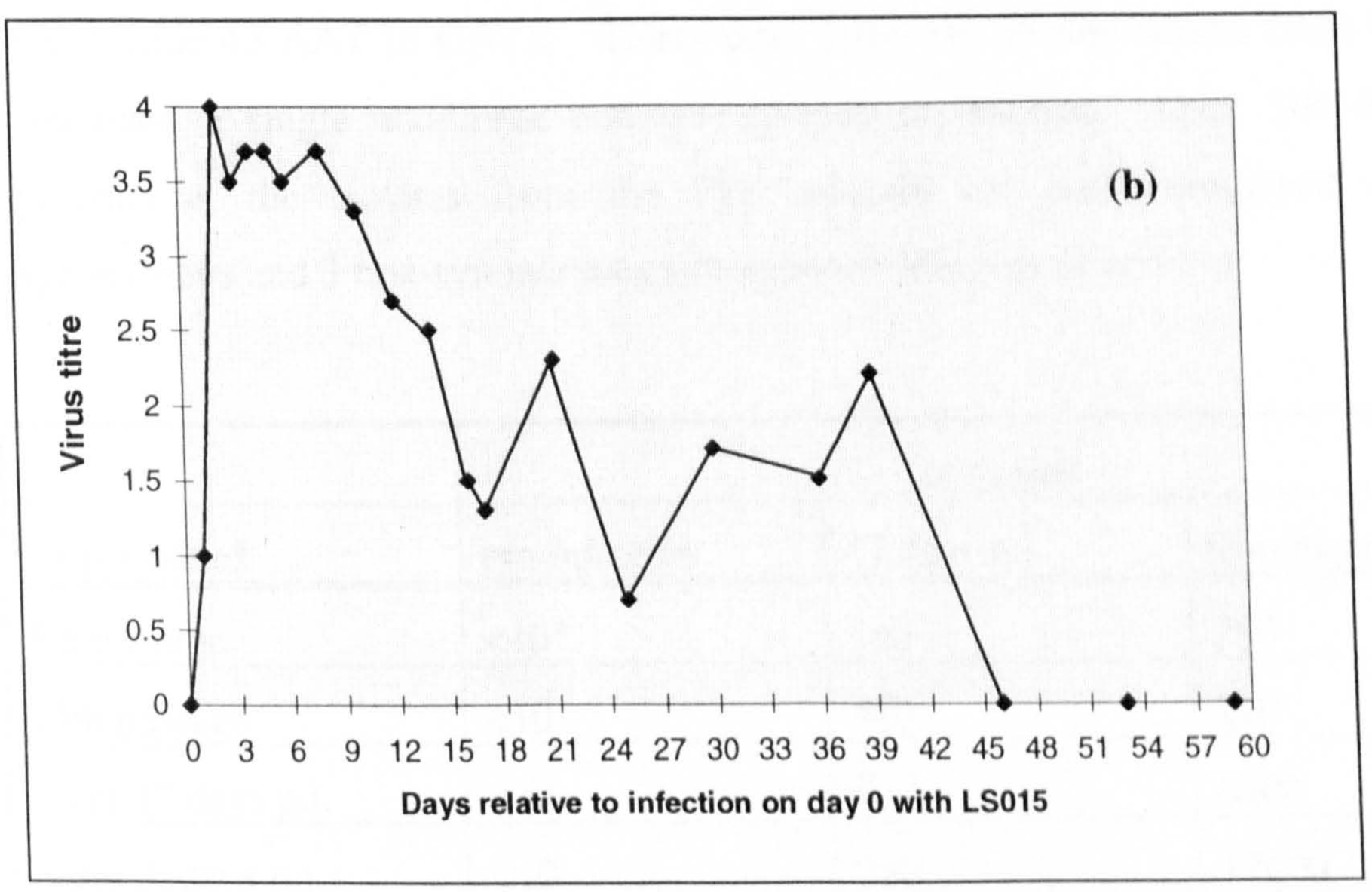


Figure 5; (a) Rectal temperature profile and (b) oropharyngeal virus titre for cat Oscar. In (a), a temperature of 39.5°C is indicated as approximately normal.

VN profiles using the same antisera.

Although the 4th passage virus used to infect Sam, Charlie and Sally was not available for analysis, all 3 cats shed a similar virus one day p.i., with identical consensus sequences in the 5'HV region (Figures. 1c and 2c). In general, clear consensus sequences were obtained from isolates from these cats implying they were relatively homogenous. This was confirmed by sequencing multiple clones of the isolates made from Sam 1 and 25 days p.i. in which 9 of 10 and 7 of 8 clones respectively, were identical to the consensus sequence for the isolate (data not presented).

In Sam and Charlie, who was vaccinated, by day 25 p.i., the consensus sequences were still identical and had evolved with 6 synonymous and 5 non-synonymous nucleotide substitutions (Figures. 1c and 2c). Although most of the changes observed were different to those seen in Oscar's isolates, one was the same (nucleotide 43 AAT to GAT). Thirty days later, the 55 day isolate from Charlie contained a single additional non-synonymous substitution. Over 106 days of persistence, the isolates from the FIV infected cat Sally contained only 1 synonymous and 3 non-synonymous substitutions (Figures 1c and 2c).

	antisera†		
virus isolate‡	pre-infection	17 days p.i.	9 weeks p.i.
5th passage	<10*	160	780
95th passage	<10	390	220
oscar 17 days p.i.	<10	470	6400
oscar 21days p.i.	<10	550	17600
oscar 39 days p.i.	<10	220	2080

Table 2; Virus neutralisation results. † antisera was obtained from cat Oscar, prior to, 17 days and 9 weeks p.i. with 5th passage LS015. ‡ 5th passage LS015 was passed a further 90 times in cell culture to generate 95th passage virus. It was also used to infect cat Oscar on day 0. Virus was reisolated from Oscar on days 17, 21 and 39 p.i. *titres are expressed as the reciprocal of the mean for duplicated 50% end points.

Comparison of consensus sequence from sequential isolates from Sam, Charlie and Sally revealed regions for which dn>ds. For the 1 and 25 day p.i. isolates from Sam and Charlie, this window was between nucleotides 22 and 84 (Figure 6). For all other comparisons (Charlie 25 and 55 days p.i.; Sally 1 and 25 days p.i.; Sally 25 and 106 days p.i.), the highlighted windows were between nucleotides 22 and 144 (corresponding to amino acids 8-48 underlined in Figure 2c). Here, non-synonymous substitutions occurred in the absence of synonymous substitutions (data not presented).

Estimates for the rate of nucleotide sequence change were carried out for Sam, Charlie and Sally, since these populations appeared highly homogenous and therefore unambiguous consensus sequences were available. The greatest rate of change was 1.07 substitutions per nucleotide per year (s/n/y) over the first 25 days p.i. in Sam and Charlie. In contrast, the rate of change for isolates from the FIV-infected cat Sally was only 0.10 s/n/y over the same period. Subsequently, the consensus sequence evolved over 30 days in Charlie and 81 days in Sally at 0.16 and 0.12 s/n/y respectively (these latter Figures assume that the two nucleotides whose sequence was unattainable in both of the latest isolates from Charlie and Sally (nts 146 & 147; Figure 1c) were the same as in the 25 day p.i. isolates).

A comparison of deduced amino acid sequences for all isolates is shown in Figure 2. The majority of changes (between amino acids 11 and 39 for cell culture-passaged virus and between 15 and 41 for virus passaged in cats) were within the 5'HV as defined by Seal *et al.* (1993) with amino acid sites 22, 27 and 31 being consistently variable. In addition, in cell culture there was complete replacement of threonine with isoleucine at amino acid 11, and in cats, the generation of aspartate at amino acid 15 during persistent infection in Oscar, Sam and Charlie .

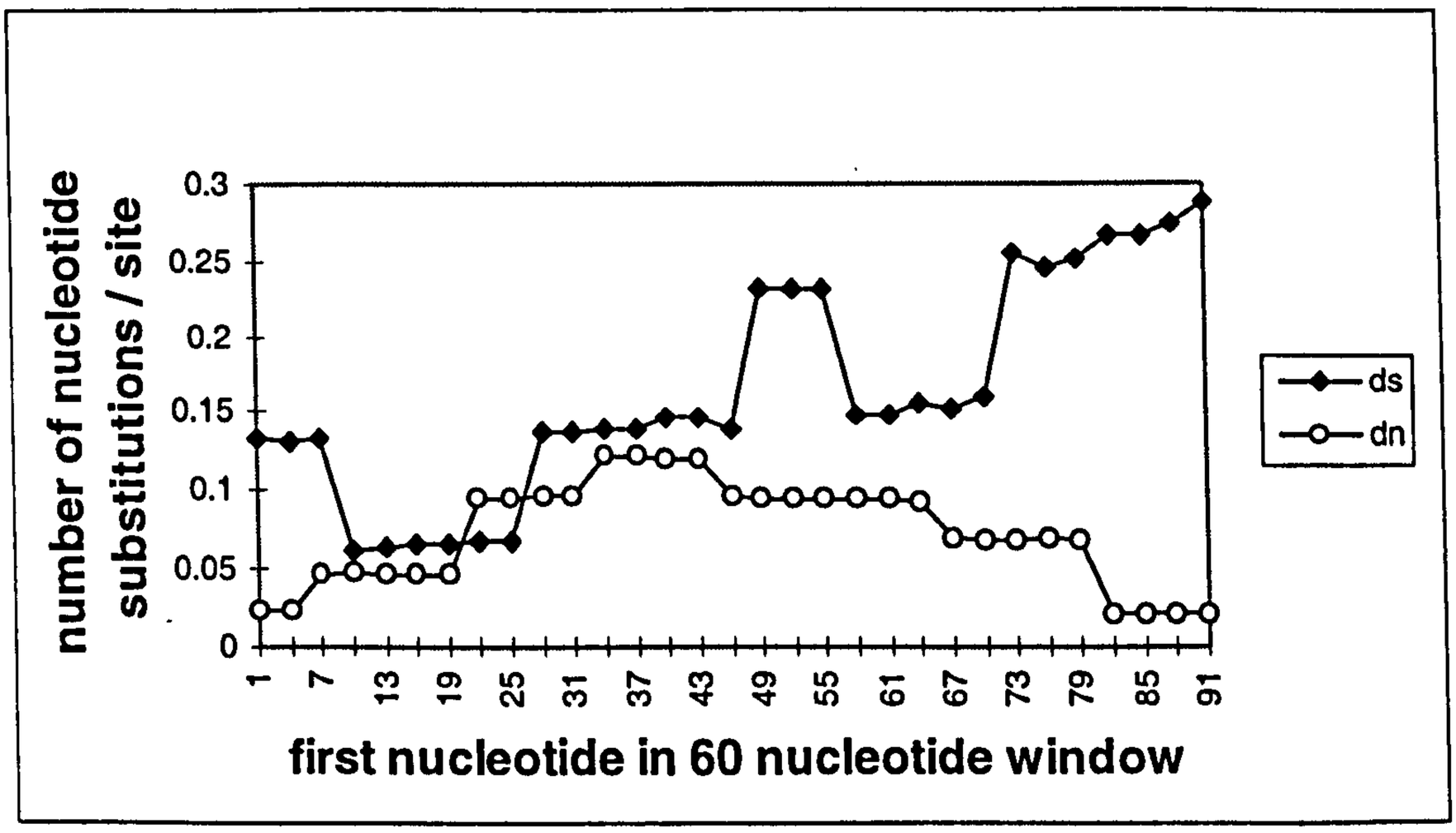


Figure 6; Sixty nucleotide, sliding window analysis of dn and ds (wina C++ version 0.3 (Endo *et al.*, 1996) using consensus sequences for isolates from either Sam or Charlie (sequence was identical), 1 and 25 days p.i.

DISCUSSION.

Feline calicivirus is unique amongst the caliciviruses in that it has a well defined carrier state (Povey *et al.*, 1973; Wardley & Povey, 1977b). This study aimed to characterize in detail sequence changes in a hypervariable region of the capsid gene of viruses obtained sequentially from persistently infected cats and compare these to changes occuring in virus passaged in cell culture. This region of the genome was selected as it is an important target for virus neutralising antibody responses (Guiver *et al.*, 1992; Milton *et al.*, 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997).

In common with other RNA viruses (Holland *et al.*, 1992) and, as has recently been shown for a calicivirus of rabbits (Gould *et al.*, 1997), the LS015 strain of FCV used in these studies was found to consist of a viral quasispecies, containing a mixed population of closely related sequences. Broadly speaking, this population could be divided into two relatively distinct but still overlapping subpopulations. All sequences of clones in the 5' HV region were greater than 92% similar, whereas distinct strains of FCV are generally only 57-79% similar (Seal *et al.*, 1993; Seal, 1994; Radford *et al.*, 1997 and unpublished data). This suggests that the LS015 strain used in these studies was a true quasispecies rather than a mixed population of different strains. Biologically uncloned, low passage virus was used in these studies as it was thought more likely to reflect virus encountered under natural conditions.

Following replication both in cell culture and in cat Oscar, this quasispecies apparently evolved at the nucleotide level, by two distinct mechanisms. Firstly, there was a loss of some sequences, largely manifest by the loss of one of the subpopulations within 5th passage LS015. Secondly, surviving virus also appeared to acquire novel sequence which tended to increase population variability. However, it is possible that such novel sequences may have arisen from continued selection of rare sequences already present in the starting population. Overall, these opposing forces of sequence loss and sequence gain tended to generate more homogenous populations of virus, particularly in cat Oscar. It is possible that eventual clearance of infection may be associated with a gradual reduction in quasispecies variability and with it, reduced adaptability of the population to the host immune response.

In cat Oscar, where cloned sequences were available for comparison with input virus, this evolution in nucleotide sequence led to both silent substitutions and changes in amino acids, most of which were observed in the 5' HV region of the capsid gene, rather than within conserved flanking sequence. Such changes may be indicative of selection for variants from within the quasispecies that are resistant to neutralisation, thus favouring viral persistence. In support of this, virus isolated from Oscar during the course of infection showed a significant change in virus neutralisation profile, especially around 17-21 days p.i.. VN responses to LS015 have been shown in previous studies to peak by that time (Knowles *et al.*, 1991) and so it is probable that such virus would have been subject to immune selection pressures. Other workers have also noted a change in neutralisation profile in FCVs isolated sequentially from persistently infected cats (Wardley, 1976; Johnson, 1992; Pedersen & Hawkins, 1995).

Studies with isolates from Sam, Charlie, and Sally, using consensus sequences, showed similar evolution in the 5' HV region in that both synonymous and non-synonymous substitutions were seen. Although input virus was not available for these cats, all three shed virus 1 day p.i. with identical nucleotide sequence in the 5'HV, suggesting either that this was the sequence of the input virus or that this virus was rapidly selected, being best adapted to establishing early infection. By day 25, Sam and Charlie were still shedding similar virus. Since Charlie was vaccinated and Sam was not, it was perhaps surprising that virus isolated from Charlie did not evolve more rapidly than virus isolated from Sam. However, both cats were housed together and therefore cross-infection may have taken place.

Nucleotide substitution rates and amino acid phylogenetic trees showed that virus from Sally appeared to evolve much more slowly than that from Sam and Charlie. Sally was FIV infected and it is possible that the immunosuppression associated with FIV infection (Willett *et al.*, 1997) would lead to less immune selection pressure on FCV. Indeed, Sally was part of a group of FIV infected cats which showed significantly longer duration of FCV shedding, and lower VN reponses to FCV after challenge, than non-FIV infected cats (Dawson *et al.*, 1991). Limited or reduced sequence evolution has also been reported for hepatitis C virus in

immunocompromised patients (Kumar *et al.*, 1994; Mazza *et al.*, 1996; Odeberg *et al.*, 1997).

Replication of virus in cell culture was also associated with the accumulation of both synonymous and non-synonymous nucleotide substitutions, and some of these changes affected the same codons as seen in virus from cats. It might be expected that a greater proportion of the changes in cell culture would be synonymous, due to lack of immune selection pressure. However, comparison of dn and ds ratios for representative cloned sequence from 5th and 95th passage virus (data not presented) with consensus sequence from Sam and Charlie did not seem to support this.

Interestingly, these non-synonymous changes during cell culture did not appear to alter significantly (> 4-fold) the neutralisation profiles of 5th and 95th passage virus. This suggests that the amino acid changes that were present were not of marked antigenic significance. Rather, they may have been associated with other selective pressures operating in cell culture (e.g. those acting on host cell receptor recognition domains, which in other viruses may be found within such hypervariable regions (Domingo *et al.*, 1993; Berinstein *et al.*, 1995)). In contrast, viruses isolated from persistently infected cats did show marked antigenic change on VN tests, and it therefore follows that the changes found only in these isolates (e.g. the change to aspartate at amino acid 15) may be critical antigenically and have evolved in order to evade the immune response. Alternatively, the observed changes in neutralisation profiles may relate to changes elsewhere in the genome rather than within the 5'HV region.

Some clones in both cell culture-passaged virus and in Oscar contained deletions and stop codons which may suggest the presence of defective viruses. It has been suggested that the presence of defective interfering particles (DIPs) within viral quasispecies may promote viral persistence by down-regulating viral lytic replication (O'Hara *et al.*, 1984; Rossi *et al.*, 1988; Martell *et al.*, 1992; Adami *et al.*, 1995; Saïb *et al.*, 1995; Chen *et al.*, 1996). DIPs have been reported for nearly all major groups of RNA viruses (Perrault, 1981; Holland *et al.*, 1990). However, there is currently little evidence of their presence within the *Caliciviridae* (Jensen & Coates, 1976). If most circulating FCV populations do contain DIPs then this would provide an alternative or supplementary mechanism to antigenic variation for FCV

persistence. However, the possibility that these mutants are artefacts of the methodology used cannot be ruled out.

In conclusion, we have demonstrated the quasispecies nature of FCV and the evolution *in vitro* and *in vivo* of part of the viral capsid considered to be a target for virus neutralisation by the host immune response. These studies demonstrate how viral populations may interact with the host and other viruses such as FIV. Further studies, including the determination of the antigenic consequences of sequence changes in the hypervariable region examined in this study, and the extension of this work to other regions of the capsid, may lead to a better understanding of the mechanisms involved in FCV persistence.

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MANUSCRIPT 2

SHORT COMMUNICATION

Mapping of a B cell epitope in the capsid gene of feline calicivirus by generation of a neutralising monoclonal antibody escape mutant.

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ABSTRACT.

The linear epitope of the feline calicivirus (FCV) neutralising monoclonal antibody IG9 has previously been mapped to a 37 amino acid region within the 5' hypervariable region (HVR) of the FCV capsid gene (Milton *et al.* (1992), Journal of General Virology 73, 2435-2439). In order to further characterise this epitope, an escape mutant was produced by replicating virus in sub-neutralising concentrations of IG9 in cell culture. After three rounds of such replication, the progeny FCV isolate showed a greater than 320 fold reduction in sensitivity to neutralisation by IG9 when compared to the parent isolate. Sequence analysis of the 5'HVR for parent and progeny viruses demonstrated a single non-synonymous nucleotide substitution responsible for a T-I mutation that mapped within the previously characterised 37 amino acid region. This mutation was shown to be stable upon further replication of the virus in the absence of IG9.

Feline calicivirus is an important oral and respiratory pathogen of cats (reviewed by Gaskell & Dawson, 1994) and belongs to the family *Caliciviridae* (Cubitt *et al.*, 1995). It has a positive sense, single stranded RNA genome of approximately 7.7kb in length coding for three open reading frames (ORFs) (Neill, 1990; Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Oshikamo *et al.*, 1994; Sosnovtsev & Green, 1995; Glenn, 1997). ORF1 at the 5' end of the genome encodes the non-structural proteins. This is followed by ORF2 which encodes the major capsid protein. ORF3 at the 3'end of the genome, encodes a small protein which, by analogy to another calicivirus, may be a minor structural protein (Wirblich *et al.*, 1996). The major capsid protein has been divided on the basis of sequence conservation between different FCV isolates and other caliciviruses into relatively conserved (A,B,D and F) and more variable (C and E) regions (Neill, 1992; Seal *et al.*, 1993). Region E has been further divided into a central conserved domain separating 5' and 3' hypervariable regions (HVRs) (Seal *et al.*, 1993).

As well as FCV, the *Caliciviridae* also contains important pathogens of humans (Caul, 1996a, b), pigs (House & House, 1992) and lagomorphs (Chasey *et al.*, 1992; Chasey, 1997). Unusual amongst the *Caliciviridae*, FCV induces a persistent infection (Povey *et al.*, 1973; Wardley & Povey, 1977b) in which antigenic variation may play a role by allowing viral escape from host immunity (Johnson, 1992). Some of the epitopes responsible for antibody-mediated neutralisation are believed to reside in the major capsid protein, particularly around the 5'HVR (amino acids 426-460 (all numbered according to published sequence for FCV isolate F9 (Carter *et al.*, 1992a)) (Guiver *et al.*, 1992; Milton *et al.*, 1992; Neill *et al.*, 1997; Tohya *et al.*, 1997). In particular, mutants of FCV isolate F4 that escape neutralisation by four monoclonal antibodies (mAbs) map to residues 441, 448, 449 and 455 (Tohya *et al.*, 1997), and the linear epitopes of two mAbs (IG9 and 4E7) that neutralise FCV isolate F9 map between amino acids 422-458 (Milton *et al.*, 1992).

In this study, we generated an F9 escape mutant showing increased resistance to IG9. By comparing the sequence in the 5'HVR for the wild type virus and the escape mutant, we were able to further localise the IG9 epitope.

Production of mAb IG9 has been described elsewhere (Carter *et al.*, 1989) and was kindly provided by Dr. M.Carter. IG9 has previously been shown to neutralise isolate

F9 (Carter *et al.*, 1989). Virus neutralisations were performed on CRFK cells (Crandell *et al.*, 1973) essentially according to Dawson *et al.*, 1993b.

F9 was plaque purified three times using a growth medium (Dawson, 1991); 1% low melting point agarose (Appligene) overlay. The resultant virus (F9IG9$^+$) was neutralised by IG9 at a dilution of 1:640. An IG9 escape mutant (F9IG9$^-$) was produced by attempting to grow F9IG9$^+$ in successive 2-fold dilutions of IG9. Virus which was able to grow in the highest concentration of IG9 was amplified in growth medium containing half this IG9 concentration. This process was performed three times to produce F9IG9$^-$ which was not neutralised by a 1:20 dilution of IG9. The stability of the IG9 resistant phenotype was assessed by passing F9IG9$^-$ three times in the absence of IG9. The resulting isolate, designated F9IG9$^-$-pass, was still resistant to neutralisation by IG9 at a final concentration of 1:20.

Methods used to isolate RNA from FCV isolates, synthesise complementary DNA and amplify the region corresponding to the 5'HVR by the polymerase chain reaction have been described previously (Radford *et al.*, 1997). Amplicons were sequenced according to manufacturers instructions (ABI prism dye terminator cycle sequencing ready reaction kit; Perkin-Elmer) using previously described techniques (Radford *et al.*, 1997).

Results of sequence analysis of the 5'HVR of F9IG9$^+$, F9IG9$^-$ and F9IG9$^-$-pass are shown in figures 1 and 2. A single non-synonymous nucleotide substitution in F9IG9$^-$ (Figure 1) was responsible for a threonine to isoleucine substitution at amino acid 449 (Figure 2). This substitution persisted in F9IG9$^-$-pass which also contained another non-synonymous mutation resulting in the substitution of aspartate for asparagine at amino acid 459 (Figure 2). Whilst these substitutions were in a similar region to those observed previously during FCV replication (Radford *et al.*, 1998), they did not occur at the same sites.

This suggests that amino acid 449 is critical to the formation of the IG9 linear epitope. This region coincides with that shown by Tohya et al., to be important for linear epitope formation in FCV isolate F4 (Tohya *et al.*, 1997), and appears to confirm that the 5'HVR is an important site for antibody-mediated virus neutralisation. The presence of such epitopes within HVRs is seen in other RNA viruses (Goudsmit *et al.*, 1989; Weiner *et al.*, 1992; Pancino *et al.*, 1993) and is believed to reflect the surface

localisation and lack of structural constraints on such regions. The variability of this region between different FCV isolates may provide the basis of the observed differences in antigenicity between FCVs (Povey, 1974; Knowles *et al.*, 1990) which have previously been exploited as a means of typing FCV isolates (Dawson *et al.*, 1993a, b).

The presence of epitopes within HVRs may also facilitate virus escape from immunity within the host. Such epitope plasticity has been implicated in promoting persistent infections of human immunodeficiency virus and hepatitis C virus (Nara *et al.*, 1990; Simmonds *et al.*, 1990; Wolfs *et al.*, 1991; Weiner *et al.*, 1992; Kato *et al.*, 1993, 1994). Indeed, we have shown elsewhere that the 5'HVR of FCV undergoes extensive sequence evolution in persistently infected cats (Radford *et al.*, 1998). However, the correlation between sequence evolution and epitope function in FCV has not been shown *in vivo*. In this study we have generated an escape mutant in cell culture from a plaque-purified, and therefore relatively homogenous, parent isolate with relative ease. Within the host, isolates may not be subject to such initial restrictions on variability as imposed by plaque purification. It therefore is likely that viral replication within the host would provide a greater opportunity for the generation of escape mutants, both within epitopes in the 5'HVR and elsewhere. However, in order to evade the polyclonal nature of a host immune response, which may also involve cytotoxic T lymphocytes (CTLs), escape mutants in the host will probably require changes in more than a single epitope. The ability of FCV to escape polyclonal antisera in cell culture and the role of CTLs in FCV neutralisation are not known.

The persistence of the amino acid 449 mutation in the absence of IG9 suggests that this mutation does not convey any obvious reduction in fitness on viruses that possess it and further suggests that sequence in the 5'HVR is not subject to strict functional constraints. It is interesting to note that a second non-synonymous mutation occurred during the production of F9IG9$^-$-pass at amino acid 459. This marks the very 3'end of the 5'HVR (amino acids 426-460). We have shown previously that replication of virus in cell culture in the absence of antibodies is associated with the accumulation of mutations in the 5'HVR (Radford *et al.*, 1998). The significance of such mutations is uncertain but may reflect further adaptation of the isolate to replication in cell culture.

```
                6571                                                    6620
                 |                                                       |
F9IG9- pass  .......... .......... .......... .......... ..........
     F9IG9-  .......... .......... .......... .......... ..........
     F9IG9+  TGGCCTGACA CCACAATTCC TGGGGAGTTG ATACCAGCTG GTGATTACGC

                6621                                                    6670
                 |                                                       |
F9IG9- pass  .......... .......... .......... ........t. ..........
     F9IG9-  .......... .......... .......... ........t. ..........
     F9IG9+  AATCACCAAT GGTACTGGCA ATGACATCAC CACGGCTACA GGATATGACA

                6671                                                    6720
                 |                                                       |
F9IG9- pass  .......... .......g.. .......... .......... ..........
     F9IG9-  .......... .......... .......... .......... ..........
     F9IG9+  CTGCTGATAT AATTAAGAAC AATACCAACT TTAAGGGCAT GTACATATGT
```

Figure 1; nucleotide sequence from the 5'HVR of F9IG9^{+}, F9IG9^{-} and F9IG9^{-}-pass. Nucleotides are numbered according to previously published F9 sequence (Carter *et al.*, 1992a, b).

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                420                                                     469
                 |                                                       |
F9IG9- pass  .......... .......... .........i .........d ..........
     F9IG9-  .......... .......... .........i .......... ..........
     F9IG9+  WPDTTIPGEL IPAGDYAITN GTGNDITTAT GYDTADIIKN NTNFKGMYIC
                                    *        **       *
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Figure 2; putative amino acid sequence from the 5'HVR of F9IG9^{+}, F9IG9^{-} and F9IG9^{-}-pass. Residues 426-460 represent the 5'HVR. Amino acids 422-458 (in bold) correspond to the region in which the IG9 epitope has been mapped previously (Milton *et al.*, 1992). Mutation of amino acids 441, 448, 449 and 455 (indicated by *) has been shown by others to be associated with escape from antibody-mediated neutralisation (Tohya *et al.*, 1997). Amino acids are numbered according to previously published F9 sequence (Carter *et al.*, 1992b).

The fine mapping of B cell epitopes such as reported here, will be important to understand the significance of virus evolution, particularly within persistently infected cats and may facilitate the rational design of future subunit vaccines.

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Manuscript 3

Preliminary mapping of B-cell epitopes in the capsid gene of feline calicivirus.

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ABSTRACT.

In order to map linear B-cell epitopes in the major capsid protein of feline calicivirus (FCV), we have constructed an expression library containing random, short (100-300bp) fragments of the FCV strain F9 capsid gene. Analysis of this library has shown it to be representative of the region of the capsid gene that encodes the mature capsid protein. This library was screened using polyclonal antisera from a cat that had been challenged experimentally with F9 to identify immunoreactive clones containing B-cell epitopes. Initial screening has identified five clones that react positively to feline antisera in immunoblots. FCV derived sequence from these clones all map to the 5' hypervariable region of the capsid.

INTRODUCTION.

Feline calicivirus (FCV) belongs to the family *Caliciviridae* (Cubitt *et al.*, 1995). It contains a single-stranded, positive sense RNA genome of approximately 7.7kb. Comparison of full genomic sequences with other caliciviruses and to the related *Picornaviridae* has identified three open reading frames encoding from the 5' end of the genome, the non-structural proteins, the major capsid protein and a putative minor structural protein (Neill, 1990; Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Oshikamo *et al.*, 1994; Sosnovtsev & Green, 1995; Glenn, 1997). The capsid gene has been divided into both variable regions (C and E) and relatively conserved regions (A, B, D and F) (Neill, 1992; Seal *et al.*, 1993; Seal, 1994; Green *et al.*, 1995). Region E has been further divided into 5' and 3' hypervariable regions (HVRs) seperated by a conserved central domain (Seal *et al.*, 1993).

FCV is an important acute, oral and respiratory pathogen of domestic cats (Gaskell & Dawson, 1994). Following recovery from clinical disease, animals may develop a persistent, inapparent infection, and represent a reservoir of infection to susceptible cats (Povey *et al.*, 1973; Wardley, 1976). The mechanisms of this persistent infection are uncertain. However, the isolation of antigenically distinguishable isolates from persistently infected cats has led to the suggestion that antigenic variants arising during the course of persistent infection allow viral escape from the host immune response (Wardley, 1976; Johnson, 1992; Pedersen & Hawkins, 1995).

Vaccination against FCV is widespread (Gaskell & Dawson, 1994). Currently used vaccines are based upon attenuated or inactivated whole virus. Although generally effective at preventing clinical disease, cats may develop subclinical infection under the protection of vaccine-induced immunity (Gaskell *et al.*, 1982) and there is some evidence to suggest that, under certain circumstances, pre-existing immunity may promote the establishment of persistent infection (Dawson *et al.*, 1991). Despite vaccination, the prevalence of FCV within the cat population has remained high (Harbour *et al.*, 1991; Coutts *et al.*, 1994), at levels similar to those reported prior to the introduction of vaccination (Wardley *et al.*, 1974). FCV-related disease post-vaccination is not uncommon and may be associated with both field virus and vaccine virus (Dawson *et al.*, 1993a; Radford *et al.*, 1997).

Following infection with FCV, serum virus neutralising (VN) antibodies develop from approximately seven days post-infection (Kahn *et al.*, 1975; Knowles *et al.*, 1991). The levels of such VN antibodies correlate well with protection against homologous challenge (Povey & Ingersoll, 1975). There is also production of IgG and IgA associated mucosal immunity (Knowles *et al.*, 1991), and serum immunofluorescence, complement fixation, complement fixation inhibition and agar gel precipitation antibodies (Gillespie *et al.*, 1971; Olsen *et al.*, 1974; Wardley, 1974). Although major histocompatibility complex-restricted cytotoxic activity of peripheral blood T lymphocytes has been demonstrated in vaccinated cats, the significance of cytotoxic T lymphocytes to FCV protection is not known (Tham & Studdert, 1987).

Several studies have sought to identify the immunologically important regions of the FCV major capsid protein. These studies have demonstrated that the 5' HVR contains B-cell epitopes as neutralising monoclonal antibody (mAb) epitopes map to this region (Milton *et al.*, 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997), and synthetic peptides corresponding to this region induce the formation of neutralising polyclonal antisera (Guiver *et al.*, 1992). In particular, mutations affecting codons 441-455 are associated with escape from neutralising mAb reactivity in FCV isolate F4 (Tohya *et al.*, 1997). No neutralising epitopes have been mapped in conserved regions of the FCV genome. However, these studies either used mouse- or rabbit-derived antibodies or failed to examine the entire capsid gene. Therefore, no methodical search has been carried out for linear B-cell epitopes recognised by feline antibodies in the mature FCV capsid.

The aim of this study was to construct an expression library containing random, short (100-300bp) fragments of an FCV capsid gene, suitable for screening using antisera from an experimentally infected cat. We have identified immunoreactive clones and these have been mapped within the capsid. The significance of these findings to persistent infections and future rational vaccine design are discussed.

MATERIALS AND METHODS.

Construction of plasmid p1BSF9.

The construction of pF9VAC,which contains the mature capsid protein coding region of FCV strain F9 (nucleotides 5686-7329 (Carter *et al.*, 1992a)), has been described elsewhere (Glenn, 1997) and was kindly provided by Dr. Mark Glenn. To facilitate subsequent DNA manipulation, the F9 capsid gene was excised from pF9VAC using *BamHI* and *HindIII* (Boehringer Mannheim) and gel purified according to manufacturers instructions (QIAquick gel extraction kit; Qiagen). The recovered fragment was ligated (T4 DNA ligase; Boehringer Mannheim) into suitably prepared pBluescript (Stratagene) and transformed into competent cells (Ligator; R&D systems). Clones containing the correctly sized insert were selected following plasmid isolation and RE digestion. The insert in one such clone, designated p1BSF9, was further characterised by sequence analysis to confirm it contained the appropriate region of the F9 capsid using both standard plasmid-based primers and two further primers within the capsid gene (Table 1 and Appendix 1).

Primer	Sequence (5'-3')	Binding site in F9*
p1BSF9-1L	GGTTCTCTATCTCTGGCTCTGG	5966-5987
p1BSF9-2L	GACTGGTCTATGGTATTACAGGG	6839-6817

Table 1; Sequencing primers for p1BSF9. *Binding sites are relative to published FCV strain F9 sequence (Carter *et al.*, 1992). See also appendix 1.

Construction of lambda expression library λF9CAP.p1.

Random overlapping fragments of p1BSF9 of 100-300bp in length were generated by bovine pancreatic DNaseI (Boehringer Mannheim) digestion using standard protocols (Sambrook *et al.*, 1989). Briefly, each 15µl reaction contained approximately 1µg of p1BSF9 in 50mM Tris HCl (pH7.6); 10mM $MnCl_2$; 0.01% BSA. The correct degree of digestion was achieved by serial 10-fold dilutions of DNaseI. All reactions were performed for 1minute at 14°C. Reactions were terminated by the addition of 5µl 50mM EDTA. Products were separated on 1.5% agarose gels. Fragments of the correct size range from seven successful reactions were excised from the gel and purified (QIAquick gel extraction kit; Qiagen) to give a final yield of approximately 500ng DNA.

This DNA was blunt ended using the Klenow fragment of DNA polymerase I (Stratagene) in a reaction volume of 10µl containing 1u Klenow; 50mM Tris HCl (pH7.5); 7mM $MgCl_2$; 1mM dithiothreitol; 20mM NaCl; 20µM of each deoxynucleotide triphosphate (dNTP). The reaction was incubated at 25°C for 15minutes. The blunt-ended DNA was purified and concentrated by phenol chloroform extraction and ethanol precipitation, and ligated to EcoRI linkers (5'-CCGGAATTCCGG-3'; Stratagene) using standard protocols (Sambrook *et al.*, 1989). DNA ligase was heat-inactivated at 65°C for 15minutes and DNA digested with *EcoRI*. The products of digestion were separated on a 1.5% agarose gel and DNA of 100-300bp in size was excised, gel purified (QIAquick gel extraction kit; Qiagen) and concentrated by phenol chloroform extraction.

The DNA was ligated into the lambda gt11 expression vector pre-cut with EcoRI and ligated DNA packaged according to manufacturers instructions (Lambda gt11 system and Packagene; Promega).

All subsequent manipulations of lambda were performed according to manufacturers' protocols unless otherwise stated. The resulting library was designated λF9CAP. Initial screening of λF9CAP was performed by blue-white plaque selection according to manufacturer's instructions. The library was amplified using standard techniques

and aliquoted to produce working stocks designated λF9CAP.p1 which were used in all subsequent experiments.

Characterisation of inserts within λF9CAP.p1.

Insert size. The size of individual inserts within λF9CAP was determined by polymerase chain reaction (PCR) amplification across the λgt11 EcoRI cloning site using commercially available primers (Lambda gt11 forward and reverse sequencing primers; Promega)

Nine distinct plaques from λF9CAP were separately picked and stored in 1ml phage buffer (20mM Tris-HCl, pH7.5; 100mM NaCl; 10mM $MgSO_4$), 20µl chloroform at 4°C. Subsequently, 3µl of plaque phage buffer was added to 17µl ddH_2O and heated at 100°C for 5 minutes to release bacteriophage DNA into solution ready for amplification. The PCR reaction was performed in a total volume of 50µl containing the 20µl boiled DNA preparation, 1.25U Taq DNA poymerase & 1x PCR buffer (Gibco BRL), 1.5mM $MgCl_2$, 50mM each dNTP and 125nM of each of gt11 forward and reverse sequencing primers (Promega). Each reaction was overlaid with 50ul of mineral oil (Sigma). Thermal cycling conditions consisted of DNA denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, primer annealing at 40°C for 1 minute and primer extension at 72°C for 3 minutes. A final extension was performed at 72°C for 5 minutes. A negative control of water was processed simultaneously.

Insert origin. *In situ* hybridisation using three probes corresponding to the 5', middle and 3' regions of the mature FCV capsid coding region was used to confirm that λF9CAP.p1 contained DNA of FCV origin and that the library was equally representative of the whole of the coding region.

Briefly, p1BSF9 was digested with restriction enzyme pairs *NheI/ NcoI*, *NcoI/ NdeI* and *NdeI/ BamHI* to generate three fragments equivalent to the 5', middle and 3' regions of the mature FCV capsid coding region (see appendix 1). Fragments of the predicted sizes were gel purified (QIAquick gel extraction kit, Qiagen).

Approximately 25-50ng of each fragment was used as template to synthesise α32-P dCTP labelled probes (Prime-it RmT random primer labelling kit, Stratagene) and labelled probe separated from unincorporated nucleotides and primers using sephadex G50 columns (ProbeQuant G-50 micro columns, Pharmacia Biotech) according to manufacturers instructions.

Target DNA was prepared by plating out λF9CAP.p1 in order to produce approximately 300 discrete lambda plaques on a standard 90mm petri dish. Plaque blots were prepared in triplicate using Hybond-N nylon membrane (Amersham). The first blot was performed for 1 minute, the second for 2 minutes and the third for four minutes. Blots were processed essentially according to manufacturers instructions. Briefly, membranes were placed colony-side up on successive filter papers soaked firstly in denaturing solution (1.5M NaCl; 0.5M NaOH) for 7 minutes, then twice in neutralising solution (1.5M NaCl; 0.5M Tris-HCl pH7.2; 1mM EDTA) for 3 minutes and finally washed twice in 2xSSPE (0.36M NaCl; 20mM $NaPO_4$; 2mM EDTA pH7.7) for 5 minutes. Plaque lifts were dried for 5-10 minutes at 65°C and DNA fixed by wrapping the membranes in cling film and placing them DNA-side down on a ultraviolet transilluminator for 2 minutes.

All hybridisations were carried out in a micro-4 hybridisation oven (Hybaid), using stringent wash conditions and according to manufacturers instuctions. Following the final wash stage, membranes were wrapped in cling film and developed by autoradiography.

Antiserum.

Antiserum from a specific pathogen free cat infected with FCV F9 was kindly provided by Dr. Susan Dawson (Dawson *et al.*, 1993b). Aliquots of antisera were preabsorbed to reduce non-specific binding. The protocols used for preadsorbing test antisera and immunologically screening λF9CAP.p1 were kindly provided Dr. Sean Donnelly (Promega, UK). Briefly, a 7ml overnight culture of lambda host cells was centrifuged to pellet the bacteria. The supernatant was discarded, and the cells were resuspended in approximately 3ml TBS (137mM NaCl; 2.7mM KCl; 25mM Tris-base; pH 8.0)) and sonicated. Primary antisera (250µl) was added to 4ml of 1:20

TBS diluted lysate and incubated at 4°C overnight. Absorbed antisera was centrifuged at 15,000g for 5 minutes at room temperature to remove cellular debris, made up to 50 ml in 0.5% (w/v) BSA (bovine serum albumin) in TBS and stored at -20°C ready for use.

Immunological screening of λF9CAP.p1.

Hybond-N nylon membrane (Amersham) filters were soaked in 10mM IPTG (Sigma) and air dried. All wash stages were performed at room temperature with gentle agitation unless otherwise stated.

Approximately 2000 pfu of λF9CAP.p1 were plated out in 90mm petri dishes using standard protocols (Promega). As negative controls, antisera against F9 was also used to screen recombinant lambda gt11 containing a non-FCV derived insert as supplied by the manufacturer (Promega). Plates were incubated at 42°C for approximately 4 hours until plaques were just visible, overlaid with the IPTG impregnated membranes and incubated overnight at 4°C. Membranes were orientated to the plates using needle holes to allow future plaque identification, removed from the plates and washed twice in PBS (154mM NaCl; 3mM KCl; 9mM Na_2HPO_4; 1.65mM KH_2PO_4) for 5 minutes. Membranes were blocked for 1 hour in 1% (w/v) BSA in PBS and washed twice in 0.05% Tween-20 (Sigma) in PBS (PBS-T).

All subsequent washes and incubations were carried out in micro-4 hybridisation oven (Hybaid), individual solutions being prewarmed to working tempeature prior to addition to the membranes. Membranes were incubated with 10ml preabsorbed primary antisera for 1 hour at 37°C. After washing three times in PBS-T for 5 minutes per wash, membranes were incubated in 10ml mouse monoclonal anticat IgG biotin conjugate (Sigma) diluted 1:6000 in 0.5% (w/v) BSA in PBS-T. Membranes were washed three times in PBS-T for 5 minutes per wash and incubated for 10 minutes in extravidin peroxidase (Sigma) diluted 1:2000 in 0.5% (w/v) BSA in PBS-T. Membranes were washed twice in PBS-T and twice in PBS, 5 minutes/ wash, and incubated at room temperature with gentle agitation in a petri dish containing freshly prepared 3-3' diaminobenzadine tetrahydrochloride (Sigma) prepared according to

manufacturers instructions until plaques became visible. Membranes were washed twice in water to terminate the reaction and air dried.

Positive plaques were picked by aligning membranes to the original plates, stored in 1ml phage buffer, and subjected to two further rounds of immunological screening prior to sequencing.

Preparation and sequencing of DNA from positive plaques.

Plaques that reacted positively with antisera after three rounds of immunological screening were amplified to confluence on six 90mm petri dishes using an agarose overlay according to manufacturer's protocols. Subsequently, each plate was overlaid with 3ml of SM buffer (0.01% (w/v) gelatin; 50mM Tris-HCl pH 7.4; 100mM NaCl; 8mM $MgSO_4$) and incubated for 2 hours at room temperature with gentle agitation. The SM buffer/ phage suspension from the six plates was pooled and centrifuged at 2,000g to pellet cellular debris and 10ml supernatant was used for DNA isolation according to manufacturers instructions (Wizard lambda preps DNA purification system, Promega).

Isolated DNA was digested with *BamHI*, phenol chloroform extracted and ethanol precipitated. DNA concentration was estimated (Genequant II, Pharmacia Biotech), and diluted to 200ng/µl in ddH_2O. Sequencing was performed according to manufacturer's instructions (ABI prism dye terminator cycle sequencing ready reaction kit; Perkin-Elmer).

Sequence analysis software.

FCV capsid gene sequences were obtained from GenBank (Table 1). Sequence comparisons (PILEUP and PLOTSIMILARITY) were performed using version 8 of the Wisconsin package (1994), Genetics Computer Group (Deveraux *et al.*, 1984). A 60 nucleotide, sliding window analyses of synonymous substitutions per synonymous site (ds) and non-synonymous substitutions per non-synonymous site (dn) of FCV capsid gene sequences was performed using WINA C++ version 0.3 (Endo *et al.*, 1996).

Virus	GenBank accession number	Reference
LS015	NS	Unpublished sequence, see Glenn *et al.*, 1997.
F65	NS	Unpublished sequence, see Glenn *et al.*, 1997.
A4	NS	Unpublished sequence, see Glenn *et al.*, 1997.
JOK63	NS	Unpublished sequence, see Glenn *et al.*, 1997.
LS012	NS	Unpublished sequence, see Glenn *et al.*, 1997.
CFI/68	M32819	Neill *et al.*, 1991.
Urbana	L40021	Sosnovtsev & Green, 1995.
255	U07130	Seal & Neill, 1994.
LLK	U07131	Seal & Neill, 1994.
F9	M86379	Carter *et al.*, 1992b.
NADC	L09718	Seal *et al.*, 1993.
KCD	L09719	Seal *et al.*, 1993.
F4	D90357	Tohya *et al.*, 1991.
2280	X99445	Geissler *et al.*, 1997.
KS109	X99446	Geissler *et al.*, 1997.
KS20	X99447	Geissler *et al.*, 1997.
KS40	X99448	Geissler *et al.*, 1997.
KS8	X99449	Geissler *et al.*, 1997.

Table 1; Origins of capsid gene sequences used for analysis by PLOTSIMILARITY (Deveraux *et al.*, 1984) and WINA C++ version 0.3 (Endo *et al.*, 1996). NS; not submitted.

RESULTS.

Sequence derived from p1BSF9, of the FCV F9 mature capsid coding region (nucleotides 5686-7329 of the F9 genome (Carter *et al.*, 1992a)), is shown in appendix 1.

Results of polymerase chain reaction analysis of nine randomly picked plaques from λF9CAP.p1 are shown in figure 1. Amplicon sizes range from approximately 180-310bp which is equivalent to lambda gt11 insert sizes of 90-220bp (PCR amplification across the cloning site of λgt11 adds approximately 90 nucleotides of vector-derived DNA to the insert). Probes representing the 5', middle and 3' regions of the F9 capsid gene each reacted positively with approximately 10% of plaques in λF9CAP.p1 (data not presented). This is in broad agreement with the proportion of p1BSF9 that was FCV derived and suggested λF9CAP.p1 was equally representative of the mature capsid coding region.

First round immunoscreening identified approximately 10 immunoreactive plaques per 2000 pfu. The feline anti-F9 sera did not react positively to any plaques in the lambda negative control. Sequence was obtained from five of the immunoreactive plaques after two further rounds of plaque purification (Appendix 1). A comparison of putative amino acid sequences for these five clones (L2, L3, L4, L5 and L7) and the capsid sequence of F9 from p1BSF9 is presented in figure 2. Conserved (B,D and F) and variable (C and E) regions are indicated (Neill, 1992; Seal *et al.*, 1993).

The five immunoreactive clones sequenced mapped between amino acids 390-487 of the capsid (Figures 2 and 3). Two contained identical sequence (L4 and L5). All five contained a part of the 5' HVR (amino acids 438-450) that contains amino acid residues known to be important for epitope fromation in another FCV isolate (Tohya *et al.*, 1997). Immunoreactive clone L2 contained sequence that mapped to HVR C as well as the 5'HVR (Figure 2).

A variability plot based on FCV capsid sequences is presented in figure 3 and highlites the variable nature of region C and the 5' and 3' HVRs. Sixty nucleotide sliding window analysis of available capsid gene sequences failed to demonstrate regions where dn exceeded ds. Since WINA performs all possible pairwise

comparisons within a group of aligned sequences, only a representative output of the programme is shown in appendix 2.

DISCUSSION.

It is important to identify those regions of viruses that closely interact with host proteins as these regions may determine the pathogenesis and clearance of the virus. For caliciviruses, such regions are largely unknown, and information that is available is limited to B cell epitopes (Guiver *et al.*, 1992; Milton *et al.*, 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997; Viaplana *et al.*, 1997).

Current evidence suggests that the 5' HVR contains the immunodominant region of the FCV capsid gene. However, these studies have either used non-feline antibodies (Milton *et al.*, 1992; Shin *et al.*, 1993; Tohya *et al.*, 1997) or have not analysed the whole of the FCV capsid coding region (Guiver *et al.*, 1992). In this study, we have constructed a lambda expression library that allows the identification of short (100-300bp) regions of the FCV strain F9 capsid gene that encode linear B cell epitopes. This library was screened using antisera obtained from an experimental cat previously infected with F9.

Preliminary sequence of five immunoreactive clones appears to confirm earlier work in implicating the 5'HVR as the immunodominant region of the FCV capsid, as all five clones partially overlapped the 5'HVR. One clone also contained sequence from HVR C. Sequence common to all five clones (amino acids 438-450) largely corresponded to residues known to be critical to monoclonal antibody recognition of linear epitopes in another FCV isolate (amino acids 441-455; Tohya *et al.*, 1997)). No clones contained sequence mapping to the 3'HVR. This region has been implicated in the formation of conformational epitopes (Tohya *et al.*, 1997) and therefore suggestes that, as expected, the methods used here are only applicable to the identification of linear B cell epitopes.

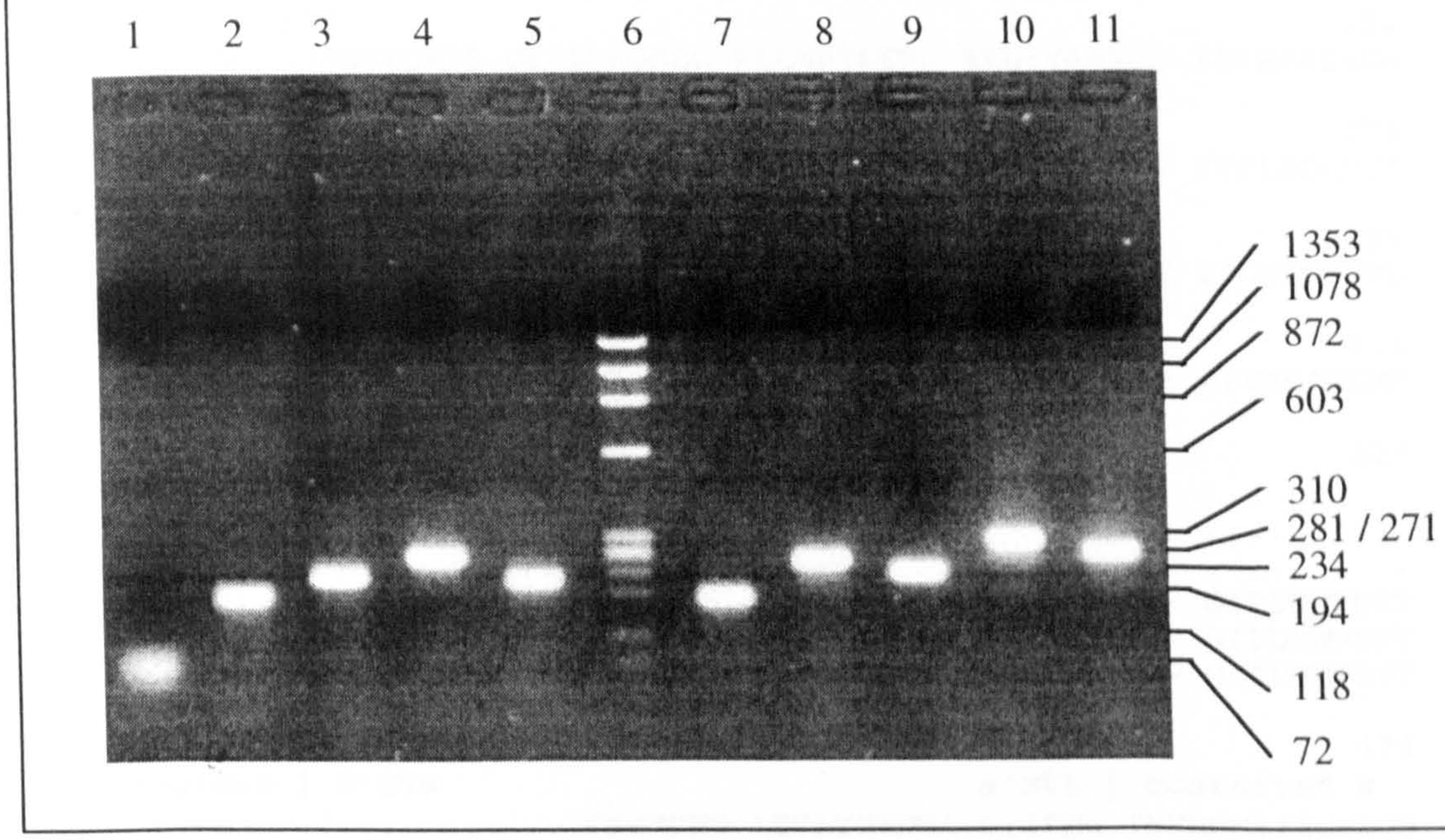

Figure 1; PCR amplification across the cloning site of nine randomnly picked plaques (lanes 2-5 and 7-11) from λF9CAP.p1. Lane 1; negative control. Lane 6; *HaeIII* digested ΦX174 molecular weight marker (Boehringer Mannheim). Amplicon sizes range from approximately 180-310bp.

```
          125                                                        174
          | region B
P1BSF9    ADDGSTTAPE QGTMVGGVIA EPSAQMSTAA DMATGKSVDS EWEAFFSFHT

          175                                                        224
P1BSF9    SVNWSTSETQ GKILFKQSLG PLLNPYLEHL AKLYVAWSGS IEVRFSISGS

          225                                                        274
P1BSF9    GVFGGKLAAI VVPPGVDPVQ STSMLQYPHV LFDARQVEPV IFCLPDLRST

          275                                                        324
P1BSF9    LYHLMSDTDT TSLVIMVYND LINPYANDAN SSGCIVTVET EPGPDFKFHL

          325                                                        374
P1BSF9    LKPPGSMLTH GSIPSDLIPK TSSLWIGNRY WSDITDFVIR PFVFQANRHF

          375                                                        424
                          region B |  C  | region D
L4/L5     .......... ..........  ..... .............. ..........
   L7     .......... ..........  ..... .............. ..........
   L3     .......... ..........  ..... .........DYIVP GIPDGWPDTT
   L2     .......... .....PISVTIT EQNGA KLGIGVATDYIVP GIPDGWPDTT
P1BSF9    DFNQETAGWS TPRFRPISVTIT EQNGA KLGIGVATDYIVP GIPDGWPDTT

          425                                                        474
regionD | 5'HVR                                  5'HVR | conserved E
 L4/L5 . .......... ...TNGTGND ITTATGYDTA DIIKNN TNFRGMYICGSLQR
    L7 . .......AGD YAITNGTGND ITTATGYDTA DIIKNN TNFRGMYICGSLQR
    L3 I PGELIPAGD YAITNGTGND ITTATGYDTA DIIKNN TNFRG.........
    L2 I PGELIPAGD YAITNGTGND ITTATG.... ...... ..............
P1BSF9 I PGELIPAGD YAITNGTGND ITTATGYDTA DIIKNN TNFRGMYICGSLQR

          475                                                        524
            conserved E | 3'HVR                              3'HVR | region
F
 L4/L5    AWG........... ...... .......... ..................... .
    L7    AWGDKKISNTAFI. ...... .......... ..................... .
    L3    .............. ...... .......... ..................... .
    L2    .............. ...... .......... ..................... .
P1BSF9    AWGDKKISNTAFIT TATLDG DNNNKINPCN TIDQSKIVVFQDNHVGKKA Q

          525                                                        574
P1BSF9    TSDDTLALLG YTGIGEQAIG SDRDRVVRIS TLPETGARGG NHPIFYKNSI

          575                                                        624
P1BSF9    KLGYVIRSID VFNSQILHTS RQLSLNHYIL PPDSFAVYRI IDSNGSWFDI

          625                                                        671
P1BSF9    GIDSDGFSFV GVSGFGKLEF PLSASYMGIQ LAKIRLASNI RSPMTKL*
```

Figure 2; Putative amino acid sequences from five immunoreactive clones (L2, L3, L4, L5 and L7) compared to F9 amino acid sequence from p1BSF9. Regions B, C, D, E and F (Neill, 1992; Seal *et al.*, 1993) are indicated. Region A is that part of the capsid precursor cleaved off to generate the mature capsid (Carter *et al.*, 1992). Sequence shared by all 5 clones (aa 438-450) is in bold. Underlined sequence (aa 441-455) represents a region containing residues critical to epitope formation in another FCV isolate (Tohya *et al.*, 1997). Amino acids are numbered according to FCV isolate F9 (Carter *et al.*, 1992).

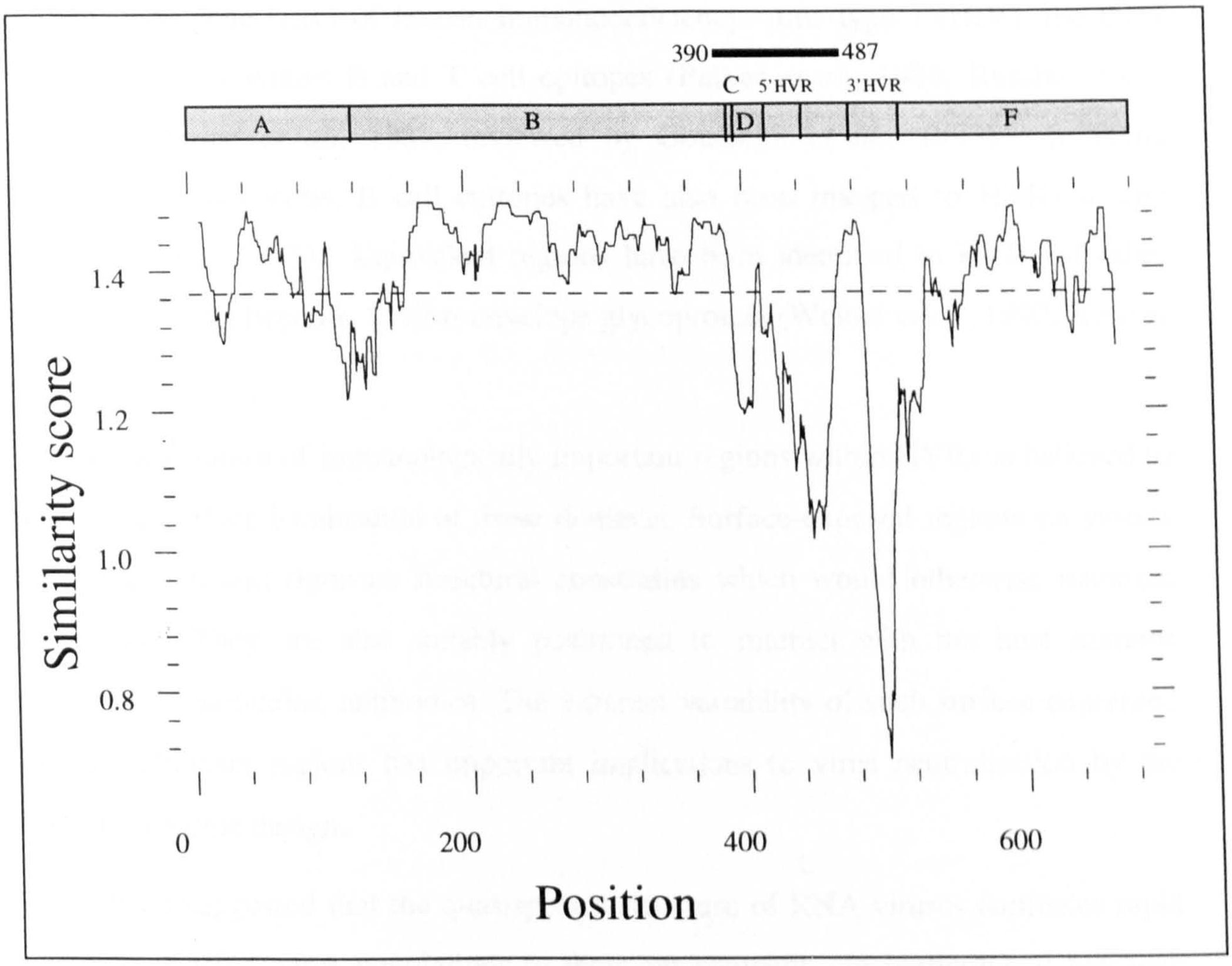

Figure 3; Variability plot derived from FCV capsid amino acid sequences (Table 1) using the PLOTSIMILARITY programme and a sliding window size of 10 amino acids (Deveraux *et al.*, 1984). The horizontal dotted line represents average similarity over the entire capsid with areas of greater than average variability below the line. Superimposed above are the relative positions of regions A-F as defined previously (Neill, 1992; Seal *et al.*, 1993). Region E is represented by 5' and 3'HVRs sepeated by a relatively conserved domain. Amino acids 390-487 indicated by the horizontal bar represents the region covered by lambda clones L2, L3, L4, L5 and L7.

The presence of immunodominant regions within viral HVRs is well recognised. In the envelope gene (*env*) of human immunodeficiency virus type I (HIV), the third variable region contains B and T cell epitopes (Palker *et al.*, 1988; Rusche *et al.*, 1988; Javaherian *et al.*, 1989; reviewed by Goudsmit *et al.*, 1993). In feline immunodeficiency virus, B cell epitopes have also been mapped to HVRs in *env* (Pancino *et al.*, 1993). Equivalent regions have been identified in HVRs of other viruses including hepatitis C virus envelope glycoprotein (Weiner *et al.*, 1992; Kato *et al.*, 1993).

The co-localisation of immunologically important regions within HVRs is believed to reflect the surface localisation of these domains. Surface-exposed regions on viruses are subject to less rigorous structural constraints which would otherwise minimise variability. They are also suitably positioned to interact with the host immune response, in particular, antibodies. The inherent variability of such surface expressed immunodominant regions has important implications to virus neutralisation by the host and vaccine design.

It has been suggested that the quasispecies structure of RNA viruses facilitates rapid adaption of RNA virus populations to their environment. In particular, it has been suggested for HIV and HCV that sequence evolution within HVRs may allow escape from the host immune response thereby facilitating the establishment and maintenance of persistent infections (Nara *et al.*, 1990; Simmonds *et al.*, 1990; Wolfs *et al.*, 1991; Weiner *et al.*, 1992; Kato *et al.*, 1993, 1994). Such continuous adaptation of virus to host may be associated with positive selection of non-synonymous (amino-acid changing) sequence evolution, such that in comparisons of HVRs between different isolates, dn may exceed ds (Simmonds *et al.*, 1990; Pancino *et al.*, 1993). The failure in this study to identify such regions within the FCV capsid gene may reflect the high degree of background variability within the HVRs and the relatively large window size used (60 nucleotides).

The lack of apparent functional constraints on epitopes residing in HVRs has two important implications relevant to FCV. Firstly, variability at antigenic sites reflects at the nucleotide level, the wide spectrum of antigenic profiles seen in different FCV isolates (Povey, 1974; Kalunda *et al.*, 1975; Knowles *et al.*, 1990; Dawson *et al.*,

1993a, b). Since most FCV vaccines are traditionally based upon a single isolate (Dr. R.Gaskell, personal communication), it is probable that vaccine induced immunity will not protect against all FCV isolates (Dawson *et al.*, 1993 a, b; Pedersen & Hawkins, 1995).

Secondly, variability of epitopes within an isolate may facilitate the selection of esape mutants that are resistent to neutralisation by the host immune response (Weiner *et al.*, 1992). This is true whether the immune response has been naturally acquired post-challenge or artificially induced post-vaccination and may promote vaccine failures and the establishment and maintenance of persistent infection.

Therefore, one approach to increase the cross-reactivity of vaccine-induced immunity and to reduce the risk of viral escape from vaccine-induced immunity would be the use of mixed vaccines containing several HVRs. Such vaccines would tend to induce protection against a larger spectrum of field isolates. This approach has been considered with both conventional multivalent vaccines based on whole virus (Dawson *et al.*, 1993b) and subunit, recombinant vaccines (DeSilver *et al.*, 1997). In the latter case, a recombinant polypeptide containing five HVRs from different FCV isolates was produced in a baculovirus expression system (MHV-vaccine). The MHV-vaccine was able to induce virus neutralising antibodies and some degree of protection to subsequent challenge with a single isolate. It did not alter duration of shedding of challenge FCV relative to the control groups. However, as no persistently infected animals were produced in this study, possibly as a result of the challenge isolate used, the effect of the MHV-vaccine on long-term virus shedding was not able to be tested (DeSilver *et al.*, 1997).

Despite the association of B cell epitopes and HVRs, conserved regions may also contain immunoreactive domains. Such epitopes make rational candidates for future vaccine design as neutralising immune responses targeting conserved epitopes should be broadly cross-reactive and reduce the risk of viral escape mutant formation. We have currently identified regions of 40-62 amino acids that react positively to feline antisera from a FCV challenged cat. As well as containing sequence from the 5'HVR, all of the immunodominant clones identified in this study also contain sequence that maps to outside the 5'HVR, covering sequence from the 3' end of conserved region

B to the 3' end of the conserved central domain of region E. Future work will aim to precisely identify the epitopes within this region by using sets of overlapping octa- and nano-peptides. We are also currently screening λF9CAP.p1 for immunoreactive clones that do not map to the 5'HVR by probing duplicated blots with either F9 antisera or oligonucleotide probes corresponding to the 5'HVR.

ACKNOWLEDGEMENTS.

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Appendix 1; FCV derived nucleotide sequence from p1BSF9 and L2, L3, L4, L5 and L7. The p1BSF9 sequence corresponds to nucleotides 5686-7329 of the published F9 sequence (Carter *et al.*, 1992). Sequencing primers p1BSF9-1L and p1BSF9-2L are indicated in bold. Internal restriction sites (*NcoI* and *NdeI*) used to generate probes corresponding to the 5', middle and 3' regions of the mature FCV capsid coding region are indicated (see text). Other restriction sites (*NheI* and *BamHI*) used were in pBluescript and are not shown.

```
                1                                                   50
P1BSF9  GCGGATGACG GGTCAACCAC AGCACCCGAG CAAGGAACAA TGGTTGGCGG

               51                                                  100
P1BSF9  CGTCATCGCT GAACCCAGCG CCCAGATGTC AACAGCTGCT GATATGGCCA

              101                                                  150
P1BSF9  CCGGGAAAAG CGTTGATTCT GAGTGGGAGG CATTCTTCTC CTTTCACACC

              151                                                  200
P1BSF9  AGCGTCAATT GGAGTACATC TGAAACCCAA GGAAAGATTC TCTTCAAACA

              201                                                  250
P1BSF9  ATCCTTAGGC CCTTTGCTCA ACCCATATCT AGAACACCTT GCTAAGCTAT

              251                                 p1BSF9-1L ->     300
P1BSF9  ATGTTGCGTG GTCTGGGTCG ATTGAGGTTA GGTTCTCTAT CTCTGGCTCT

              301                                                  350
P1BSF9  GGTGTCTTTG GTGGGAAGCT CGCAGCTATT GTTGTACCTC CTGGGGTTGA

              351                                                  400
P1BSF9  TCCAGTGCAG AGTACTTCGA TGCTACAATA CCCCCATGTT TTGTTTGATG

              401                                                  450
P1BSF9  CTCGTCAGGT GGAACCAGTT ATCTTCTGTC TTCCTGATCT AAGAAGCACC

              451                                                  500
P1BSF9  CTGTACCACC TTATGTCTGA CACTGACACT ACATCCTTGG TCATTATGGT

              501                                                  550
P1BSF9  GTACAATGAT CTCATCAATC CCTATGCCAA TGATGCCAAC TCTTCTGGGT

              551                                                  600
P1BSF9  GTATTGTCAC TGTCGAGACA GAACCTGGCC CTGACTTCAA GTTTCACCTC

              601                                                  650
P1BSF9  CTTAAGCCAC CCGGATCTAT GCTAACCCAT GGCTCTATCC CTTCTGATTT
                                          NcoI
              651                                                  700
P1BSF9  AATTCCCAAA ACATCTTCGC TCTGGATCGG TAACCGCTAC TGGTCAGACA

              701                                                  750
P1BSF9  TAACTGATTT TGTGATTCGG CCGTTTGTCT TCCAAGCAAA TCGTCATTTT

              751                                                  800
   L4   .......... .......... .......... .......... ..........
   L5   .......... .......... .......... .......... ..........
   L7   .......... .......... .......... .......... ..........
P1BSF9  GACTTTAATC AAGAGACCGC AGGGTGGAGC ACACCACGGT TTCGGCCTAT
   L2   .......... .......... .......... .......... .....CCTAT
   L3   .......... .......... .......... .......... ..........

              801                                                  850
   L4   .......... .......... .......... .......... ..........
   L5   .......... .......... .......... .......... ..........
   L7   .......... .......... .......... .......... ..........
P1BSF9  ATCTGTTACC ATTACTGAAC AGAACGGAGC AAAATTGGGC ATTGGGGTGG
   L2   ATCTGTTACC ATTACTGAAC AGAACGGAGC AAAATTGGGC ATTGGGGTGG
   L3   .......... .......... .......... .......... ..........
```

```
          851                                                       900
    L4    .......... .......... .......... .......... ..........
    L5    .......... .......... .......... .......... ..........
    L7    .......... .......... .......... .......... ..........
P1BSF9    CAACAGATTA CATAGTGCCT GGAATCCCTG ATGGCTGGCC TGACACCACA
    L2    CAACAGATTA CATAGTGCCT GGAATCCCTG ATGGCTGGCC TGACACCACA
    L3    .....GATTA CATAGTGCCT GGAATCCCTG ATGGCTGGCC TGACACCACA

          901                                                       950
    L4    .......... .......... .......... .........A CCAATGGTAC
    L5    .......... .......... .......... .........A CCAATGGTAC
    L7    .......... .......... .GCTGGTGAT TACGCAATCA CCAATGGTAC
P1BSF9    ATTCCTGGGG AGTTGATACC AGCTGGTGAT TACGCAATCA CCAATGGTAC
    L2    ATTCCTGGGG AGTTGATACC AGCTGGTGAT TACGCAATCA CCAATGGTAC
    L3    ATTCCTGGGG AGTTGATACC AGCTGGTGAT TACGCAATCA CCAATGGTAC

          951                                                      1000
    L4    TGGCAATGAC ATCACCACGG CTACAGGATA TGACACTGCT GATATAATTA
    L5    TGGCAATGAC ATCACCACGG CTACAGGATA TGACACTGCT GATATAATTA
    L7    TGGCAATGAC ATCACCACGG CTACAGGATA TGACACTGCT GATATAATTA
P1BSF9    TGGCAATGAC ATCACCACGG CTACAGGATA TGACACTGCT GATATAATTA
    L2    TGGCAATGAC ATCACCACGG CTACAGGATA .......... ..........
    L3    TGGCAATGAC ATCACCACGG CTACAGGATA TGACACTGCT GATATAATTA

         1001                                                     1050
    L4    AGAACAATAC CAACTTTAGG GGCATGTACA TATGTGGTTC GCTCCAGCGT
    L5    AGAACAATAC CAACTTTAGG GGCATGTACA TATGTGGTTC GCTCCAGCGT
    L7    AGAACAATAC CAACTTTAGG GGCATGTACA TATGTGGTTC GCTCCAGCGT
P1BSF9    AGAACAATAC CAACTTTAGG GGCATGTACA TATGTGGTTC GCTCCAGCGT
    L2    .......... .......... .......... .......... ..........
    L3    AGAACAATAC CAACTTTAGG GGCA..... ..........  ..........
                                          NdeI
         1051                                                     1100
    L4    GCCTGGGGT. .......... .......... .......... ..........
    L5    GCCTGGGGT. .......... .......... .......... ..........
    L7    GCCTGGGGTG ATAAGAAAAT TTCCAACACT GCCTTTATCA C.........
P1BSF9    GCCTGGGGTG ATAAGAAAAT TTCCAACACT GCCTTTATCA CCACTGCCAC
    L2    .......... .......... .......... .......... ..........
    L3    .......... .......... .......... .......... ..........

         1101                                  <- p1BSF9-2L       1150
P1BSF9    CCTAGATGGT GACAACAACA ACAAGATCAA TCCCTGTAAT ACCATAGACC

         1151                                                     1200
P1BSF9    AGTCAAAGAT CGTCGTGTTT CAAGACAACC ATGTTGGAAA GAAAGCGCAA

         1201                                                     1250
P1BSF9    ACCTCAGACG ATACATTGGC CCTGCTTGGT TACACTGGCA TTGGTGAGCA

         1251                                                     1300
P1BSF9    GGCCATCGGG TCTGATAGGG ACCGGGTTGT GCGCATCAGC ACTCTCCCTG

         1301                                                     1350
P1BSF9    AAACTGGTGC TCGAGGCGGT AACCACCCAA TTTTCTACAA GAACTCCATT

         1351                                                     1400
P1BSF9    AAATTGGGAT ATGTAATTAG GTCTATTGAT GTCTTTAATT CACAAATCTT

         1401                                                     1450
P1BSF9    GCACACTTCC AGACAGTTAT CGCTAAATCA TTACATACTC CCACCTGATT

         1451                                                     1500
P1BSF9    CTTTTGCCGT CTATAGAATA ATTGACTCAA ATGGCTCGTG GTTTGATATT

         1501                                                     1550
P1BSF9    GGAATTGATA GTGATGGGTT CTCTTTTGTT GGTGTTTCTG GCTTTGGTAA

         1551                                                     1600
P1BSF9    ATTAGAATTT CCCCTTTCTG CCTCCTACAT GGGAATACAA TTGGCAAAGA

         1601                                                1644
P1BSF9    TCCGGCTTGC CTCTAACATT AGGAGTCCCA TGACTAAGTT ATGA
```

Appendix 2; Sixty nucleotide sliding window analysis of synonymous substitutions per synonymous site (ds) and non-synonymous substitutions per non-synonymous site (dn) using WINA C++ version 0.3 (Endo *et al.*, 1996).

Sample output file is for comparison of FCV strains LS015 and F9. Position identifies the first nucleotide in the window. Size refers to the number of nucleotides in the window. Inf in the ds column indicates a ds value that can not be determined due to the presence of an infinite in the calculation.

position	ds	dn	size
1	0.299	0.043	60
4	0.2716	0.0907	60
7	0.3831	0.091	60
10	0.5604	0.0897	60
13	0.7489	0.0897	60
16	1.0298	0.0893	60
19	1.7376	0.088	60
22	1.7376	0.088	60
25	Inf	0.1113	60
28	Inf	0.1113	60
31	Inf	0.113	60
34	Inf	0.113	60
37	Inf	0.1113	60
40	Inf	0.088	60
43	3.2178	0.0887	60
46	3.2178	0.0887	60
49	3.2178	0.0887	60
52	2.0508	0.09	60
55	2.7082	0.0657	60
58	2.4142	0.066	60
61	2.7082	0.0657	60
64	Inf	0.0213	60
67	Inf	0.0214	60
70	Inf	0.0214	60
73	Inf	0.0214	60
76	Inf	0.0217	60
79	2.2834	0.0221	60
82	2.2834	0.0221	60
85	3.2958	0	60
88	Inf	0	60
91	Inf	0	60
94	Inf	0.0216	60
97	Inf	0.0214	60
100	Inf	0.0214	60
103	Inf	0.0214	60
106	Inf	0.0214	60
109	Inf	0.0211	60
112	Inf	0.0211	60
115	Inf	0.0216	60
118	Inf	0.0218	60
121	Inf	0.0217	60
124	Inf	0.0216	60
127	Inf	0.0216	60
130	Inf	0.0219	60
133	Inf	0.0216	60
136	Inf	0.0216	60
139	Inf	0.0214	60
142	Inf	0.0217	60
145	2.2834	0.0221	60
148	1.7269	0.0217	60
151	2.2083	0.0214	60
154	Inf	0	60
157	Inf	0	60
160	1.8177	0.0217	60

position	ds	dn	size
163	1.1373	0.0219	60
166	0.7899	0.0222	60
169	0.5716	0.0226	60
172	0.4693	0.0222	60
175	0.6126	0.0222	60
178	0.485	0.0221	60
181	0.485	0.0221	60
184	0.6126	0.0222	60
187	0.7899	0.0222	60
190	0.4693	0.0222	60
193	0.4408	0.0226	60
196	0.4408	0.0226	60
199	0.4693	0.0222	60
202	0.6603	0.0219	60
205	0.7489	0.0214	60
208	0.7489	0.0214	60
211	0.7489	0.0214	60
214	0.6664	0.0742	60
217	0.7716	0.0759	60
220	0.8432	0.0516	60
223	0.8848	0.0512	60
226	0.9837	0.0504	60
229	1.1119	0.0497	60
232	0.9837	0.0504	60
235	0.8025	0.0497	60
238	0.7631	0.0501	60
241	0.9313	0.0508	60
244	0.6956	0.0508	60
247	0.6807	0.051	60
250	0.7823	0.0499	60
253	0.8708	0.0492	60
256	1.4757	0.0485	60
259	1.8526	0.0716	60
262	1.0763	0.0955	60
265	0.9567	0.1212	60
268	0.9567	0.1212	60
271	0.8634	0.123	60
274	0.6735	0.0914	60
277	0.7324	0.09	60
280	0.8817	0.1161	60
283	0.9745	0.1143	60
286	1.3341	0.1143	60
289	1.6479	0.1161	60
292	Inf	0.1143	60
295	Inf	0.117	60
298	Inf	0.117	60
301	Inf	0.1174	60
304	Inf	0.1156	60
307	Inf	0.1158	60
310	Inf	0.1195	60
313	Inf	0.1195	60
316	Inf	0.1215	60
319	Inf	0.1008	57
322	Inf	0.0785	54

position	ds	dn	size
325	Inf	0.051	54
328	Inf	0.0519	54
331	Inf	0.0519	54
334	Inf	0.0253	54
337	Inf	0.0257	54
340	Inf	0	54
343	Inf	0	54
346	Inf	0	54
349	Inf	0	54
352	Inf	0	54
355	Inf	0	54
358	Inf	0	54
361	Inf	0	54
364	Inf	0	54
367	Inf	0	54
370	Inf	0	54
373	Inf	0	54
376	Inf	0	54
379	Inf	0	57
382	Inf	0	60
385	Inf	0.0229	60
388	Inf	0.0465	60
391	Inf	0.0458	60
394	Inf	0.0451	60
397	Inf	0.0451	60
400	Inf	0.0688	60
403	Inf	0.0688	60
406	Inf	0.0698	60
409	Inf	0.0698	60
412	Inf	0.0682	60
415	Inf	0.0682	60
418	Inf	0.0946	60
421	2.855	0.0946	60
424	Inf	0.0931	60
427	Inf	0.0917	60
430	Inf	0.0917	60
433	Inf	0.091	60
436	Inf	0.091	60
439	27.0327	0.091	60
442	2.7082	0.089	60
445	1.5011	0.0657	60
448	2.7082	0.0432	60
451	2.7082	0.0432	60
454	Inf	0.0435	60
457	Inf	0.0435	60
460	Inf	0.0211	60
463	Inf	0.0208	60
466	Inf	0.0208	60
469	Inf	0.0208	60
472	Inf	0.0213	60
475	Inf	0.021	60
478	Inf	0	60
481	Inf	0	60
484	Inf	0	60

position	ds	dn	size
487	Inf	0	60
490	Inf	0	60
493	Inf	0	60
496	27.0327	0	60
499	Inf	0.0423	60
502	Inf	0.0426	60
505	Inf	0.0429	60
508	1.6479	0.0429	60
511	Inf	0.0429	60
514	1.6479	0.0429	60
517	1.301	0.0423	60
520	1.1281	0.0429	60
523	1.0013	0.0435	60
526	0.7489	0.0435	60
529	0.903	0.0441	60
532	0.6872	0.0441	60
535	0.6126	0.0451	60
538	0.5913	0.0455	60
541	0.4279	0.0462	60
544	0.4279	0.0462	60
547	0.7037	0.0462	60
550	0.7037	0.0704	60
553	0.7587	0.0693	60
556	0.9313	0.0698	60
559	0.9313	0.0458	60
562	1.2071	0.0458	60
565	2.3516	0.0462	60
568	Inf	0.0462	60
571	Inf	0.0469	60
574	Inf	0.0476	60
577	Inf	0.0473	60
580	2.9189	0.0473	60
583	Inf	0.0473	60
586	Inf	0.0473	60
589	Inf	0.0469	60
592	Inf	0.0462	60
595	Inf	0.0458	60
598	Inf	0.0462	60
601	Inf	0.0455	60
604	Inf	0.0455	60
607	1.9969	0.0451	60
610	1.6479	0.0226	60
613	2.0637	0.0229	60
616	1.3438	0.0231	60
619	1.0397	0	60
622	0.7662	0	60
625	0.6566	0	60
628	0.6566	0	60
631	0.6566	0	60
634	0.7037	0	60
637	0.794	0	60
640	0.794	0	60
643	0.6355	0	60
646	0.6566	0	60

position	ds	dn	size
649	0.794	0	60
652	0.9178	0	60
655	1.1504	0	60
658	1.4039	0	60
661	1.2648	0	60
664	1.2963	0.0241	60
667	1.3656	0.0239	60
670	1.3656	0.0239	60
673	1.3656	0.0239	60
676	2.2543	0.0235	60
679	3.431	0.0232	60
682	Inf	0.0232	60
685	2.2543	0.0235	60
688	1.956	0.023	60
691	1.956	0.023	60
694	1.3844	0.023	60
697	1.3844	0.023	60
700	1.3844	0.023	60
703	2.5592	0.0226	60
706	2.1924	0.0228	60
709	Inf	0.0228	60
712	3.3665	0.0225	60
715	2.1249	0.0221	60
718	1.4083	0.0221	60
721	1.4083	0.0221	60
724	2.7852	0	60
727	1.3615	0	60
730	2.7852	0	60
733	1.5781	0	60
736	1.3615	0	60
739	1.2786	0	60
742	1.0763	0	60
745	1.4594	0	60
748	1.7984	0	60
751	1.2786	0	60
754	1.7984	0	60
757	1.9969	0	60
760	2.1249	0	60
763	2.1249	0	60
766	2.1249	0	60
769	1.4083	0	60
772	1.7186	0	60
775	1.7186	0	60
778	Inf	0	60
781	Inf	0	60
784	Inf	0	60
787	Inf	0	60
790	Inf	0	60
793	Inf	0	60
796	Inf	0	60
799	Inf	0	60
802	Inf	0.0225	60
805	Inf	0.0225	60
808	Inf	0.0226	60

position	ds	dn	size
811	Inf	0.0225	60
814	Inf	0.0223	60
817	Inf	0.0225	60
820	Inf	0.0226	60
823	Inf	0.0226	60
826	Inf	0.0222	60
829	Inf	0.0221	60
832	Inf	0.0219	60
835	Inf	0.0219	60
838	Inf	0.0219	60
841	Inf	0.0224	60
844	Inf	0.0224	60
847	Inf	0.0224	60
850	Inf	0.0221	60
853	Inf	0.0455	60
856	Inf	0.0448	60
859	Inf	0.0448	60
862	2.2834	0.0221	60
865	1.0763	0.0221	60
868	0.8817	0.0218	60
871	0.6735	0.0218	60
874	0.6018	0.0223	60
877	0.4618	0.0223	60
880	0.4618	0.0223	60
883	0.6018	0.0223	60
886	0.5622	0.0226	60
889	0.5813	0.0225	60
892	0.5622	0.0226	60
895	0.6677	0.023	60
898	0.5278	0.023	60
901	0.5622	0.0226	60
904	0.6018	0.0223	60
907	0.6018	0.0453	60
910	0.6018	0.0453	60
913	0.4932	0.022	60
916	0.4546	0.0224	60
919	0.4693	0.0222	60
922	0.5018	0.0219	60
925	0.6603	0.0219	60
928	0.6238	0.0221	60
931	0.6018	0.0223	60
934	0.6018	0.0223	60
937	0.6735	0.0218	60
940	0.9255	0.0217	60
943	0.8423	0.022	60
946	0.9255	0.0217	60
949	1.1058	0.022	60
952	1.1058	0.022	60
955	1.1058	0.022	60
958	1.1058	0.022	60
961	0.7739	0.0223	60
964	0.7739	0.0223	60
967	0.874	0	60
970	1.0638	0	60

position	ds	dn	size
973	0.9745	0	60
976	1.0892	0	60
979	1.3438	0	60
982	1.2071	0	60
985	1.2071	0	60
988	1.5902	0	60
991	2.0637	0	60
994	2.3516	0	60
997	2.9189	0	60
1000	1.8637	0	60
1003	1.7464	0	60
1006	1.7464	0	60
1009	1.5555	0	60
1012	1.5555	0	60
1015	1.8426	0	60
1018	2.0578	0	60
1021	2.0578	0	60
1024	2.0578	0	60
1027	1.6861	0.0218	60
1030	1.1213	0.0219	60
1033	1.1887	0.0218	60
1036	0.7818	0.0223	60
1039	0.7818	0.0223	60
1042	0.8517	0.0219	60
1045	0.8517	0.0219	60
1048	0.709	0.0216	60
1051	0.6803	0.0218	60
1054	0.6539	0.0219	60
1057	0.5343	0.0216	60
1060	0.6355	0.0221	60
1063	0.709	0.0216	60
1066	0.6943	0.044	60
1069	0.6668	0.0444	60
1072	0.7244	0.0437	60
1075	0.7244	0.0437	60
1078	1.0153	0.0434	60
1081	1.6911	0.0428	60
1084	1.4113	0.0434	60
1087	1.2667	0.0216	60
1090	1.2667	0.0216	60
1093	1.4696	0.0213	60
1096	2.0421	0.0209	60
1099	2.0421	0.0209	60
1102	3.7326	0.0212	60
1105	1.6911	0.0211	60
1108	2.9189	0.0431	60
1111	Inf	0.0437	60
1114	Inf	0.0437	60
1117	Inf	0.044	60
1120	Inf	0.0438	60
1123	Inf	0.0675	60
1126	Inf	0.0682	60
1129	Inf	0.0688	60
1132	Inf	0.0688	60

position	ds	dn	size
1135	Inf	0.0688	60
1138	Inf	0.0931	60
1141	Inf	0.1184	60
1144	Inf	0.1184	60
1147	Inf	0.1184	60
1150	Inf	0.1165	60
1153	Inf	0.1193	60
1156	Inf	0.1212	60
1159	Inf	0.1203	60
1162	Inf	0.1203	60
1165	Inf	0.1468	60
1168	2.3516	0.1212	60
1171	1.5847	0.1474	60
1174	1.3184	0.145	60
1177	0.9523	0.1462	60
1180	0.9978	0.145	60
1183	0.7587	0.1193	60
1186	0.5913	0.0939	60
1189	0.5716	0.0946	60
1192	0.4279	0.0954	60
1195	0.3041	0.0969	60
1198	0.3041	0.0715	60
1201	0.3121	0.05	57
1204	0.4157	0.05	57
1207	0.339	0.0488	57
1210	0.4279	0.0496	57
1213	0.4647	0.1247	57
1216	0.3494	0.1533	57
1219	0.3398	0.1546	57
1222	0.2496	0.1533	57
1225	0.2463	0.1252	57
1228	0.2463	0.1252	57
1231	0.3597	0.1236	57
1234	0.4441	0.1263	57
1237	0.5716	0.1855	57
1240	0.4314	0.1872	57
1243	0.5537	0.1872	57
1246	0.6999	0.1872	57
1249	0.7253	0.2498	57
1252	0.8817	0.2522	57
1255	1.1221	0.2522	57
1258	1.3878	0.2546	57
1261	1.5391	0.2406	60
1264	1.4873	0.3165	60
1267	1.7726	0.3986	60
1270	1.4873	0.4305	60
1273	1.3223	0.3263	60
1276	2.1454	0.2856	60
1279	3.0323	0.2804	60
1282	Inf	0.2804	60
1285	Inf	0.364	60
1288	Inf	0.364	60
1291	Inf	0.3347	60
1294	Inf	0.3331	60

position	ds	dn	size
1297	Inf	0.293	60
1300	Inf	0.293	60
1303	Inf	0.2877	60
1306	Inf	0.3208	60
1309	Inf	0.2583	60
1312	Inf	0.2877	60
1315	Inf	0.3179	60
1318	Inf	0.3179	60
1321	2.2661	0.3871	60
1324	1.8075	0.3034	60
1327	2.0219	0.239	60
1330	1.6479	0.2135	60
1333	2.0219	0.2099	60
1336	1.3484	0.2099	60
1339	1.2831	0.2405	60
1342	1.3721	0.2385	60
1345	1.3341	0.1654	60
1348	0.9745	0.1654	60
1351	1.4397	0.1641	60
1354	1.1281	0.1622	60
1357	1.3844	0.1389	60
1360	1.3844	0.1389	60
1363	Inf	0.141	60
1366	Inf	0.1139	60
1369	Inf	0.1132	60
1372	Inf	0.0898	60
1375	3.7326	0.0654	60
1378	1.4696	0.0659	60
1381	1.7376	0.0211	60
1384	1.7376	0.0211	60
1387	1.7376	0.0211	60
1390	Inf	0.0426	60
1393	Inf	0.0432	60
1396	Inf	0.0432	60
1399	Inf	0.0215	60
1402	1.6861	0.0218	60
1405	2.6224	0.0219	60
1408	2.9964	0.0444	60
1411	2.3791	0.0447	60
1414	1.9413	0.0452	60
1417	1.3395	0.0452	60
1420	1.1887	0.0474	57
1423	0.7752	0.0497	54
1426	0.8137	0.0532	51
1429	0.6655	0.0566	48
1432	0.6655	0.0566	48
1435	0.7083	0.1041	48
1438	0.9607	0.1041	48
1441	0.9607	0.1041	48
1444	0.9098	0.1381	48
1447	0.6228	0.141	48
1450	0.4904	0.1389	48
1453	0.4904	0.1389	48
1456	0.6626	0.1389	48

position	ds	dn	size
1459	0.7251	0.1361	48
1462	0.7251	0.1361	48
1465	0.5097	0.1374	48
1468	0.7428	0.1031	48
1471	0.824	0.1327	48
1474	1.1629	0.1327	48
1477	2.5755	0.1301	48
1480	1.7984	0.1219	51
1483	1.3438	0.1156	54
1486	1.3341	0.1499	57
1489	1.6479	0.168	60
1492	Inf	0.1923	60
1495	Inf	0.1511	60
1498	Inf	0.1488	60
1501	Inf	0.1488	60
1504	Inf	0.1248	60
1507	Inf	0.123	60
1510	Inf	0.1239	60
1513	Inf	0.122	60
1516	Inf	0.1239	60
1519	Inf	0.1239	60
1522	Inf	0.1239	60
1525	Inf	0.1248	60
1528	Inf	0.1248	60
1531	3.2579	0.102	60
1534	1.6099	0.102	60
1537	1.3721	0.1036	60
1540	1.8547	0.1297	60
1543	Inf	0.1297	60
1546	2.2834	0.0924	60
1549	1.9969	0.0688	60
1552	1.2071	0.0458	60
1555	1.0892	0.0465	60
1558	0.9952	0.0473	60
1561	0.9547	0.0476	60
1564	0.9547	0.0476	60
1567	1.2071	0.0476	60
1570	1.7118	0.0233	60
1573	1.4894	0.0236	60
1576	2.1249	0.0236	60
1579	1.7706	0.024	60
1582	1.3298	0.024	60
1585	1.1504	0.0236	60
1588	1.3844	0.0239	60
1591	1.5633	0.0477	60
1594	1.8221	0.047	60
1597	1.9082	0.0713	60
1600	1.3637	0.0468	60
1603	1.0516	0.0468	60
1606	1.4515	0.0464	60
1609	1.6822	0.0457	60
1612	3.0666	0.0457	60
1615	Inf	0.0461	60
1618	Inf	0.0461	60

position	ds	dn	size
1621	3.0666	0.0457	60
1624	3.0666	0.0457	60
1627	1.4515	0.0464	60
1630	1.2882	0.0472	60
1633	1.7464	0.0472	60
1636	1.2882	0.0472	60
1639	1.7464	0.0472	60
1642	2.1249	0.0464	60
1645	2.1249	0.0464	60
1648	1.3844	0.0467	60
1651	1.3061	0.0232	60
1654	1.3061	0.0232	60
1657	0.9952	0	60
1660	1.4282	0	60
1663	2.855	0	60
1666	2.3516	0	60
1669	2.0637	0	60
1672	1.6479	0	60
1675	1.3615	0	60
1678	1.0225	0	60
1681	0.9313	0	60
1684	0.9313	0	60
1687	1.2786	0	60
1690	0.9978	0	60
1693	0.9978	0	60
1696	1.4083	0	60
1699	1.1709	0	60
1702	1.4083	0	60
1705	1.4083	0	60
1708	1.6479	0	60
1711	1.5157	0	60
1714	1.5157	0	60
1717	1.6479	0	60
1720	1.4083	0	60
1723	1.0486	0	60
1726	0.8065	0	60
1729	0.7739	0	60
1732	0.7301	0	60
1735	0.7587	0	60
1738	0.7301	0	60
1741	0.9313	0	60
1744	0.9313	0	60
1747	0.9745	0	60
1750	1.0486	0	60
1753	0.8065	0	60
1756	0.5813	0	60
1759	0.6677	0	60
1762	0.6677	0	60
1765	0.6254	0	60
1768	0.6254	0	60
1771	0.7798	0	60
1774	0.9745	0	60
1777	0.9359	0	60
1780	0.9359	0	60

position	ds	dn	size
1783	0.8681	0	60
1786	0.884	0	60
1789	0.9178	0	60
1792	0.9359	0	60
1795	0.9359	0	60
1798	0.9745	0	60
1801	0.8399	0	60
1804	0.8399	0	60
1807	0.6254	0	60
1810	0.6459	0	60
1813	0.6913	0	60
1816	0.7739	0	60
1819	0.6476	0	60
1822	0.4932	0	60
1825	0.5107	0	60
1828	0.6735	0	60
1831	0.5107	0	60
1834	0.377	0	60
1837	0.5107	0	60
1840	0.5497	0	60
1843	0.7324	0	60
1846	0.7845	0	60
1849	0.5604	0	60
1852	0.5393	0	60
1855	0.5018	0	60
1858	0.6417	0.0498	60
1861	0.6417	0.0498	60
1864	0.6682	0.0495	60
1867	0.6682	0.0495	60
1870	0.8429	0.0498	60
1873	0.7722	0.0506	60
1876	1.0048	0.0506	60
1879	1.0582	0.0502	60
1882	1.0582	0.0502	60
1885	1.0048	0.0506	60
1888	0.9144	0.0514	60
1891	0.9144	0.0514	60
1894	0.8404	0.0521	60
1897	0.6632	0.0521	60
1900	0.5367	0.0517	60
1903	0.4114	0.0517	60
1906	0.5199	0.0521	60
1909	0.5548	0.0514	60
1912	0.5741	0.051	60
1915	0.5741	0.051	60
1918	0.485	0	60
1921	0.6126	0	60
1924	0.4546	0	60
1927	0.4546	0	60
1930	0.3206	0	60
1933	0.3206	0	60
1936	0.3206	0	60
1939	0.3121	0	60
1942	0.3206	0	60

position	ds	dn	size
1945	0.339	0	60
1948	0.339	0.0455	60
1951	0.371	0.0445	60
1954	0.371	0.0445	60
1957	0.3961	0.0438	60
1960	0.371	0.0445	60
1963	0.4099	0.0465	57

MANUSCRIPT 4

The use of sequence analysis of a feline calicivirus (FCV) hypervariable region in the epidemiological investigation of FCV related disease and vaccine failures[†].

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ABSTRACT.

A reverse transcriptase polymerase chain reaction (PCR) was used to amplify a 235bp hypervariable region of the feline calicivirus (FCV) genome which encodes part of the capsid protein. Sequence from this region was used to compare viruses used in three attenuated vaccines to viruses isolated from vaccinated cats with clinical signs of FCV-infection (vaccine failures). All three vaccine viruses contained sequence similar to that published for FCV strain F9 (Carter *et al.* (1992), Virology 190, 443-448). Howèver, two of the three vaccines contained a separate sequence which was 20.67% distant (number of nucleotide substitutions per 100 bases) from F9. Sequence derived from isolates obtained from vaccine failures fell into two categories. Most were distinct (21.33-38.00% distant) from vaccine sequence. However, in some cases, sequences were sufficiently similar to the vaccines' (0.00-5.33% distant) to suggest that the isolate may have originated from the vaccine. In addition, comparison of sequence determined for isolates from the same disease outbreak showed them to be closely related (0.00-1.33% distant), whereas epidemiologically unrelated isolates were 20.67-38.00% distant.

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INTRODUCTION.

Feline calicivirus (FCV) is a pathogen of cats and belongs to the family caliciviridae (Burroughs & Brown, 1974). Other important viruses in this family infect humans, rabbits, pigs and sealions (Cubitt *et al.*, 1995). FCV has a single stranded, positive sense RNA genome of approximately 7.7kb (Carter *et al.*, 1992a; Sosnovtsev & Green, 1995)with three open reading frames, the second of which encodes the single major capsid protein (Neill *et al.*, 1991).

FCV strains are sufficiently closely related to be considered as a single serotype (Povey, 1974; Kalunda *et al.*, 1975). Nevertheless, antigenic differences do exist allowing most FCV isolates to be distinguished serologically(Povey, 1974; Kalunda et al., 1975). Following FCV infection, clinical signs are predominantly those of mild, acute upper respiratory tract disease (URTD) including oral ulceration, oculonasal discharge and pyrexia (Kahn & Gillespie, 1971; Wardley & Povey, 1977b). However, differences in pathogenicity between isolates produce a spectrum of disease from subclinical infection to severe URTD (Povey & Hale, 1974). Some strains induce a febrile, 'limping syndrome' (Pedersen *et al.*, 1983; Dawson *et al.*, 1994), and FCV infection has also been associated with chronic gingivitis and stomatitis (Thompson *et al.*, 1984; Knowles *et al.*, 1989).

Vaccination against FCV is widespread in the UK. Most vaccines available are live, attenuated and are based on a single isolate (F9) (Bittle *et al.*, 1960) given subcutaneously (Gaskell & Dawson, 1994). Vaccination protocols usually consist of first and second vaccines given three weeks apart to kittens followed by annual boosters (Gaskell & Dawson, 1994). Despite being generally effective at preventing disease, cats occasionally may develop FCV related clinical signs after vaccination (vaccine failures) and FCV can be isolated from some of these cases (Church, 1989; Dawson *et al.*, 1993a). The origin of such vaccine failure isolates is unclear although they may be either field strains or vaccine-derived. Indeed, it has been shown experimentally that vaccine virus can cause disease if given oronasally (Pedersen & Hawkins, 1995) and in some cases, subcutaneously vaccinated cats may shed vaccine virus from the oropharynx (Bennett *et al.*, 1989; Pedersen & Hawkins, 1995).

FCV vaccine failure has been classified clinically as vaccine reactions (clinical signs within 21 days of the last vaccination) or vaccine breakdowns (clinical signs later than 21 days after the last vaccination) (Dawson *et al.*, 1993a) . Serological typing of FCV isolates from some apparent vaccine reactions has implicated vaccine virus as the cause of disease, although the majority of cases appeared to be caused by antigenically distinct strains of field virus (Dawson *et al.*, 1993a). However, interpretation of serological typing may be difficult as some FCV strains are difficult to distinguish serologically (Kalunda *et al.*, 1975; Dawson *et al.*, 1993b) and the neutralisation patterns of FCV strains may change on replication *in vitro* or *in vivo* (Pedersen & Hawkins, 1995).

Comparison of nucleotide and amino acid sequences from the capsid protein genes of caliciviruses allow the capsid to be divided into conserved (regions A, B, D & F) and hypervariable (C and E) regions (Neill, 1992; Seal *et al.*, 1993; Seal, 1994; Green *et al.*, 1995). We have used a reverse transcriptase, nested polymerase chain reaction to amplify a region of the FCV genome at the 5' end of hypervariable region E which encodes amino acids 426-460 (Seal *et al.*, 1993) of the immature capsid. This region is believed to be an important target for virus neutralising antibodies (Guiver *et al.*, 1992; Milton *et al.*, 1992; Tohya *et al.*, 1997). The aim of this study was to investigate the role of attenuated vaccines in vaccine failures by determining the nucleotide sequences of this region for vaccine failure isolates and comparing these to the sequences of the vaccine viruses used. Because RNA viruses replicate as populations with closely related but distinct nucleotide sequences or quasispecies (Domingo *et al.*, 1985; Holland *et al.*, 1992), PCR products were directly sequenced to gain a consensus or majority sequence for each isolate. For heterogenous isolates which contained obvious sequence ambiguities in the consensus sequence, multiple clones were sequenced.

MATERIALS AND METHODS.

Viruses.

Vaccine failure isolates were obtained from oropharyngeal swabs sent by veterinarians for diagnostic purposes essentially according to Wardley *et al.* 1974 and used at low passage. Diagnosis of FCV was by demonstration of characteristic cytopathic effect (Povey & Johnson, 1971) (cpe) on confluent monolayers of feline embryo-derived cells (ICN Biomedicals Ltd.). Vaccine viruses were obtained from the manufacturers and used at passage 10 (vaccineA), unknown passage (vaccine B) and passage 1 (vaccine C). Virus stocks were prepared by growing virus in CRFK cells (Crandell *et al.*, 1973) until cpe was complete (18-36h) and stored at -80°C until RNA isolation was performed. The clinical details and vaccination history for the cats from which viruses were isolated are given in Table 1.

virus	vaccine brand used	stage of vaccination	cats age	days after vaccination clinical signs first seen	clinical signs
G83	A	1st	<6 months	6 days	lameness, pyrexia, URTD
G228	A	1st	<6 months	8 days	oral ulceration, URTD
G229	A	1st	<6 months	8 days	URTD
K582	A	1st	6 years	<21 days	URTD
L097	A	ND	ND	ND	URTD
M135	A	booster	18 months	3.5 months	oral ulceration, dyspnoea, pyrexia
M138	A	2nd	1 year	9 months	oral ulceration and pyrexia
M141[a]	-	-	8 years	-	oral ulceration and pyrexia
E314	B	1st	<6 months	6 days	lameness and pyrexia
G303	B	1st	<6 months	7 days	lameness and pyrexia
G308	B	1st	<6 months	<21 days	URTD
G332	B	1st	<6 months	4 days	lameness
G334	B	1st	<6 months	1 day	oral ulceration and URTD
L277	C	1st	<6 months	4-5 days	URTD
L278	C	1st	<6 months	4-5 days	URTD

Table 1; Clinical data on FCV isolates used in this study. ND - no data. URTD - upper respiratory tract disease. Viruses bracketed together were isolated from cats in the same household. **a;** virus M141 was from a cat hospitalised in the same premises at the same time as the cats from which viruses M135 and M138 were isolated.

RNA isolation and cDNA synthesis.

RNA was isolated from 100µl of stock virus according to the manufacturer's instructions (RNA Isolator; Genosys) and dissolved in 33µl of RNase free water (Sigma). cDNA was prepared by oligodT primer extension using all of the RNA prepared above (Ready-To-Go, Pharmacia). Mock-infected CRFK cells were processed simultaneously as negative controls.

Amplification of cDNA by nested polymerase chain reaction.

Nested polymerase chain reaction (PCR) was performed using *Pfu* DNA polymerase (Stratagene) and oligonucleotide primers (*Table 2*; Kings College, London) in 50µl reaction volumes. Thermal cycling conditions for PCR consisted of DNA denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, primer annealing at 40°C for 1 minute and primer extension at 72°C for 3 minutes. A final extension was performed at 72°C for 5 minutes. First round amplification was carried out using 5µl of cDNA in 1.5µM of each of primers 1 and 2 and 100µM of each deoxynucleotide triphosphate (dNTP) (Advanced Biotechnologies Ltd). Second round nested amplification used 1µl of the first round PCR reaction as template in 240nM of each of primers 4 and 5 and 200µM of each dNTP.

PCR products were purified using the Wizard PCR preps DNA purification system (Promega).

Primer	Sequence (5'-3')	binding site in F9
1	CCCTTTGTGTTCCAAGCAAATCG	6406-6428
2	CCTCTCCGATACCAGTGTATCC	6934-6913
4	TTGCAACTGATTATATTGTTCCTGG	6533-6557
5	GCAGTGTTGGATATTTTCTTGTCACC	6767-6742

Table 2; Primer sequences used for PCR and sequencing and their binding sites in FCV strain F9 (Carter *et al.*, 1992a).

Sequence analysis.

To determine a consensus or majority sequence for each isolate, direct sequencing was performed with primers 4 and 5 (*Table 2*) using the conditions specified by the manufacturer (ABI prism dye terminator cycle sequencing ready reaction kit; Perkin Elmer).

To resolve ambiguities in the direct sequence, purified PCR products of vaccine A and B were ligated into pCR-script SK(+) (Stratagene) and transformed into competent cells (Ligator; R&D systems) according to the manufacturers' instructions. Individual plasmid clones containing PCR product inserts were selected by restriction enzyme digestion (Boehringer Mannheim) and sequenced using a dideoxy chain termination method essentially according to Sanger *et al.*, (1977).

Sequence analysis software.

Distance calculations and sequence comparisons were performed using programmes DISTANCE, PILEUP and PRETTY from version 8 of the Wisconsin package (1994), genetics computer group (Deveraux *et al.*, 1984). Phylogenetic analysis was performed using SEQBOOT, DNADIST, NEIGHBOR and CONSENSE from the Phylip package (Felsenstein, 1989).

RESULTS.

PCR amplification of the hypervariable region and sequence determination.

Using reverse-transcriptase-PCR we amplified a hypervariable region of the FCV capsid gene from 15 vaccine failures and three vaccines. Direct sequencing of the PCR products from vaccine failure isolates and vaccine C gave pure consensus sequence (*Figure 1*) indicating that these isolates contained a relatively homogenous population of viruses. However direct sequence of vaccines A and B yielded a large number of sequencing ambiguities. By sequencing multiple clones from these vaccines, it was shown each exists as a mixed population consisting of two distinct sequences (*Figure 1i* and *1ii*), a majority and minority sequence, as determined by the number of clones with each sequence.

Figure 1i; Comparison (PRETTY and PILEUP (Deveraux *et al.*, 1984)) of nucleotide sequences from vaccine A to vaccine A failure isolates. Vaccine A contained a mixed population represented by two cloned sequences, * indicating that obtained least frequently.

```
                1                                                  50
M135/8/41         A       T       C   GTCA   C C       T       T  C  T
     G228         G  T    A  T    C   AACT   C A    T      C    TA
     G229         A  TC   A  T    C   AACT   C A    T      C    TA
     L097         A                C   AAC C     A    T  T   A    T
     K582         A                C   A ACAC C          T  G    TA     T
      G83                                                          T
Vaccine A* TGGCCTGACA CCACAATTCC TGGGGAGTTG ATACCAGCTG GCGATTACGC
Vaccine A             A  C  CT       A  C A   C            T  C  T

               51                                                  100
M135/8/41  C  A      A    AGA   G       T  TG      A    GA    AC
     G228     TTTAGCG     GG A     C  T        A  T   C TG   A        TT
     G229     TTTAGCG     GG A     C  T        A  T   C TG   A        TT
     L097  T  A      G    GGAC  G       T        A  T   C AG   AG       TG
     K582     T  A      AA A AA         T   A    A  TAAA GT CA        AG
      G83
Vaccine A* AATCACCAAT GGTACTGGCA ATGACATCAC CACGGCTACA GGATATGACA
Vaccine A             CG C  T   C     TGT    A   CGT  A  TC

              101                                                  150
M135/8/41  AG       G     C  A                      AAA             C
     G228   G  CAC  C        A  T G A      T       AA              C
     G229   G  CAC  C        A  T G A      T       AA              C
     L097      C   G  C                 C  T  T   C AA  T          C  C
     K582  T   AAGCT     C     T   C  A  T       A T              T
      G83                                        A
Vaccine A* CTGCTGATAT AATTAAGAAC AATACCAACT TTAGGGGCAT GTACATATGT
Vaccine A         CGA    C  A                  C
```

Figure 1ii; Comparison (PRETTY and PILEUP (Deveraux *et al.*, 1984)) of nucleotide sequences from vaccine B to vaccine B failure isolates. Vaccines B contained a mixed population represented by two cloned sequences, * indicating that obtained least frequently.

```
                1                                                  50
E314
G303
G332
G334                                                            A
G308              T   C  A   C  ATCAA A  G G T  T          GT
Vaccine B* TGGCCTGACA CAACCATCTC TGAGGAGCTA ATCCCAGCTG GTGACTATGC
Vaccine B             C  A  TC    G    T G   A          C  T  C

               51                                                 100
E314
G303                                                  A
G332
G334
G308      T  TGCA AAG AT  G       T  CAC T  T   GCG C   A   TG
Vaccine B* AATCACCAAT GGCGCCGGTA ACGACATTGT CACAGCTCGT GAATTCGACA
Vaccine B               TA T  C   T     CAC    G   ACA  G  AT

               101                                                150
E314
G303
G332
G334
G308   A  A GT       TGT              T   T AAA             C
Vaccine B* CTGCTGACGA AATCAAAAAC AATACCAACT TCAGGGGCAT GTACATATGT
Vaccine B         TAT    T  G                T
```

Figure 1iii; Comparison (PRETTY and PILEUP (Deveraux *et al.*, 1984)) of nucleotide sequence from vaccine C to vaccine C failure isolates.

```
                1                                                    50
     L277                                      A        A
     L278                                      A        A
Vaccine C  TGGCCTGACA CCACAATTCC TGGGGAGTTG ATACCAGCTG GCGATTACGC

               51                                                   100
     L277            A G                                  G   A
     L278                                                 G   A
Vaccine C  AATCACCAAT GGTACTGGCA ATGACATCAC CACGGCTACA GGATATGACA

              101                                                   150
     L277                    GG
     L278                    GG
Vaccine C  CTGCTGATAT AATTAAAAAC AATACCAACT TTAGGGGCAT GTACATATGT
```

Comparison of vaccine sequences to each other and to a published sequence for a vaccine-derived strain of F9 (Carter *et al.*, 1992a) showed each vaccine contained an F9-like sequence (consensus vaccine C, minority vaccine A and majority vaccine B; *Table 3i*). However, the alternative sequences in vaccines A and B (majority vaccine A and minority vaccine B sequence) were both 20.67% distant (uncorrected distances - number of nucleotide substitutions per 100 bases) from F9, and were identical to each other (*Table 3i*).

Comparison of vaccine failure isolates to each other and to vaccines

Distances calculated by comparing vaccine failure isolates to each other ranged from 0.00-38.00% (*Table 3ii*). However, within this, two distinct groups were evident. Firstly, a group of relatively similar sequences (distances of 0.00-6.00%) including those of isolates from cats sharing the same accomodation either within a household (G228/229-1.33%; L277/278-1.33%; M135/138-0.00%) or within a hospital (M141 was isolated from a cat that was admitted to the same hospital at the same time as the cats from which M135 and M138 were isolated and all three isolates shared the identical sequence). Secondly, there was a group of sequences quite different from one another (distances of 20.67-38.00%) which were derived from isolates that had no known geographical relationship.

Sequence comparisons and distance calculations for each vaccine with the vaccine failure isolates made after the use of that vaccine are shown in *Figure 1* and *Table 3iii-v* respectively. Distance calculations again fell into categories of similar sequences (0.00-5.33%) and relatively dissimilar sequences (21.33-38.00%). For vaccine A, of the six vaccine failure isolates examined, all except G83 contained sequence that was 21.33-38.00% different from both cloned vaccine sequences (*Table 3iii*). Vaccine reaction isolate G83 was only 1.33% different from the minority sequence of the vaccine. For vaccine B, G308 was 33.33% and 35.33% different from the majority and minority vaccine sequences respectively (*Table 3iv*). The four other vaccine B reactions were <1.0% different from the minority vaccine sequence. L277 and L278 were 5.33% and 4.00% different from the consensus sequence for vaccine C respectively (*Table 3v*).

The phylogenetic relationship of the isolates in this study to each other and to F9 are shown in *Figure 2*. Isolates fall into three relatively distinct groups: (1) those with

Table 3; Uncorrected distances (DISTANCE (Deveraux *et al.*, 1984)) based on comparison of nucleotide sequence from the hypervariable region for (i) Vaccine viruses to each other and published F9 sequence (Carter *et al.*, 1992a). (ii) Vaccine failure isolates to each other. (iii) Vaccine A associated failures. (iv) Vaccine B associated failures. (v) Vaccine C associated failures.

(i)

	F9	VacA	VacA	VacB	VacB*	VacC
F9[b]	0.00	20.67	0.67	0.67	20.67	0.67
VacA		0.00	21.33	21.33	1.33	20.67
VacA			0.00	0.00	21.33	0.67
VacB				0.00	21.33	0.67
VacB*					0.00	20.67
VacC						0.00

(ii)

	G83	G228	G229	K582	L097	M135	E314	G303	G308	G332	G334	L277	L278
G83	0.00	32.00	32.67	31.33	26.00	23.33	21.33	22.00	32.00	21.33	22.00	6.00	4.67
G228		0.00	1.33	33.33	28.00	33.33	36.67	37.33	28.67	36.67	36.00	32.00	32.00
G229			0.00	33.33	28.00	33.33	37.33	38.00	29.33	37.33	36.67	32.67	32.67
K582				0.00	32.67	34.67	36.67	36.00	34.67	36.67	36.00	32.00	32.00
L097					0.00	25.33	33.33	34.00	28.67	33.33	34.00	27.33	27.33
M135[a]						0.00	24.00	24.67	26.67	24.00	24.67	25.33	25.33
E314							0.00	0.67	35.33	0.00	0.67	20.67	20.67
G303								0.00	36.00	0.67	1.33	21.33	21.33
G308									0.00	35.33	36.00	33.33	34.67
G332										0.00	0.67	20.67	20.67
G334											0.00	21.33	21.33
L277												0.00	1.33
L278													0.00

(iii)

	VacA	VacA*
G83	21.33	1.33
G228	37.33	32.00
G229	38.00	32.67
K582	36.67	31.33
L097	33.33	27.33
M135[a]	24.67	24.67

(iv)

	VacB	VacB*
E314	21.33	0.00
G303	22.00	0.67
G308	33.33	35.33
G332	21.33	0.00
G334	22.00	0.67

(v)

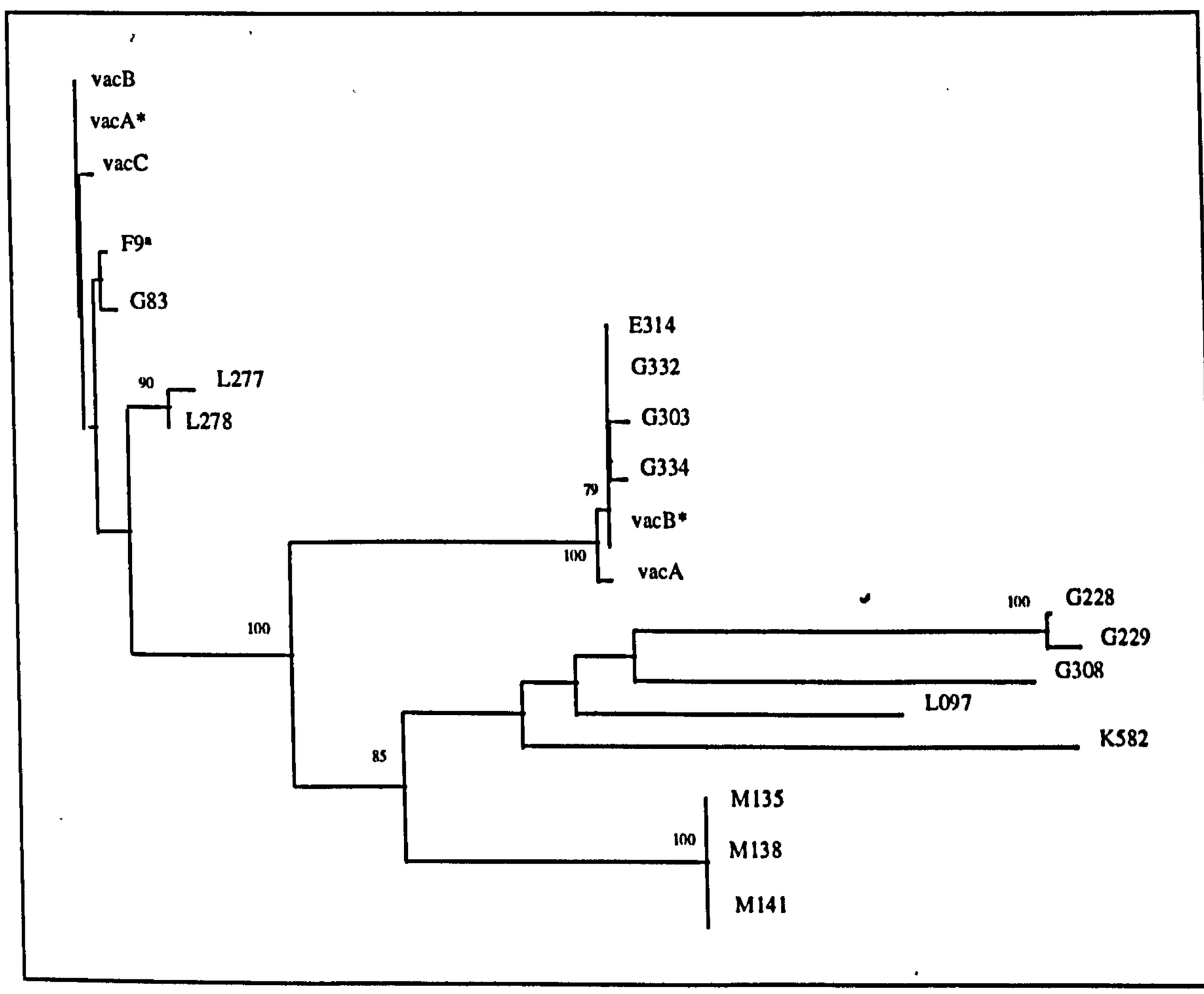
	VacC
L277	5.33
L278	4.00

[a]Distance calculations for M138 and M141 are not shown and are the same as for M135 as all three isolates are identical.

*Sequences obtained least frequently by cloning of vaccine A and B representing the minority sequence in each population.

Figure 2; Phylogenetic analysis (KIMURA DISTANCE, NEIGHBOR-JOINING; PHYLIP (Felsenstein, 1989)) of sequences from this study.

All sequences were obtained by direct sequencing of PCR products except for vaccines A and B where sequencing of clones was necessary to overcome sequencing ambiguities. *represents the cloned sequence from vaccines A and B obtained least frequently (minority sequence). [a]Published F9 sequence (Carter *et al.*, 1992a). Bootstrap values greater than 75 are given as figures at the relevant nodes and were calculated on 100 data sets (SEQBOOT (Felsenstein, 1989)).



F9-like sequences, which includes all three vaccines, the vaccine C failures (L277/278) and one vaccine A failure (G83); (2) those with sequences very similar to minority vaccine B sequence, which includes most of the vaccine B failures and the majority vaccine A sequence; and (3) a group of isolates with sequences relatively distant from one another and from vaccine, which includes most of the vaccine A failures and one vaccine B failure (G308).

DISCUSSION

Comparison of sequences from a hypervariable region of the FCV capsid gene for isolates used in this study gave distances ranging from 0.00 to 38.00%. This is broadly in agreement with previously published sequences of FCV (0.67-40.00 (Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Seal *et al.*, 1993; Seal, 1994; Sosnovtsev & Green, 1995)). However, in the present study, the FCV isolates fell into two relatively distinct groups; those with similar sequences and low distance values (0.00-6.00%) and those less related sequences with higher distance values (20.67-38.00%).

Geographically related isolates associated with shared accomodation all had low distance values implying the circulation of individual isolates between in-contact individuals. Low distance values between the sequences of M141, M135 and M138 may represent cross-infection at a time when all three animals from which the isolates were made were in the same veterinary hospital. This demonstrates that sequence from the hypervariable region may be useful in the epidemiological investigation of FCV-related disease.

All three vaccines from this study were based on F9-like isolates. However, both vaccine A and B contained a second and quite distinct isolate represented by the majority vaccine A and minority vaccine B sequences. Whether the different sequences represent a quasispecies (Domingo *et al.*, 1985; Holland *et al.*, 1992) or the co-existence of two distinct isolates within each vaccine is uncertain. The origin of the majority vaccine A and minority vaccine B sequence is not clear although the possibility that it was present within the original F9 isolate (Bittle *et al.*, 1960) cannot be excluded.

Isolate F9 was originally chosen as a vaccine candidate based on the ability of antisera raised against it to neutralise a large proportion of field isolates (Kalunda *et al.*, 1975; Knowles *et al.*, 1990). The hypervariable region of the capsid is believed to be very important for immune recognition (Guiver *et al.*, 1992; Milton *et al.*, 1992; Tohya *et al.*, 1997) and the presence of two quite distinct hypervariable sequences within some F9-like strains may explain its broadly cross-reactive nature. The relative proportions of each sequence within vaccine A and vaccine B may not reflect that administered in the vaccine as virus isolates grown in cell culture in competition may displace one another at different times (Woodbury *et al.*, 1995). Similar shifts in population makeup may also have occured when vaccine virus was passaged in cell culture prior to use in this study. Such factors may represent a source of vaccine batch variability to the manufacturer and may explain observed differences in the cross-reactivity of different F9-based vaccines (Dawson *et al.*, 1993b).

Vaccine failure isolates fell into one of two groups on the basis of similarity to vaccine virus. One group included those with sequences 21.33-38.00% distant from vaccine's, and by inference represents field virus. G228/229, K582 and G308 had been classified on clinical grounds as vaccine reactions in that they were all isolated within 21 days of first vaccination. The animals in these cases were either incubating the virus when vaccinated or were infected soon after vaccination. The cats from which M135 and M138 were isolated were fully vaccinated. These isolates therefore may represent a failure of the vaccine to induce a protective immune response either due to incorrect vaccine administration or lack of antigenic cross-reactivity between these isolates and the vaccine. Despite being classed as a single serotype, FCV isolates do vary antigenically (Povey, 1974; Kalunda *et al.*, 1975) and antisera raised against F9 fails to neutralise all FCV field isolates *in vitro* (Knowles *et al.*, 1990; Dawson *et al.*, 1993b). Further characterisation of isolates such as M135 and M138 may enable the development of better polyvalent vaccines.

Sequence from the second group of vaccine failure isolates (G83, E314, G303, G332/334 and L277/278) all showed low (0.00-5.33%) distances from vaccine derived sequences. This suggests that virus present within the vaccine may have caused the disease seen in the vaccinated animals. However, comparisons were made using sequence from a small

region of the capsid gene and other differences may exist elsewhere in the virus genome. It is also possible that vaccine-like sequences exist in the field.

Although distance values were low (0.00-5.33%) between isolates that were epidemiologically closely related either geographically or through the possibility of vaccine-induced disease, these related isolates do show some sequence variation. This variation may be caused by nucleotide subtitutions introduced during viral replication or due to selection of particular isolates from within a quasispecies both *in vitro* and *in vivo*. Because the hypervariable region is believed to contain important antigenic determinants, such changes may be driven by immune selection pressures within the cat. Sequence changes in the order of 6.66% between isolates made 25 days apart from a single cat and 2.00% due to 3 passages of virus in cell culture have been observed previously (author's unpublished data).

Previous attempts to compare vaccines to vaccine failures have used both polyclonal one way neutralisation tests (Dawson *et al.*, 1993a) and an enzyme-linked immuno-flow-assay using monoclonal antibodies raised to F9 (McArdle *et al.*, 1996). The polyclonal neutralisation patterns broadly agreed with our results in implicating vaccine B in disease after first vaccination although these findings were not substantiated by monoclonal antibody analysis. However, immunologically-based typing methods may give different results to sequence-based methods since single nucleotide substitutions may change antibody reactivity.

The mechanism by which virus present within FCV vaccines may induce disease is unknown but may include the inadvertant oronasal administration of the vaccine due to aerosolisation or local leakage of the vaccine to the skin surface subsequently licked by cats (Povey, 1977). Alternatively, the vaccine may not have been fully attenuated and may spread after subcutaneous innoculation to the oropharynx. The mixed viral populations present within vaccines in this study may contain isolates of varying pathogenicity and competition *in vitro* or *in vivo* (Woodbury *et al.*, 1995) may result in the outgrowth of attenuated virus by more pathogenic types resulting in vaccine failures. It is also possible that the vaccine may mutate *in vivo* with subsequent reversion to virulence. Such mutations in the 5' non-coding region of the genome of polio virus vaccine are well documented (Guillot *et al.*, 1994) and are associated with loss of the attenuated phenotype and reversion to neurovirulence. These revertants are thought to be

the cause of low level vaccine-associated paralytic poliomyelitis in vaccinees or their contacts (Nkowane *et al.*, 1987). Finally host factors such as immunosuppression may play a role. Recently, a measles vaccine virus strain has been implicated in a case of measles pneumonitis in an individual with human immunodeficiency virus infection (Angel *et al.*, 1996).

In conclusion, we have been able to group isolates on the basis of sequence similarity into those that are epidemiologically related and those that appear not to be. In particular we have shown a potential role for vaccine-derived virus in FCV-related disease. Little is known about the attenuation process in FCV or sites within the FCV genome that are involved in virulence. Further study of isolates from vaccine failures may facilitate the development of safer vaccines in the future.

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MANUSCRIPT 5

A comparison of serological and sequence-based methods for the typing of feline calicivirus isolates from vaccine failures.

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ABSTRACT.

Typing of feline calicivirus (FCV) has previously been performed by exploiting antigenic differences between the majority of isolates. However, due to possible difficulties in interpreting such methods, we recently established a new technique for FCV typing based upon sequence analysis of a hypervariable region of the capsid gene (Radford *et al.* (1997), Vaccine 15, 1451-1458). In this study, we compare the ability of these two methods to characterise FCV isolates from vaccine failures following the use of commercial, live-attenuated FCV vaccines. Using virus neutralisation, isolates showed a spectrum of relatedness to the vaccine. Depending on the criterion adopted for identity, 23-54% of 26 vaccine failure isolates appeared similar to vaccine virus. Comparison of isolates by sequence analysis showed isolates fell into one of two categories; 35% of isolates had similar sequence to vaccine (0-5.3% distant) the remainder having dissimilar sequence to the vaccines (21.3-38.7% distant). Sequence analysis identified a small number of FCV isolates that appeared to contain two distinct FCVs. Comparison of serological and molecular typing results gave the same typing result in 65-73% of individual cases. Recommendations for future FCV typing are discussed.

INTRODUCTION.

Feline calicivirus (FCV) is a major cause of oral and upper-respiratory tract disease in domestic cats (reviewed by Gaskell & Dawson, 1994). The virus belongs to the family *Caliciviridae* (Cubitt *et al.*, 1995) which also includes important pathogens of humans (Norwalk-like viruses and classic human caliciviruses) and other animals (rabbit haemorrhagic disease virus, European brown hare syndrome virus, vesicular exanthema of swine virus). FCV has a single stranded, positive sense RNA genome of approximately 7.7kb (Neill, 1990; Neill *et al.*, 1991; Tohya *et al.*, 1991; Carter *et al.*, 1992a; Oshikamo *et al.*, 1994; Sosnovtsev & Green, 1995; Glenn, 1997). The genome is predicted to encode three open reading frames (ORFs) (Figure 1) encoding the non-structural proteins (ORF1), the single major capsid protein (ORF2) and a small putative minor structural protein (ORF3)(Wirblich *et al.*, 1996). Comparisons of capsid gene sequences from different FCV isolates allow the capsid to be divided into relatively conserved (regions A, B, D, and F) and more variable (regions C and E) regions (Figure 1) (Neill, 1992; Seal *et al.*, 1993; Seal, 1994). Variable region E may be further divided into a 5' hypervariable region (HVR) and 3'HVR (Figure 1) (Seal *et al.*, 1993). Divergent FCV isolates are generally 20-40% variable at the nucleotide level within the 5'HVR (Radford *et al.*, 1997).

Vaccination against FCV is widespread in the UK domestic cat population (Gaskell & Dawson, 1994). The majority of vaccines used are live, attenuated and given subcutaneously. Typical vaccination protocols include first and second vaccinations at 9 and 12 weeks of age followed by annual boosters. Despite being generally effective at preventing disease, there are four potential drawbacks of such vaccines. Firstly, they do not prevent subsequent FCV infection (Gaskell *et al.*, 1982). Secondly, if vaccine virus is inadvertently given by the incorrect route (such as by vaccine aerosolisation at the time of administration), then it may be capable of inducing disease (Povey, 1977; Pedersen & Hawkins, 1995). Thirdly, there is some evidence to suggest that, even if given by the correct subcutaneous route, the vaccine virus may be shed from the oropharynx of some cats (Bennett *et al.*, 1989; Pedersen & Hawkins, 1995). Finally, the majority of vaccines available in the U.K. have been

based upon a single FCV isolate (originally F9 (Bittle *et al.*, 1960) or F9-like). Therefore, since *in vitro* FCVs show considerable variability in their virus neutralisation profiles (Kalunda *et al.*, 1975; Knowles *et al.*, 1990), vaccine induced immunity may not protect against challenge with all FCV isolates encountered in the field.

Following FCV vaccination, FCV associated disease is well recognised (Church, 1989; Dawson *et al.*, 1993a). It is important in such vaccine failures to determine whether the virus involved is a field virus or derived from the vaccine. Currently, typing of such isolates is performed by comparison of virus neutralisation profiles for vaccine virus and vaccine failure virus (Dawson *et al.*, 1993a). However, the results of this method may not be easy to interpret since not all FCVs can be distinguished serologically (Kalunda *et al.*, 1975; Dawson *et al.*, 1993b) and passage in cell culture may alter the antigenic characteristics of the virus (Pedersen & Hawkins, 1995). More recently, we have described a technique based upon comparison of sequence from the 5' HVR of the capsid gene (Radford *et al.*, 1997). By sequencing multiple molecular clones, we demonstrated that some vaccines appear to include more than one isolate as they contained two sequences that were >20% different from each other. We showed that vaccine failure isolates were either similar (0.0-5.3%) or different (21.3-38.0%) to vaccine derived sequences implying the vaccine failure isolates may be either derived from the vaccine or from a field isolate respectively (Radford *et al.*, 1997). The aim of this study was to compare the results of vaccine failure typing performed by both virus neutralisation and by sequence analysis.

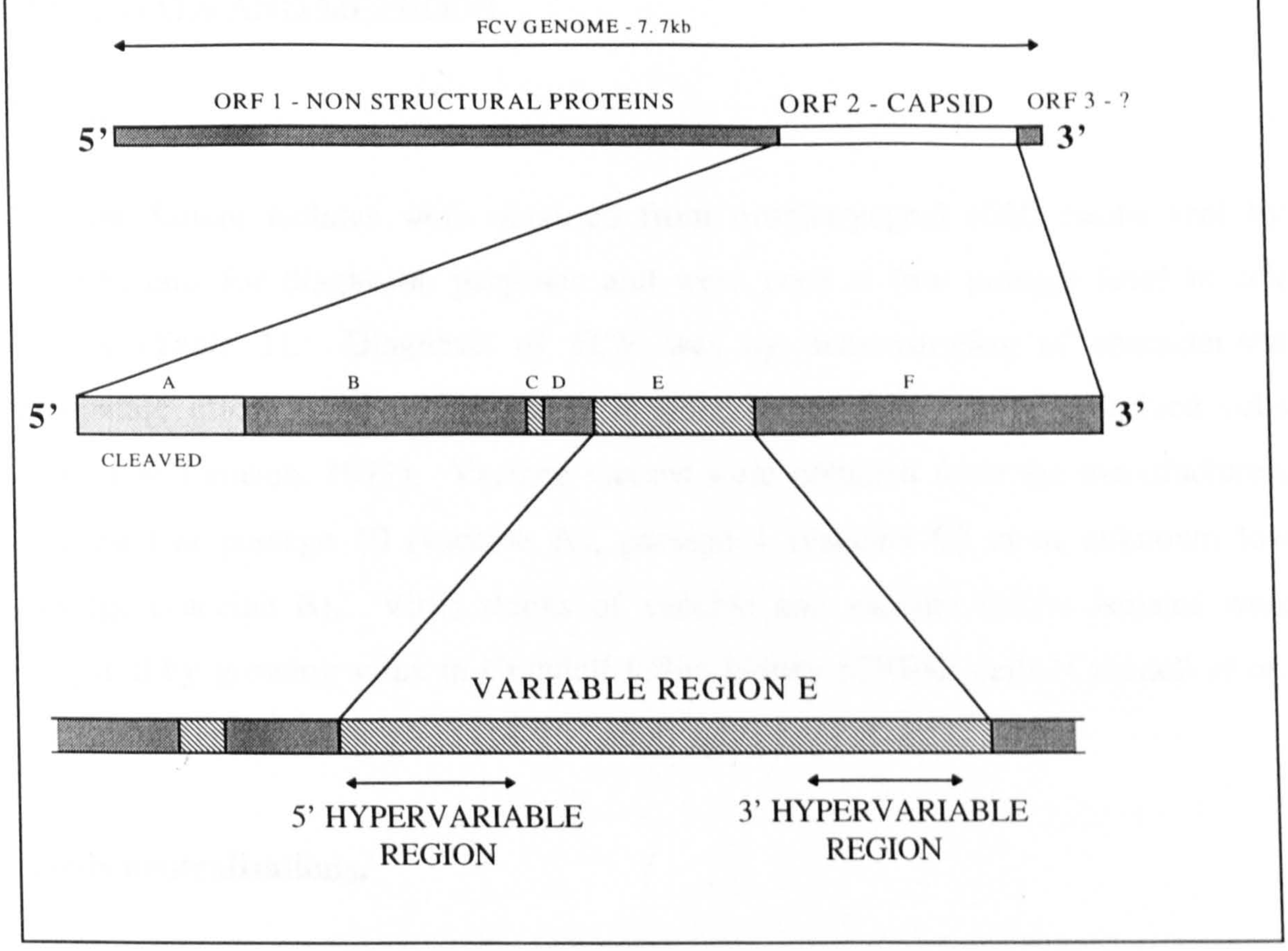

Figure 1; Genome organisation and capsid variability of FCV. The genome encodes the non-structural proteins at the 5' end in a single open reading frame (ORF1). The major capsid gene is encoded by ORF2. A third small ORF at the 3' end of the genome encodes a putative minor structural protein. ORF2 has been divided into regions A-F on the basis of sequence variability between different FCV isolates (Neill, 1992; Seal *et al.*, 1993; Seal, 1994; Glenn, 1997). Regions A,B,D and F are relatively conserved whilst regions C and E are more variable. Region E has been further divided into a 5' and 3' hypervariable region.

MATERIALS AND METHODS.

Viruses.

Vaccine failure isolates were obtained from oropharyngeal (OP) swabs sent by veterinarians for diagnostic purposes and were used at low passage level in cell culture (Table 1). Diagnosis of FCV was by demonstration of characteristic cytopathic effect (cpe) on confluent monolayers of feline embryo-derived cells (Povey & Johnson, 1971). Vaccine viruses were obtained from the manufacturers and used at passage 10 (vaccine A), passage 1 (vaccine C) or at unknown low passage (vaccine B). Virus stocks of vaccine and vaccine failure isolates were prepared by growing virus in Crandell feline kidney (CRFK) cells (Crandell *et al.*, 1973) until cpe was complete (18-36 hours) and stored at -80°C.

Virus neutralisations.

Parent vaccine and vaccine failure viruses were compared by virus neutralisation (VN) tests against a panel of antisera raised to isolates used in this laboratory (Table 2). Antisera production and VN tests were carried out according to Dawson *et al.*, (1993) and were performed by Dr. S.Dawson. This method has been shown to be approximately 97% repeatable (Dawson *et al.*, 1993a, b). VN titres were expressed as the $\log_2$ of the reciprocal of the 50% end point. To compare isolates, the VN titre of a given antisera was determined against the two isolates to be compared. For each pair of isolates, excluding those where one or more of the antibody titre differences were <1 or <2, the sum of the differences was also calculated.

Where all four different antisera used to type any pair of isolates gave an end point, the four individual titre differences were also summed.

Two separate definitions of identity were ultimately used (Table 3). For the high stringency definition of identity, all four comparable VN results for vaccine and vaccine failure isolates were to be within $\log_2 2$ of each other. The less stringent definition of identity allowed the difference between one of the four VN comparisons to be greater than $\log_2 2$.

virus	vaccine brand used	stage of vaccination	cats age	time after last vaccination clinical signs first seen	clinical signs
*E314[1]	B	1st	<6 months	6 days	lameness and pyrexia
E315[1]	B	1st	<6 months	8 days	lameness
F208[2]	A	1st	<6 months	5 days	lameness
F211[2]	A	1st	<6 months	6 days	lameness
F227[2]	A	1st	<6 months	5 days	lameness
*G83[3]	A	1st	<6 months	6 days	lameness, pyrexia, URTD
G85[3]	A	1st	<6 months	6 days	lameness, pyrexia, URTD
*G228[4]	A	1st	<6 months	8 days	oral ulceration, URTD
*G229[4]	A	1st	<6 months	8 days	URTD
*G303	B	1st	<6 months	7 days	lameness and pyrexia
*G308	B	1st	<6 months	<21 days	URTD
G320	A	2nd	<6 months	12 days	lameness, respiratory distress & collapsing
G323	A	1st	<6 months	4 days	pyrexia and weakness
*G332	B	1st	<6 months	4 days	lameness
*G334	B	1st	<6 months	1 day	oral ulceration and URTD
*K582	A	1st	6 years	<21 days	URTD
*L097	A	ND	ND	ND	URTD
*L277[5]	C	1st	<6 months	4-5 days	URTD
*L278[5]	C	1st	<6 months	4-5 days	URTD
*M135	A	booster	18 months	3.5 months	oral ulceration, dyspnoea, pyrexia
M256	A	2nd	<6 months	3 days	pyrexia, lingual ulceration
M364[6]	A or C	ND	ND	ND	ND
M366[6]	A or C	ND	ND	ND	ND
M382	A	booster†	12 years	4 days	lethargy, oral ulceration and stomatitis
M400[7]	A	ND	ND	ND	URTD
M403[7]	A	ND	~ 1 year	31 days	URTD

Table 1; Clinical data on FCV isolates used in this study. ND - no data. URTD - upper respiratory tract disease. Isolates identified by the same superscript number (**1-7**) were from the same household. * indicates those sequences reported previously (manuscript 4; Radford *et al.*, 1997). † denotes booster was given approximately 5 years after last vaccine (manufacturers recommended maximum interval between vaccines is one year).

Isolate	Origin of isolate	Reference
Vaccine A	commercial vaccine*	
Vaccine B	commercial vaccine†	
Vaccine C	commercial vaccine*	
F9	mild respiratory disease	Bittle *et al.*, 1960
Bulmer13	lameness	Dawson, 1991
LS015	chronic stomatitis	Knowles, 1988
LS027	chronic stomatitis	Knowles, 1988
F65	lameness	Dawson, 1991

Table 2; Laboratory FCV isolates used for the production of antisera. *Systemic, live-attenuated vaccines based on F9 (Bittle *et al.*, 1960) or †F9-like isolates of FCV.

Table 3; Comparison of molecular and serological methods of typing vaccine failure isolates. Those isolates identified by the same superscript number **(1-7)** were from the same household.

a; Vaccine failures occured following the use of one of three commercial vaccines, A, B and C.

b; PCR products were sequenced directly to produce a consensus sequence. Where consensus sequences contained ambiguities, multiple molecular clones were sequenced.

c; Uncorrected nucleotide distances between sequences derived from vaccine and vaccine failures. For vaccines A and B, which contained two distinct sequences, the distance values for the vaccine failure sequence relative to each vaccine derived sequence is given. For E315, M382 and M403, the range of distances is given for sequences obtained from individual clones relative to the vaccine derived sequence.

d; Using a maximum variability limit of 5.3%, isolates were defined as similar (+) or dissimilar (-) to the vaccine virus.

e; Isolates were compared to their appropiate vaccine virus by VN using four antisera - anti F9, anti LS015, anti Bulmer 13 and anti 'other'. To compare isolates, the VN titre (log_2 of the reciprocal of the 50% end point) of a given antisera was determined against the two isolates to be compared, and the difference calculated.

f; 'Other' antisera was against the relevant parent vaccine except when anti LS027 (*) and anti F65 (#) were used.

g; Where all four different antisera used to type any pair of isolates gave an end point, the four individual titre differences were also summed. ND; not determined due to presence of a greater-than or less-than in the values to be summed.

h; Where serotyping agrees with sequence based typing, background is shaded.

i; The high stringency definition of identity. All four comparable VN results are within $log_2 2$ of each other.

j; The less stringent definition of identity. The difference between one of the four comparable VN results is allowed to be greater than $log_2 2$.

Table 3.

Isolate	Vaccine[a]	Consensus /Clone[b]	Percent[c]		Identity[d]	Antisera[e]					Identity[h]	
						F9	Other[f]	LS015	Bulmer13	Sum[g]	All[i]	3[j]
E314[1]	B	consensus	21	0	+	**0.6**	**1**	**1**	**2**	4.6	+	+
E315[1]	B	clone	30-33	29	-	>5	>9	5	>6	ND	-	-
F208[2]	A	consensus	21	30	-	6	2.4	5	5	18.4	-	-
F211[2]	A	consensus	30	33	-	7	3.4	5	5	20.4	-	-
F227[2]	A	consensus	28	32	-	3	>4	4	5.4	ND	-	-
G83[3]	A	consensus	21	1	+	6	**0.6**	3	3	12.6	-	-
G85[3]	A	consensus	29	33	-	**1**	**0**	**1**	4	6	-	+
G228[4]	A	consensus	37	32	-	**2**	**0**	4	3	9	-	-
G229[4]	A	consensus	38	33	-	3	**1**	**2**	**2**	8	-	+
G303	B	consensus	22	1	+	**0**	**2**	**1**	**0**	3	+	+
G308	B	consensus	33	35	-	**2**	**0**	**2**	**2**	6	+	+
G320	A	consensus	33	35	-	5	3	**1**	**0**	9	-	-
G323	A	consensus	27	31	-	7	**1.4**	**1**	3.4	12.8	-	-
G332	B	consensus	21	0	+	**1**	**2**	**2**	**1**	6	+	+
G334	B	consensus	22	1	+	**1**	**1**	3	**1**	6	-	+
K582	A	consensus	37	31	-	**0.6**	**0.4**	**1**	**1**	3	+	+
L097	A	consensus	33	27	-	2.6	3	**1.6**	**0**	6.2	-	-
L277[5]	C	consensus	5		+	**0**	**0.6***	**0.6**	>3	ND	-	+
L278[5]	C	consensus	4		+	**1**	**0.6***	**1.4**	>3.6	ND	-	+
M135	A	consensus	25	25	-	3.4	4.6	**0.4**	**>2**	ND	-	-
M256	A	consensus	39	35	-	>5.6	**2#**	**2**	>3.6	ND	-	-
M364[6]	A	consensus	3	23	+	2.6	**0.4#**	**0**	**2**	5	-	+
M366[6]	A	consensus	3	23	+	3	**0.4#**	**0.4**	**2**	5.8	-	+
M382	A	clone	30-33	25-26	-	**1**	**0. 6#**	**1**	**1.4**	4	+	+
M400[7]	A	consensus	33	23	-	4	**1#**	**1**	>3.6	ND	-	-
M403[7]	A	clone	36-38	26-28	-	3	**1.6#**	**0.4**	**1.6**	6.6	-	+

RNA isolation and cDNA synthesis.

Total RNA was isolated from 100µl of freeze-thawed cell lysate according to the manufacturer's instructions (RNA Isolator; Genosys) and dissolved in 33µl of RNase free water (Sigma). Complementary DNA was prepared by oligo dT primer extension using all of the RNA prepared above (Ready-To-Go, Pharmacia). Negative controls consisting of mock-infected CRFK cells were processed simultaneously.

Amplification of cDNA by nested polymerase chain reaction.

The nested polymerase chain reaction (PCR) was performed to amplify a 235 nucleotide region of the capsid gene equivalent to residues 6533-6767 of FCV strain F9 (Carter *et al.*, 1992a) and including the 5'HVR. Amplification was performed using *Pfu* DNA polymerase (Stratagene) and oligonucleotide primers from Kings College, London (Table 4). Thermal cycling conditions for both rounds of nested PCR were as described previously (Radford *et al.*, 1997). Pooled PCR products were purified using the Wizard PCR preps DNA purification system (Promega).

Primer	Sequence (5'-3')	binding site*
1	CCCTTTGTGTTCCAAGCAAATCG	6406-6428
2	CCTCTCCGATACCAGTGTATCC	6934-6913
4	TTGCAACTGATTATATTGTTCCTGG	6533-6557
5	GCAGTGTTGGATATTTTCTTGTCACC	6767-6742

Table 4; Primers used for PCR and sequencing. * Binding sites relate to FCV strain F9 (Carter *et al.*, 1992a).

Sequencing.

All sequencing was performed according to conditions specified by the manufacturer (ABI prism dye terminator cycle sequencing ready reaction kit; Perkin Elmer). Direct sequencing of PCR products was performed to produce a consensus sequence for each isolate. Where consensus sequences contained multiple ambiguities implying the presence of a mixed virus population, multiple clones of PCR products were also sequenced. Briefly, purified PCR products were ligated into pCR-script SK(+) (Stratagene). Products of the ligation reaction were transformed into competent cells (Ligator, R&D systems). Plasmids were isolated according to the manufacturer's instructions (Wizard minipreps, Promega) and inserts from 5 or 6 clones sequenced using primers 4 and 5 (Table 4).

Sequence analysis software.

Uncorrected nucleotide distance calculations (number of nucleotide differences per 100 compared) and sequence alignments were performed using programmes DISTANCE, PILEUP and PRETTY from version 8 of the Wisconsin package, genetics computer group (Deveraux *et al.*, 1984). Phylogenetic analysis was performed using DNADIST and NEIGHBOR from the Phylip package (Felsenstein, 1989).

RESULTS.

Serological typing based on VN results.

When compared by VN tests, vaccine failure isolates showed a spectrum of relatedness to the parent vaccine, ranging from relatively similar to more divergent (Table 3). No isolate appeared identical to the relevant vaccine. Where calculated, total divergence (which was measured as the sum of all the titer differences for the two isolates being compared) ranged from 3.0-20.4 (Table 3). Actual VN titres are given in appendix 1.

Two different serological criterion were used to determine the relatedness between each pair of isolates (Table 3). Using the high stringency definition of identity, where all antisera titre differences had to be $\log_2$ 2 or less, 23% (6/26) isolates were

similar to the vaccine. Using the less stringent definition, which allows the difference between one of the four comparable VN results to be greater than $\log_2$ 2, 54% (14/26) of isolates tested were similar to the vaccine (Table 3).

Sequence analysis of individual isolates.

All sequences and distance calculations used in this study are shown in appendices 2 and 3 respectively.

For the majority of isolates (88%, 23/26), direct sequence analysis of PCR products gave relatively pure sequence suggesting these isolates to be relatively homogenous. However, for three vaccine failure isolates (E315, M382 and M403), sequence obtained directly from PCR products showed multiple ambiguities. These were resolved by sequencing molecular clones of the appropriate PCR products. E315 and M382 each contained two sequences that were 14% and 28% different from each other respectively (Figure 2 and Appendix 3). This is a similar situation to vaccine A and B which were both shown previously to contain two sequences 21.3% different from each other (Radford *et al.*, 1997).

M403 contained a group of sequences that were all closely related to each other, no single sequence being more than 3.0% different from others in the isolate (data not presented). In all subsequent analysis, only one sequence from M403 was used.

Comparison of isolates by sequence analysis.

Nucleotide distances for most isolates compared fell into one of two categories; either 0-5.3% different (related isolates) or 20.7-42.7% different (unrelated isolates). The related isolates fell into one of six groups phylogenetically (groups A-F, figure 2). Groups A and B contained vaccine-derived and vaccine failure sequences. Groups C and F include sequences of isolates from cats within the same household. Groups D and E contained isolates with no apparent epidemiological link.

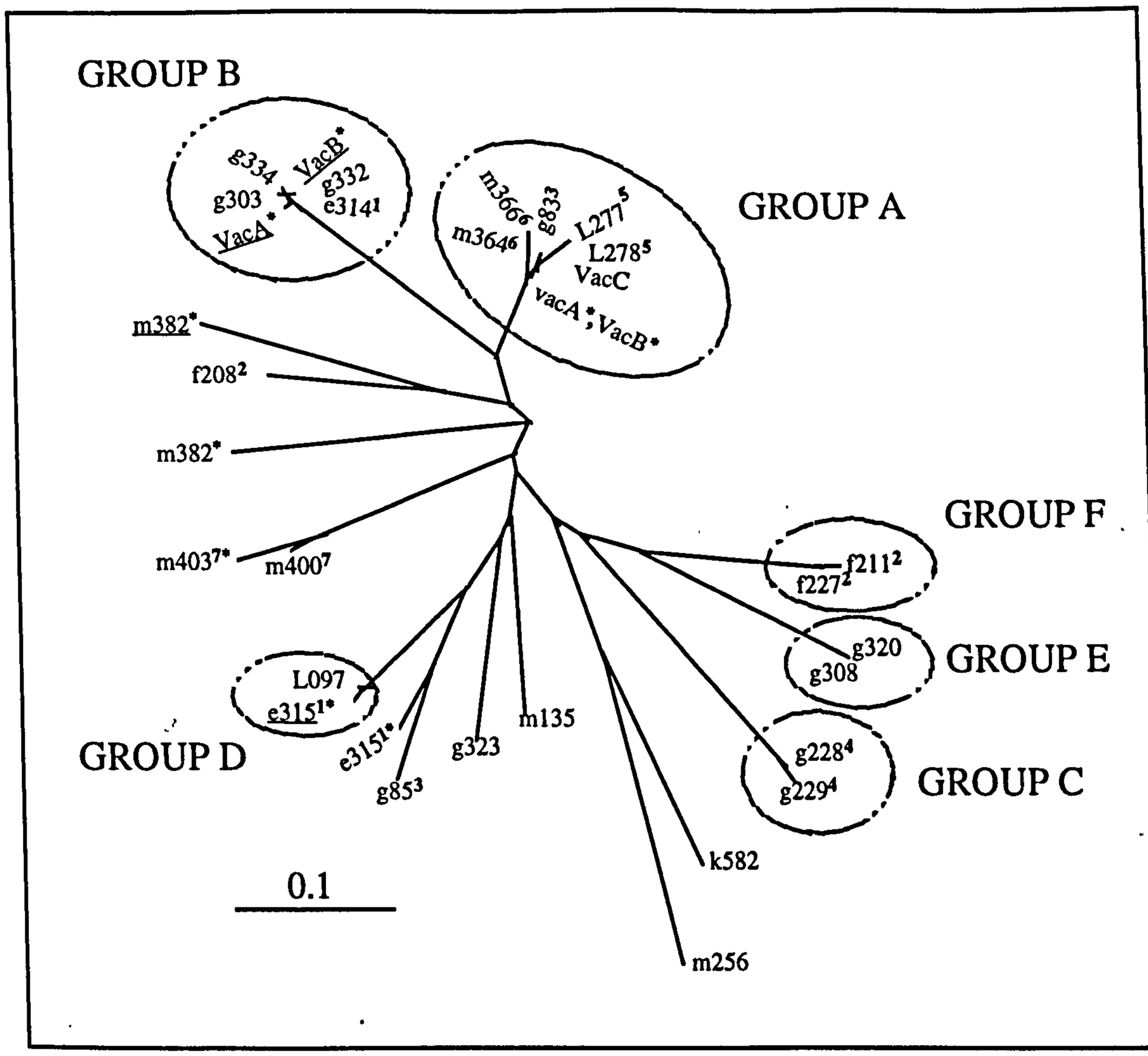

Figure 2; Phylogenetic analysis (KIMURA, NEIGHBOR-JOINING (Felsenstein, 1989)) of 5'HVR nucleotide sequences. * identifies those sequences obtained from clones. Underlined sequences from those isolates containing more than one sequence correspond to sequence as presented in appendix 2. Superscripted numbers (**1-7**) identify isolates from the same household. Closely related sequences (nucleotide distances 0-5.3%) fall into six groups, A-F. Branch lengths are drawn to scale, the bar representing a nucleotide difference of 10%.

Comparison of several sequences fell in between the apparently related and unrelated isolates (6-14% different; Figure 2 and Appendix 3). Some of these isolates were linked epidemiologically, either by household (M400/M403, 8.0%), or by the common use of similar vaccines (L277/G83, 6.0%; L277/M366, 7.3%). Other isolate relationships were less easily explained (E315/G85, 11.3%; E315/L097, 14.0%) as they were not obviously linked epidemiologically.

Distance comparisons of sequences from vaccine failure isolates and the relevant vaccine fell into two categories; those with similar sequences (0.0-5.3; 9/26) represented by groups A and B in figure 2, and those with divergent sequences (21.3-38.7%; 17/26) (Table 3 and Appendix 3).

Comparison of serological and sequence-based typing.

Comparison of results for typing vaccine failures by sequence analysis and serological analysis is shown in table 5. Comparing the more strict serological criterion (all titre differences $\log_2 2$ or less) with sequence analysis (table 3i) showed agreement in 17 of the 26 cases (65%). Of the discordancies, six of nine were represented by identity with sequence analysis and non-identity by serology. If sequence based typing is assumed to be most accurate, the sensitivity and specificity of the high stringency serological test compared to this is 0.33 and 0.82 respectively.

Comparison of sequence analysis with the less stringent serological criterion (table 3ii) showed agreement in 19 of 26 cases (73%). Of the discordancies, six of nine were represented by identity with serological analysis and non-identity by sequence analysis. Again, assuming sequence based typing to be most accurate, this gives values for sensitivity and specificity of 0.89 and 0.65 respectively for the less stringent serological test.

The grouping of isolates by the sum of their VN titre differences is shown in figure 3. In contrast to sequence analysis, no apparent overall division of isolates into those related and those unrelated to the relevant vaccine was observed. Most of the isolates that appeared closely related to the relevant vaccine by sequence analysis (distance values 0-5.3%) had total VN titre differences of 6 or less. However, one such isolate had a total VN titre difference of >10 (G83, 12.6).

(i) sequence

antisera	+	+	-
	+	3/26	3/26
	-	6/26	14/26

(ii) sequence

antisera	+	+	-
	+	8/26	6/26
	-	1/26	11/26

Table 5; Comparison of vaccine failure typing results based on serology and sequence analysis. Sequence identity was defined as nucleotide differences of 5% or less (Table 3). (i); serological identity defined as all four titre differences $\log_2 2$ or less. (ii); serological identity defined as three titre differences of $\log_2 2$ or less.

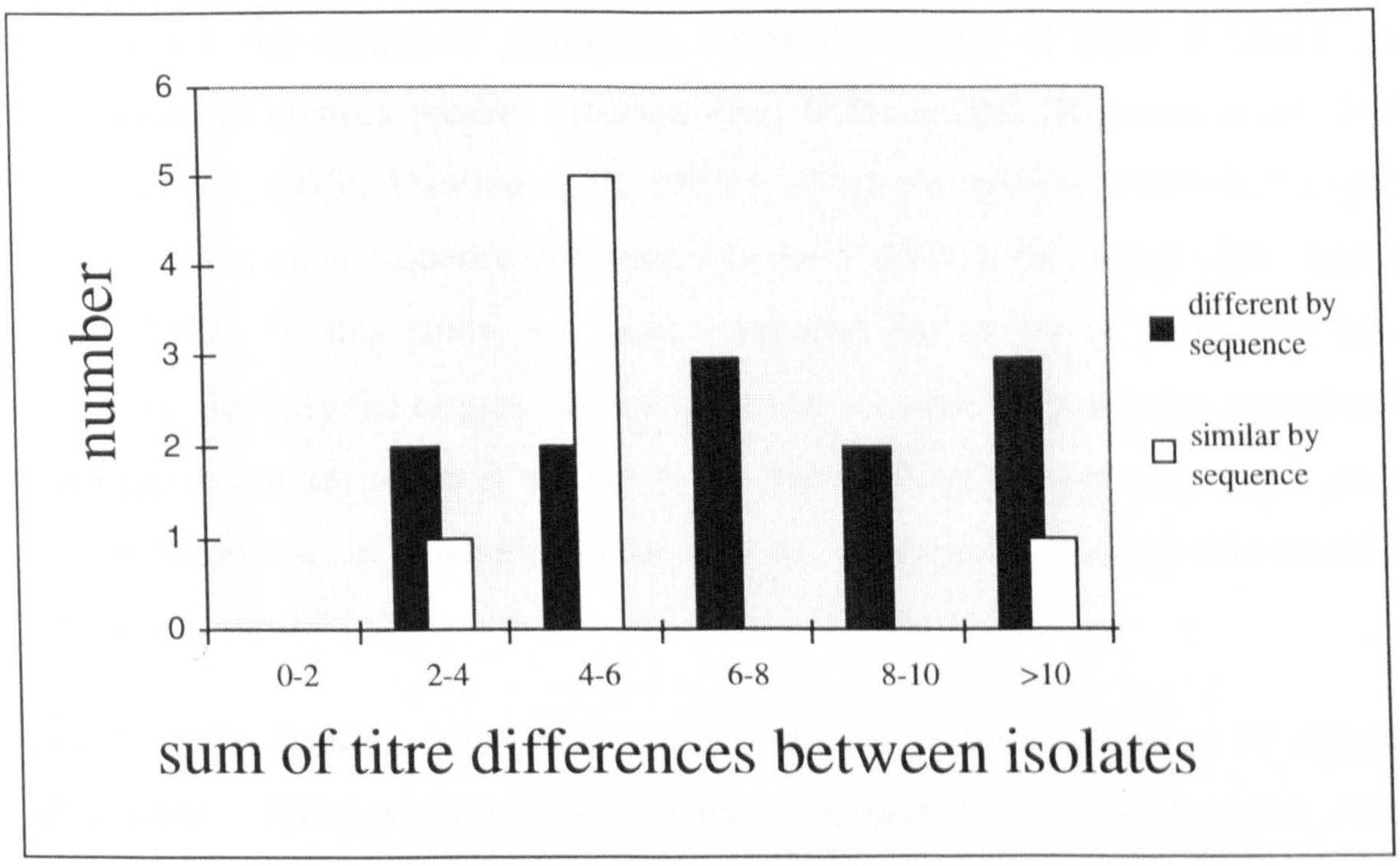

Figure 3; vaccine-failure isolates grouped according to their degree of relatedness to vaccine virus as judged by the sum of the titre differences (see text). Black bars represent viruses that appear different to vaccine by sequence analysis (distance 20.7-42.7%). White bars represent those viruses that appear similar to vaccine due to the presence of sequences 0-5.3% distance).

DISCUSSION.

The epidemiological investigation of infectious diseases is facilitated by methods which allow the typing of pathogens. Currently, typing of FCV is based upon serological differences present between most FCV isolates (Knowles *et al.*, 1990; Dawson *et al.*, 1993a; Dawson *et al.*, 1993b). We have recently established a typing protocol based upon sequence differences in the 5'HVR of the capsid gene (Radford *et al.*, 1997). In this study, we have compared the ability of these two typing methods to identify the origins of vaccine failure isolates. In particular, to determine if a vaccine failure isolate is similar to the vaccine virus, suggesting a role for the vaccine in disease, or dissimilar to the vaccine, implying the disease was associated with a field virus.

In this study, isolates compared by VN revealed a broad spectrum of antigenic relatedness. There was no clear division of isolates into those that were closely related and those those that are unrelated. It has been stated previously that the interpretation of serological data based on VN tests may be difficult. This may be due in part to the mutatibility of RNA viruses, allowing the antigenic profile of individual FCV isolates to change on replication both in cell culture and in cats (Pedersen & Hawkins, 1995; Radford *et al.*, 1998). Also, not all isolates of FCV are distinguishable by VN tests (Kalunda *et al.*, 1975; Dawson *et al.*, 1993b). This may in part, be a reflection of the limited number of polyclonal antisera used. Also, isolates sharing an immunodominant epitope may appear to be similarly neutralised by a particular antisera despite being quite distinct in other more minor epitopes.

In contrast, and as has been shown previously using a smaller number of isolates (Radford *et al.*, 1997), comparison of 5'HVR sequences from different FCVs showed that the majority of isolates fall into one of two categories. Most are highly variable when compared to one another (sequence differences ranging from 20.7-42.7%) and confirms the hypervariable nature of this region of the FCV capsid gene. A second group of isolates appear very similar (sequence differences ranging from 0-5.3%). The significance of this degree of homology is particularly apparent when the extent of variability of the 5'HVR is considered. The majority of these similar

isolates share an epidemiological link. It would therefore seem reasonable to adopt this range of sequence differences as that which strongly suggests a common recent origin for two isolates. Using this definition, sequence analysis appears to be able to characterise vaccine failure isolates as either likely to have originated from the vaccine or from a field isolate.

We have shown previously by sequence analysis that FCV vaccines may contain more than a single type of virus (Radford *et al.*, 1997). In this study, we have also shown that individual cats (E315 and M382) may be co-infected with more than a single isolate. The heterogenous nature of such complex isolates, and the interaction of divergent viruses in mixed populations (Pedersen and Hawkins, 1995; Woodbury *et al.*, 1995) may further confuse the interpretation of VN tests.

Comparison of serological typing and sequence-based typing methods agree in suggesting that vaccine virus may be responsible for some vaccine failures. However, the two methods frequently disagree in individual cases as to the origin of vaccine failure isolates. Because of the difficulties of interpreting serological data, the ability of sequence analysis to identify mixed isolates, and the demonstration that most isolates that share high sequence homology also share an epidemiological link, we propose that sequence analysis of the 5'HVR represents a superior typing method for FCVs.

In this study we have also found some sequences that, when compared, fall in between the apparently related and unrelated isolates (6-14% different). Some of these isolates share an epidemiological link and may represent divergent evolution of sequence away from that of a common ancestor which may be a field virus (e.g M400/M403, E315/E315) or a vaccine virus (L277/G83, L277/M366). Indeed, we have shown variability of FCVs within quasispecies may be as high as 8% in the same HVR (Radford *et al.*, 1998). Other isolate relationships are less easily explained (E315/G85, E315/L097) as they are not obviously linked epidemiologically.

Most isolates that share considerable sequence homology are related epidemiologically. However, we have identified some isolates of uncertain epidemiological linkage which fall into this same category of sequence relatedness

(eg G308 and G320, L097 and one of the isolates in E315). These isolates may represent either an unidentified epidemiological link or the widespread circulation of a single isolate. It is also possible that these sequences represent within-laboratory contamination, either during virus culture or PCR. However this is thought unlikely, since in the above examples, the related sequences were processed several months apart, and precautions were routinely taken to minimise the possibility of contamination occuring.

In this study, we have also shown that cat households (E314 and E315, F208, F211 and F227, G83 and G85) and individual cats (E315 and M382) may be co-infected with more than a single virus. This provides another potential mechanism for virus evolution by recombination between the genomes of divergent viruses within the same host. Recombination has not been demonstrated in FCV (Geissler *et al.*, 1997) but is well characterised in other RNA viruses (Romanova *et al.*, 1986; Lemon *et al.*, 1991; Lai, 1992).

We therefore suggest that sequence analysis from the 5'HVR represents an improved method over serological typing for FCV, particularly in vaccine reactions and disease outbreaks. Analysis of PCR products and where necessary, molecular clones, allows the complexity of certain isolates to be more fully determined. We suggest a cut-off of 5.3% as representing the maximum distance between sequences that are likely to have originated from a recent common ancestor. Divergent isolates are likely to be approximately 20-40% distant. Interpretation of isolate relatedness between 5 and 20% may be difficult. As for any PCR based technology, rigorous controls must always be in place to minimise the risk of contamination.

In vaccine breakdowns, disease is seen later post-vaccination, and is perhaps more likely to be associated with isolates not neutralised by vaccine-induced immunity rather than vaccine virus itself. They may also represent disease in animals that did not respond to their last vaccination. Vaccine breakdowns are seen less commonly than vaccine reactions (Dawson et al., 1993a). In this study, only two such isolates (2 of 26) have been included (M135 and M403). The proportion of vaccine failures made up by vaccine breakdowns broadly agrees with the frequency that we receive them in our diagnostic laboratory (personal communication Dr S.Dawson). In this study, both

M135 and M403 appear distinct from vaccine virus by sequence analysis suggesting they are field viruses. Such isolates are of interest as their addition to currently monovalent FCV vaccines may improve the range of field isolates neutralised by vaccine-induced immunity. For vaccine breakdowns therefore, a dual approach based on serology and sequence analysis may be considered.

ACKNOWLEDGEMENTS.

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Isolate	Vaccine[a]	Antiserum[b]			
		F9	Other[c]	LS015	Bulmer13
E314[1]	B	**6.6/6**	**11/10**	**9/10**	**9/7**
E315[1]	B	<1/6	<1/10	5/10	<1/7
F208[2]	A	2/8	2.6/5	4/9	2/7
F211[2]	A	1/8	1.6/5	4/9	2/7
F227[2]	A	5/8	<1/5	5/9	1.6/7
G83[3]	A	2/8	3.6/3	6/9	4/7
G85[3]	A	**9/8**	**5/5**	**10/9**	11/7
G228[4]	A	**6/8**	**5/5**	5/9	4/7
G229[4]	A	5/8	**6/5**	**7/9**	**5/7**
G303	B	**6/6**	**12/10**	**9/10**	**7/7**
G308	B	**4/6**	**10/10**	**8/10**	**9/7**
G320	A	3/8	8/5	**10/9**	**7/7**
G323	A	1/8	**3.6/5**	**8/9**	3.6/7
G332	B	**5/6**	**12/10**	**8/10**	**8/7**
G334	B	**5/6**	**11/10**	7/10	**6/7**
K582	A	**5/5.6**	**6.6/7**	**4.6/3.6**	**3/2**
L097	A	3/5.6	10/7	**4.6/3**	**3.6/3.6**
L277[5]	C	**5/5**	**2/2.6***	**5/5.6**	**4/<1**
L278[5]	C	**4/5**	**2/2.6***	**7/5.6**	**4.6/<1**
M135	A	3.6/7	3/7.6	**5/4.6**	**4/<2**
M256	A	<1/6.6	**3/5#**	**7/5**	<1/4.6
M364[6]	A	4/6.6	**4.6/5#**	**5/5**	**2.6/4.6**
M366[6]	A	3.6/6.6	**4.6/5#**	**4.6/5**	**2.6/4.6**
M382	A	**7.6/6.6**	**5.6/5#**	**6/5**	**6/4.6**
M400[7]	A	2.6/6.6	**6/5#**	**6/5**	<1/4.6
M403[7]	A	3.6/6.6	**6.6/5#**	**4.6/5**	**3/4.6**

Appendix 1; Serological method of typing vaccine failure isolates. VN titres are expressed as $\log_2$ of the reciprocal of the 50% end point. Those isolates identified by the same superscript number (**1-7**) were from the same household.

a; Vaccine failures occured following the use of one of three commercial vaccines, A, B and C.

b; Isolates were compared to vaccine by VN using four antisera - anti F9, anti LS015, anti Bulmer 13 and anti 'other'. To compare isolates, the VN titre of a given antisera was determined against the two isolates to be compared.

c; 'Other' antisera was against the relevant parent vaccine except when anti LS027 (*) and anti F65 (#) were used.

Appendix 2; Sequences obtained in this study and *previously (Radford *et al.*, 1997) aligned using programmes PILEUP and PRETTY from version 8 of the Wisconsin package, genetics computer group (Deveraux *et al.*, 1984). n represents a,g,c or t. Full stop (.) represents identity to the consensus sequence. Vac A, Vac B, e315 and m382 all contained two sequences; one sequence from each isolate is underlined to allow the two sequences to be consistently distinguished (see also figure 2).

```
                1                                                  50
          m256  .....a.... .......a.. cagt...c.c ..t..c..c. .......t..
         *K582  .....a.... .c..c..t.. a.acac.c.. .....t..g. ..a....t..
          m403  .....g.... .......t.. ..ac.t.... .c...t..g. ..........
          m400  .....g.... .......t.. ..ac.t.... .....t..g. ....c.....
          e315  .....aa... .c........ aac.c....a ..t..t..a. ..........
         *L097  .....a.... .c........ aac.c....a ..t..t..a. ..........
          e315  .....a.... .c........ aac.c....a ..t..t..a. ..........
           g85  .....a.... .c........ atta...c.t .cc..t.ta. .c........
          g323  .....a..t. .t........ caccacc..a .....ta.a. ....c..t..
         *m135  .....a.... .t........ gtca...c.c .....t.... ....c..t..
         *e314  .......... ....c...t. ..a....c.a ..c..... ....c..t..
         *g332  .......... ....c...t. ..a....c.a ..c..... ....c..t..
        *Vac B  .......... ....c...t. ..a....c.a ..c..... ....c..t..
         *g334  .......... ....c...t. ..a....c.a ..c..... ..a.c..t..
         *g303  .......... ....c...t. ..a....c.a ..c..... ....c..t..
        *Vac A  .......... ....c...t. ..g.a..c.a ..c..... ....c..t..
         *L278  .......... .c.....t.. ..g......a .....a... .c........
         *L277  .......... .c.....t.. ..g......a .....a... .c........
          m364  .......... .c.....t.. ..g....... .......... .c........
          m366  .......... .c.....t.. ..g....... .......... .c........
        *Vac B  .......... .c.....t.. ..g....... .......... .c........
        *Vac A  .......... .c.....t.. ..g....... .......... .c........
        *Vac C  .......... .c.....t.. ..g....... .......... .c........
          *g83  .......... .c.....t.. ..g....... .......... ..........
          m382  .....a..t. ....t..... ..aa..a... g.g..g..a. .a..c...t.
          f208  .....a.... ....c..... ..a...a... .......... .g........
          m382  .....a..t. ....c..tgg c.aaaca... .....c.... .ca.......
         *g229  .....a..tc ....t..... aact...c.a ..t.....c. ..a.......
         *g228  .....g..t. ....t..,.. aact...c.a ..t.....c. ..a.......
          g320  ........t. .c........ atcaa.ac.. g.t..t.... ...g...t..
         *g308  ........t. .c........ atcaa.ac.. g.t..t.... ...g...t..
          f227  .....a.... .......t.. atcaag.c.. .cc.....a. ....c..t..
          f211  .....a.... .......t.. atcaag.c.. .cc.....a. ...gc..t..
     Consensus  TGGCCTGACA CAACAATCCC TG-GGAGTTG ATACCAGCTG GTGATTACGC
```

Appendix 2 continued overleaf

Appendix 2 continued

```
          51                                                        100
m256      ...tgtg... caaagcta.. ....t..a.. t..caaag.. c....c..gt
K582      ...t..a... .aaaa.aa.. ....t..a.. a...aaa.gt c.......ag
m403      ......acga ..g.a..... .......... a...c..t.g .gg......t
m400      ......ac.g ..g.a..... .......... ...cc..t.. .g.......t
e315      t..a.....g ..g.ac..g. ....t..... a.....c.ag ..g......g
L097      t..a.....g ..g.ac..g. ....t..... a.....c.ag ..g......g
e315      t..a.....g ..g.ac..g. ....t..t.. a..a..cg.. .c.......t
g85       t..a..t.g. ..a.g...g. ....t..t.. a..a..cg.. .c.......t
g323      ...t.....a ..g.a...g. .......t.. a......g.. ..t......t
m135      c..a.....a ..a.a...g. ....t..tg. ...a...ga. ..c.....c.
e314      .......... ..c..c..t. .c.....tgt ...a...cgt ....tc..c.
g332      .......... ..c..c..t. .c.....tgt ...a...cgt ....tc..c.
Vac B     .......... ..c..c..t. .c.....tgt ...a...cgt ....tc..c.
g334      .......... ..c..c..t. .c.....tgt ...a...cgt ....tc..c.
g303      .......... ..c..c..t. .c.....tgt ...aa..cgt ....tc..c.
Vac A     .......... ..c..c..t. .c.....tgt ...a...cgt ....tc..c.
L278      .......... ...a...... .......... ...g....g. ........c.
L277      .......... .a........ .......... ...g....g. ........c.
m364      .......... ...a...... .......... ...g...g.. .g......c.
m366      .......... ...a...... .......... ...g...g.. .g......c.
Vac B     .......... ...a...... .......... ...g...... .g......c.
Vac A     .......... ...a...... .......... ...g...... .g......c.
Vac C     .......... ...a...... .......... ...g...... .g......c.
g83       .......... ...a...... .......... ...g...... .g......c.
m382      c..a.....c .....a..t. ........ca t.....cga. .gc..c...t
f208      ...a.g...c a..at..... .......... t.....cg.t ..t......g
m382      t.....a... .aaata.... ....t...gt a.......a. .....c...t
g229      ...tttagcg ....g.a... .c..t..... a.....c.tg .........t
g228      ...tttagcg ....g.a... .c..t..... a.....c.tg .........t
g320      ...t..tgca aag.a...g. .c..t..... t......g.g c....c...g
g308      ...t..tgca aag.a...g. .c..t..... t......g.g c....c...g
f227      ......tcta a.caa...a. .......... t........g .g.......g
f211      ......tcta a.caa...a. .......... t......g.g .g.......g
Consensus AATCACCAAT GGTGCTGGCA ATGACATCAC CACTGCTACA GAATATGATA

          101                                                       150
m256      ....aataac .....c.... ..c..a.... .c.aaa.t.. ......t...
K582      t...aagct. ...c..g..t ..c..a.... .....a.t.. ......t...
m403      .g.....caa g..cgtt... .....t.... ...aaa.t.. ......c...
m400      .a.....caa g..c.tt... .....t.... ...aaa.t.. ......t...
e315      ....c..... c......... ..c..t.... .c.aa..t.. ......c..c
L097      ....c..... c.....g... ..c..t.... .c.aa..t.. ......c..c
e315      .a.....c.. g..c.g.... ..c....... .cg.aa.t.. ......t...
g85       .a.....c.. g..c.g.... ..c....... .c..aa.t.. ......t...
g323      .g........ ......g..t ..c..t..c. .c...a.t.. ......t...
m135      ag........ ...c...... ........c. ...aaa.... ......c...
e314      .......c.a ...c...... ........c. .c........ ..........
g332      .......c.a ...c...... ........c. .c........ ..........
Vac B     .......c.a ...c...... ........c. .c........ ..........
g334      .......c.a ...c...... ........c. .c........ ..........
g303      .......c.a ...c...... ........c. .c........ ..........
Vac A     .......c.a ...c...... ........c. .c........ ..........
L278      ........a. .....gg... ........c. .......... ..........
L277      ........a. .....gg... ........c. .......... ..........
m364      ......g.a. ....g.g... ..c.....c. .......... ..........
m366      ......g.a. ....g.g... ..c.....c. .......... ..........
Vac B     ........a. ......g... ........c. .......... ..........
Vac A     ........a. ......g... ........c. .......... ..........
Vac C     ........a. .......... ........c. .......... ..........
g83       ........a. ......g... ........c. ...a...... ..........
m382      .......... c.....g... .c........ ...a...... ......c...
f208      .a...a.... c..c.....t .c...t.... .......... .a........
m382      .g....c.aa ......g... .......... .......t.. ...t..t...
g229      .g..cac.ac .........t g.a....... ....aa.... ......c...
g228      .g..cac.ac .........t g.a....... ....aa.... ......c...
g320      .a..a.g..a ....gt.... .......... ...aaa.... ......c...
g308      .a..a.g..a ....gt.... .......... ...aaa.... ......c...
f227      .a..caca.a g......... .......... ....aa.... .c....c...
f211      .a..caca.a g......... .......... ....aa.... .c....c...
Consensus CTGCTGATGT AATTAAAAAC AATACCAATT TTAGGGGCAT GTACATATGT
```

	e 314†	g 334	g 303	Vac A	L 278	L 277	Vac C	Vac A†	g 83	m 366	m 382	f 208	m 382	e 315	L 097	e 315	g 85	g 323	m 135	m 403	m 400	f 227	f 211	g 308†	g 229	g 228	m 256	k 582
e314	0.0	0.7	0.7	1.3	20.7	20.7	20.7	21.3	21.3	23.3	30.0	28.7	32.0	33.3	33.3	30.0	32.0	31.3	24.0	34.7	31.3	32.7	33.3	35.3	37.3	36.7	38.7	36.7
g334		0.0	1.3	2.0	21.3	21.3	21.3	22.0	22.0	24.0	30.7	29.3	31.3	34.0	34.0	30.7	32.7	32.0	24.7	35.3	32.0	33.3	34.0	36.0	36.7	36.0	39.3	36.0
g303			0.0	2.0	21.2	21.3	21.3	22.0	22.0	24.0	30.7	29.3	32.7	34.0	34.0	30.7	32.7	32.0	24.7	34.7	31.3	33.3	34.0	36.0	38.0	37.3	38.0	36.0
VacA				0.0	20.7	20.7	20.7	21.3	21.3	23.3	31.3	30.0	32.0	33.3	33.3	30.0	32.7	30.7	24.7	36.0	32.7	32.0	32.7	34.7	38.0	37.3	38.7	36.7
L278					0.0	1.3	4.0	3.3	4.7	6.0	28.0	23.3	26.0	28.7	27.3	28.7	30.0	26.7	25.3	28.7	26.0	31.3	32.7	34.7	32.7	32.0	36.0	32.0
L277						0.0	5.3	4.7	6.0	7.3	28.0	24.7	26.0	28.7	27.3	28.7	30.0	26.7	25.3	28.7	26.0	32.7	34.0	33.3	32.7	32.0	36.0	32.0
VacC							0.0	0.7	2.0	3.3	26.0	20.7	25.3	28.0	28.0	28.0	28.7	28.0	24.0	26.0	23.3	27.3	28.7	32.7	32.0	31.3	34.7	32.0
VacA								0.0	1.3	2.7	25.3	21.3	24.7	28.7	27.3	28.7	29.3	27.3	24.7	26.0	23.3	28.0	29.3	33.3	32.7	32.0	35.3	31.3
g83									0.0	4.0	24.7	22.0	26.0	27.3	26.0	28.7	30.7	27.3	23.3	24.7	22.0	28.0	29.3	32.0	32.7	32.0	34.0	31.3
m366										0.0	26.7	22.7	26.7	30.0	28.7	28.7	29.3	27.3	26.0	26.7	25.3	30.0	30.0	32.0	34.0	33.3	34.7	31.3
m382											0.0	22.7	28.0	28.0	26.7	31.3	32.7	30.0	27.3	32.7	31.3	34.0	34.0	33.3	36.0	36.0	42.0	42.7
f208												0.0	30.7	28.0	28.0	29.3	31.3	28.7	30.0	30.0	29.3	28.7	28.7	33.3	32.0	32.0	38.7	32.7
m382													0.0	34.0	32.7	34.7	34.0	30.0	33.3	31.3	30.7	34.0	35.3	34.7	33.3	33.3	36.7	29.3
e315														0.0	1.3	14.0	24.0	23.3	25.3	28.7	30.7	30.0	31.3	28.7	28.0	28.0	34.7	34.0
L097															0.0	14.0	24.0	22.0	25.3	28.0	30.0	30.0	31.3	28.7	28.0	28.0	34.7	32.7
e315																0.0	11.3	20.7	23.3	28.0	24.7	30.7	30.7	30.0	30.7	30.7	32.0	33.3
g85																	0.0	26.7	23.3	28.0	28.0	28.0	28.0	30.7	32.7	32.7	32.7	33.3
g323																		0.0	22.0	28.0	27.3	30.7	30.7	30.0	32.0	32.0	35.3	30.0
m135																			0.0	30.0	28.7	28.7	28.7	26.7	33.3	33.3	35.3	34.7
m403																				0.0	8.0	25.3	26.0	30.0	33.3	32.0	38.7	32.7
m400																					0.0	26.7	27.3	32.7	35.3	34.0	35.3	32.0
f227																						0.0	1.3	23.3	29.3	29.3	36.7	34.0
f211																							0.0	22.0	30.7	30.7	36.7	35.3
g308																								0.0	29.3	28.7	34.0	34.7
g229																									0.0	1.3	32.0	33.3
g228																										0.0	32.0	33.3
m256																											0.0	27.3
k582																												0.0

Appendix 3; Distance table (nucleotide substitutions per 100 nucleotides compared) for comparisons of all sequences in this study. † To avoid duplication, identical sequences have only been represented once. Therefore e314 also represents g332 and VacB sequence, g308 also represents g320, and VacA also represents Vac B sequence. Important comparisons discussed in the text are highlighted. Vac A, Vac B, e315 and m382 all contained two sequences; one sequence from each isolate is underlined to allow the two sequences to be consistently distinguished (see also figure 2 and appendix 2).

GENERAL DISCUSSION AND FUTURE WORK.

It is known that despite widespread vaccination of cats, feline calicivirus (FCV) still persists at high levels in the domestic cat population (Harbour *et al.*, 1991; Coutts *et al.*, 1994). Reasons for this are unknown but may involve persistent infections of individual cats facilitated by intra-host antigenic variation (Johnson, 1992). Such persistent infections may be established following the challenge of both naive cats and those with pre-existing immunity from either previous challenge or vaccination. Indeed some evidence suggests that pre-existing immunity may prolong viral shedding thereby promoting persistence (Dawson *et al.*, 1991). It has also been suggested that vaccine derived FCV, which experimentally may be shed from the oropharynx of subcutaneously vaccinated cats (Bennett *et al.*, 1989; Pedersen & Hawkins, 1995), may be contributing to FCV prevalence, particularly in the form of vaccine failures. The origins of the FCVs from vaccine failures are uncertain. Whilst many are presumed to be field viruses, serotyping evidence suggests that vaccine virus may be associated with some cases (Dawson *et al.*, 1993a).

This thesis primarily aimed to utilise sensitive molecular techniques including those based on the polymerase chain reaction and sequence analysis to address two important questions.

1). What was happening to the virus in persistently infected cats and could this explain the difficulty of the host to clear FCV infection?

2). What were the origins of vaccine failure isolates? In particular, were some of these isolates from the vaccine (as suggested by serotyping) or field virus?

The region of the genome analysed (the 5' hypervariable region; 5'HVR) was chosen because of its variability between isolates (Seal *et al.*, 1993) making it a suitable candidate for a sequence-based typing method. The 5'HVR was also known to contain important linear epitopes that are responsible for antibody-mediated virus neutralisation (Guiver *et al.*, 1992; Milton *et al.*, 1992; Tohya *et al.*, 1997). Therefore, if antigenic variation was responsible for the host's apparent difficulty to

clear an FCV infection, then this variation may be mediated through sequence evolution in the 5'HVR during persistent infections.

Using sequence from this region, it was shown that FCV existed as a mixed population or quasispecies both in cats and in cell culture. This quasispecies was shown to evolve over time at the sequence level, and in the case of a persistently infected cat, also antigenically. Therefore it is possible that persistence of FCV, as for other RNA viruses, results from the preferential replication of viral mutants that are resistant to the current immune environment within the host. The repetition of this process during persistent infection leads to sequence evolution and antigenic variation. By analogy to other RNA viruses, the generation of a quasispecies is believed to be brought about by the low fidelity of the virally encoded RNA dependant RNA polymerase (Holland *et al.*, 1992).

Viruses in general utilise a high proportion of their genome. This thriftiness with their genome has developed from their small genome size and becomes more apparent as viruses get smaller. If this argument is taken to its logical conclusion it follows that each gene encodes a protein, the function of which is essential to its owner's survival strategy. Therefore, observed virus phenotypes resulting from viral protein expression should act as signposts that point to critical components of viral survival strategies.

One observed phenotype of many RNA viruses is their ability to rapidly mutate. An application of the above argument to this phenotype leads to the conclusion that error prone replication may be a critical survival strategy used by RNA viruses. This has two important conclusions for the ultimate goal of controlling RNA viruses and the diseases they cause.

Firstly, pharmacological intervention of viral replication fidelity, may represent one strategy for the control of RNA viruses. Increasing the fidelity of viral RNA polymerases may reduce the rate at which mutants are produced thereby allowing the host defences to clear infection. Alternatively, if the fidelity of RNA polymerases can be reduced, the production of functional genomes by replication of a parent virus may be reduced or theoretically abolished.

Secondly, if error prone replication is essential to many RNA viruses, it is important to understand how such viruses evolve and what effect external environmental

selection pressures have upon these virus populations. For example, do selection pressures promote virus evolution and thereby facilitate the short and long-term survival strategies of the virus? In the case of FCV, one could speculate that viral replication in cats with pre-existing vaccine induced immunity could promote higher rates of virus evolution. It is intriguing to note that in the experiment in which virus evolution was measured in persistently infected cats (manuscript 1; Radford *et al.*, 1998), vaccinated animals usually shed FCV for longer than unvaccinated ones. It could therefore be speculated that pre-existing immunity induced by vaccination may, under certain circumstances, actually promote virus evolution and facilitate persistent infections. Unfortunately, in this experiment, vaccinated and unvaccinated cats were housed together making it difficult to evaluate differential rates of virus evolution between these groups.

The demonstration of sequence evolution does not necessarily equate with escape from neutralisation. The amino acid changes observed in the 5'HVR may not alter epitope function and the antigenic changes observed in isolates taken from a persistently infected cat may be due to changes elsewhere in the genome. It is therefore necessary to define B cell epitopes more accurately and to demonstrate that sequence variants within epitopes in quasispecies and sequence evolution in epitopes over time are associated with altered epitope reactivity. Such experiments will be facilitated by the availability of an infectious FCV clone in which the effect of specific mutations upon neutralisation of whole virus may be assessed. Until epitopes are defined more precisely, the correlation between observed sequence evolution and epitope reactivity is uncertain.

In order to define such epitopes more accurately, a lambda expression library was produced and screened with antisera from a challenged cat. The isolate used in these studies was chosen as it, and isolates like it, are frequently incorporated into commercial vaccines. Due to constraints on time, these studies are only at a preliminary stage. However, analysis of immunoreactive clones appears to confirm that the region we have sequenced indeed contains the immunodominant B cell epitopes. It will be necessary to utilise overlapping peptides corresponding to this region to further characterise these epitopes. Another approach to map these epitopes has become possible following the commercial release of a phage-based peptide

expression library expressing random 7-mers on the surface of the phage coat protein (Ph.D.-7 phage display peptide library kit; New England BioLabs.). Due to the finite number of amino acids and the small size of epitopes, this phage expression library is predicted to contain most 7 amino combinations. This method may facilitate the relatively rapid identification of linear B cell epitopes in several isolates.

It is hoped that such work will identify epitopes that fall outside the hypervariable regions of the capsid gene. If such epitopes are identified and are shown to be associated with virus neutralisation, then they would make excellent candidates for recombinant vaccines. Such vaccines may be broadly cross-reactive and less likely to allow escape mutant production, than current conventional vaccines.

Currently, little is known about the role of major histocompatibility-restricted cytotoxic T lymphocytes (CTL) in the immune response to FCV (Tham & Studdert, 1987). This is partly due to the difficulty of the necessary experiments, in particular the requirement for major histocompatibility-matched target tissues. Sequence variation in CTL epitopes appears to be an important feature facilitating persistent infections in other RNA viruses (Phillips *et al.*, 1991). Future work would therefore aim to determine what part, if any, CTLs contribute to protection and clearance of FCV.

In order to address the question of vaccine virus involvement in vaccine failures, a sequence analysis of the same hypervariable region was used to compare vaccine viruses and vaccine failure isolates. Molecular typing confirmed serotyping in implicating vaccine virus in some vaccine failures.

The use of live attenuated vaccines will always be associated with the risk of vaccine virus shedding and reversion to virulence, particularly in RNA viruses, where error-prone copying of the genome during viral replication promotes virus evolution. This is classically described for live poliovirus vaccines, where mutations in the 5' non-coding region of the genome are associated with loss of attenuated phenotype and reversion to neurovirulence (Guillot *et al.*, 1994). These revertants are thought to be the cause of low level vaccine-associated paralytic poliomyelitis in vaccinees or their contacts (Nkowane *et al.*, 1987). Whilst such attenuated vaccines have proved to be excellent in control of widespread disease, it is possible that future vaccine design

should sensibly focus on inactivated vaccines, particularly where disease eradication is the goal.

Although both serotyping and sequence analysis broadly agreed in suggesting that vaccine virus could be involved in disease post-vaccination, discrepancies in the results of the two methods were often seen when they were compared for individual isolates. Somewhat unexpectedly, although with precedent in other viruses (e.g. foot-and-mouth disease virus; discussed in Woodbury *et al.*, 1995), sequence analysis demonstrated that an individual isolate from a cat or a vaccine could contain more than a single virus. The epidemiology of FCV associated disease may therefore be relatively complicated with multiple divergent viruses in both vaccines and field isolates coming together in individual cats. Due to this complexity, and the difficulty in interpreting serologically based typing methods, we suggest that for vaccine reactions where disease is seen soon after vaccination, sequence-based typing methods may be more definitive than serotyping. For vaccine breakdowns, where disease is seen later post-vaccination, and is perhaps more likely to be associated with isolates not neutralised by vaccine-induced immunity, a dual approach to typing may be considered. Such isolates are of interest as their addition to currently monovalent FCV vaccines may improve the range of field isolates neutralised by vaccine-induced immunity.

In conclusion, the studies detailed in this thesis suggest that virus evolution may play a role in persistent FCV infections and that, under some circumstances, live attenuated vaccines are the cause of vaccine failures. Persistent infections and vaccine failures may explain in part, the continued high prevalence of FCV in the domestic cat population. Future studies will aim to correlate sequence and antigenic evolution by mapping B and T cell epitopes. This may facilitate the rational design of future sub-unit vaccines based upon either conserved epitopes or multiple variable epitopes. Such vaccines should not cause disease and may reduce the generation of escape mutants that are not neutralised by host immunity.

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