Studies on the molecular biology of feline calicivirus replicaton.

by

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CONTENTS.

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ABSTRACT.

Previous studies have identified a small region of the feline calicivirus (FCV) capsid (antigenic site (ags) 4) that is recognised by antibodies in the majority FCV infected cats. In this study, we raised polyclonal antisera to ags 4 in two rabbits by peptide inoculation. These antibodies reacted well with 18 FCV isolates tested in dot immunoblotting, Western blotting and immunofluorescence suggesting that this antiserum could be a highly useful diagnostic and research tool for the specific detection of a wide range of FCV strains. The ability of the polyclonal antisera to neutralise FCV was also assessed by virus neutralisation in cell culture. There was some evidence to suggest that ags 4 may induce antibodies capable of neutralising FCV. However, neutralising titres were generally very low for one antiserum, ranging from 1 in 6 to 1 in 32 with a mean of 15, and were not detectable for the other. The potential of this site to inform future vaccine design for FCV is discussed.

The mechanism by which FCV induces cell death is not known although morphological changes, most notably cellular blebbing, have suggested a process involving apoptosis. In this study, we used several assays to characterise the effect of FCV on cells in cell culture. There was little evidence to support specific induction of apoptosis in FCV-infected cells as assessed by terminal deoxynucleoide transferase-mediated labelling of 3'-OH ends (TUNEL assay). In addition, there was no apparent upregulation of the anti-apoptotic factor Bcl-2. However, there was some suggestion that cell cycle progression was being manipulated by FCV infection as judged by levels of proliferating cell nuclear antigen (PCNA).

Despite the likely importance of the 5' end of both the genomic and sub-genomic RNA molecules to key processes in viral replication there have been no previous attempts to identify putative secondary structures within the 5`ends of the FCV full-length genome. These regions were analysed and areas containing putative secondary structures were identified using the RNAdraw programme that uses energy rules to determine optimal secondary structures for RNA molecules. We have obtained evidence for seven putative stem-loop structures that were conserved amongst all FCV isolates analysed. In contrast, only one conserved stem-loop was predicted at the 5`end of the sub-genomic RNA. However, an extensive region of sequence homology was identified between the 5' ends of both the genomic and sub-genomic RNA molecules suggesting similar mechanisms may be responsible for the replication, transcription and / or RNA packaging in both molecules.

Infectious clones have provided a valuable tool for studying the molecular biology of many viruses. In this study we have explored a new, improved technology for generating infectious clones of FCV based on long-range PCR. We have been successful in producing full-length PCR products both from RNA isolated from infectious virus and from a FCV F9 DNA genome copy. However, due to insufficient yield only the latter amplicons were cloned into a plasmid vector. Although we were unable to recover infectious virus from any of these clones, we have developed all the necessary steps required to generate infectious clones by this protocol.

A key step in the viral life cycle is the specific interaction with its cellular receptor. A virus overlay protein binding (VOPB) assay was performed to attempt to identify the putative cellular receptor for FCV. Using this approach it was not possible to detect any consistent and specific interaction between the virus and permissive cells. The possible reason for the failure of the VOPB assay, and future approaches which may lead to the identification of the FCV cellular receptor, are discussed.

INTRODUCTION

The *Caliciviridae.*

The *Caliciviridae* are a large family of viruses that cause a variety of disease syndromes (Cubitt et al., 1995), each containing a single-stranded positive sense RNA genome of approximately 7-8 kb (Clarke & Lambden, 1997). Originally, members of this family were classified within the *Picornaviridae* (Fenner, 1976). However, they were reclassified as the *Caliciviridae* due to their characteristic genome arrangements and physiochemical properties differentiating them from picornaviruses (Burroughs & Brown, 1974, Cubitt et al., 1995, Ehresmann & Schaffer, 1977, Melnick et al., 1974).

This introduction is divided into three sections.

Section 1 outlines the key features of representative members of each genus of the *Caliciviridae* and includes general background, history and clinical signs of infection.

Section 2 describes the properties, genome structure, and function of caliciviruses with particular emphasis where appropriate on feline calicivirus (FCV).

Section 3 is focused on FCV and describes the clinical and epidemiological features, of the disease, the antigenic and genetic relationships of the virus and the control of the disease by vaccination.

Section 1

1 Members of the *Caliciviridae.*

Sequence data for many caliciviruses allow the family to be divided into four genera: vesivirus, lagovirus, norovirus (previously 'Norwalk- like viruses') and sapovirus (previously 'Sapporo-like viruses') (Berke et al., 1997, Noel et al., 1997). A phylogenetic tree of the family members is shown in Fig. 1.

1.1 Genus vesivirus.

Caliciviruses belonging to the genus vesivirus include vesicular exanthema of swine virus, San Miguel sea lion virus, canine calicivirus, mink calicivirus and feline calicivirus. A characteristic of this group is that they tend to induce vesicles in their hosts which later develop into erosions and ulcers.

1.1 a) Vesicular exanthema of swine virus (VESV).

Vesicular exanthema of swine (VES) virus was first recognised during an outbreak of the disease in California in 1932 (Sawyer, 1976, Smith & Akers, 1976). The disease eventually spread to 42 states across America, and was transmitted by transporting infected animals and contaminated pig food (Sawyer, 1976). The clinical signs of VES included ulceration of feet and oral-nasal areas showing similarities to foot and

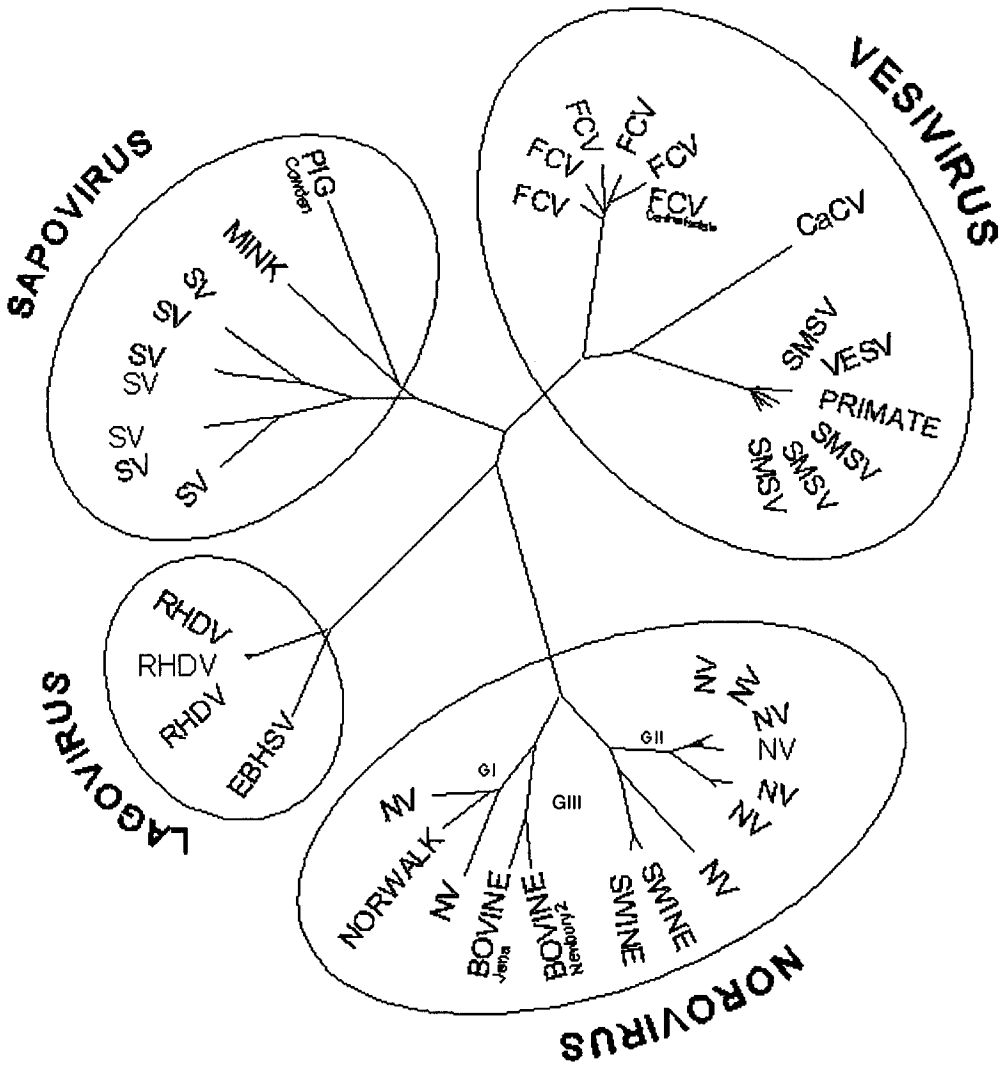


Fig. 1. Phylogenetic tree of members of the *Caliciviridae* family based on sequences from the polymerase region.

FCV = Feline calicivirus, CaCV = Canine calicivirus, SMSV = San Miguel sea lion virus, VESV = Vesicular exanthema of swine, NV = Norovirus, Norwalk = prototype NV, EBHSV = European brown hare syndrome virus, RHDV = Rabbit haemorrhagic disease virus, SV = Sapovirus.

mouth disease (House & House, 1992), thus making the diseases difficult to distinguish. To prevent further spread of VES, infected pigs were slaughtered and pig food was heat treated. Such measures led to eradication of the disease in 1959 (USDA, 1959).

It is likely that VESV originated from caliciviruses present in marine animals such as San Miguel Sea Lion Virus (SMSV) (Smith et al., 1973). Both viruses share similar physiochemical properties and SMSV produced a vesicular disease resembling VESV when administered to pigs (Breese & Dardiri, 1977, Smith et al., 1973). It was suggested that foodborne infection of pigs was likely as SMSV was also isolated from seal meat, which was used in animal feed (Sawyer et al., 1978). This evidence was supported further by serological studies of Californian sea lions, Northern fur seals and feral swine (Prato et al., 1974). Subsequently another serotype of SMSV was isolated from ocean fish, and a VES like infection was established in experimentally infected pigs (Smith et al., 1980). This study suggested fish could possibly act as a reservoir of SMSV either by spreading infection to pigs directly or via marine mammals.

1.1b) San Miguel sea lion virus.

In 1972 San Miguel sea lion virus (SMSV) was first isolated from sea lions during an outbreak of abortion off the coast of California (Smith et al., 1973, Studdert, 1978). However, there is no direct evidence to suggest that SMSV was associated with the outbreak of unexplained abortion (Smith et al., 1973). There are now 17 SMSV serotypes described and these have been designated SMSV 1-17 (Sawyer et al., 1978, Skilling et al., 1987, Smith et al., 1980, Smith et al., 1981).

As described earlier, SMSV can induce VES-like infection in domestic pigs. The relatedness of these two viruses was further shown by RNA hybridisation showing a high degree of sequence homology between the two genomes (Burroughs et al., 1978). A later study using phylogenetic analysis on non-structural genes also confirmed that the two viruses were closely related (Neill et al., 1995).

1.1c) Canine calicivirus.

The first canine calicivirus (CaCV) was isolated in 1979 from a domestic dog with glossitis (Evermann et al., 1981). Subsequently, other CaCV isolates became available from dogs with diarrhoea, enteric infections or vesicular genital disease (Crandell, 1988, Evermann et al., 1985, Sangabriel et al., 1996). Phylogenetic studies have recently been performed in order to establish the relationship of CaCV to other animal and human caliciviruses, and these studies suggest that two distinct caliciviruses may be isolated from dogs. It was shown that the Japanese strain designated CaCV no.48 isolated from a two month old dog in 1990 (Mochizuki et al., 1993), is most closely related to, but genetically distinct from, feline and San Miguel sea lion caliciviruses (Roerink et al., 1999a, Roerink et al., 1999b) (Fig. 1). Another strain, CaCV Sapporo/283, has been grouped phylogenetically with feline calicivirus (FCV) (Fig. 1), and this strain is also closely related to FCV antigenically (Hashimoto et al., 1999, Sangabriel et al., 1996). The significance of caliciviruses in canine disease remains to be fully determined.

1.1d) Mink calicivirus.

Cultivable mink caliciviruses (MCV) were first isolated from clinically normal mink, with a history of haemorrhagic pneumonia, and have been shown to be antigenically distinct from VESV, SMSV, and FCV (Evermann et al., 1983, Long et al., 1980). In addition, non-cultivable mink enteric caliciviruses (MEC) have also been detected in faeces from diarrhoeic mink and possessed typical calicivirus morphology (Guo et al., 2001). The same study compared the genetic relationships between MCV, MEC and other caliciviruses, and sequence analysis indicated that MCV are most closely related to vesiviruses and MEC to SLVs (see section 1.4).

1.1e) Feline calicivirus.

Feline calicivirus (FCV) is an important oral and respiratory pathogen of domestic cats (Gaskell & Dawson, 1998). FCV was first isolated in 1957 by Fastier during an attempt to identify the viral agent responsible for feline panleucopenia (Fastier, 1957). FCVs were originally classified as feline picornaviruses (Burki, 1965), but as mentioned earlier, FCV was later reclassified as a calicivirus (Almeida, 1968, Burroughs & Brown, 1974, Studdert et al., 1970, Zwillenberg, 1966).

FCV will be discussed in more detail in section 3.

1.2 Genus lagovirus.

The genus lagovirus is a major group of caliciviruses associated with acute hepatic and haemorrhagic diseases in their lagomorph hosts. They include rabbit haemorrhagic disease virus and European brown hare syndrome virus. They are both classified as closely related but are in distinct groups based upon phylogenetic studies of capsid gene sequences (Nowotny et al., 1997) (Fig. 1).

1.2a) Rabbit haemorrhagic disease virus (RHDV).

The first reports of rabbit haemorrhagic disease (RHD) were in China in 1984 (Liu et al., 1984). Initially it was suggested that the virus involved in this syndrome was either a parvovirus (Gregg & House, 1989), or a picornavirus (Xu & Chen, 1989). However, in 1990 the new virus, now known as RHDV, was characterised as a calicivirus after typical classical caliciviral morphology was demonstrated (Ohlinger et al., 1990, Parra & Prieto, 1990). Characteristic signs of RHDV infection include haemorrhages and necrotic hepatitis and the disease has a high mortality rate in adult rabbits (Liu et al., 1984, Meyers et al., 1991b).

In addition, a non-pathogenic strain of rabbit calicivirus has also been identified. Rabbits in Europe have tested seropositive for RHDV infection but no typical signs of disease have been observed (Capucci et al., 1996). This non-pathogenic strain has been termed rabbit calicivirus (RCV) to differentiate it from other pathogenic RHDV strains.

RHDV has spread quite rapidly across Asia and Europe and first appeared in the United Kingdom in 1992 (Fuller et al., 1993). In 1995, the virus was accidentally introduced into Australia (Cooke, 1997, Tribe, 1995), during an experimental trial for assessment as a potential biological control agent of large rabbit populations (Robinson & Westbury, 1997). The virus spread rapidly throughout southern Australia and in 1997 cases of RHD were also reported in New Zealand (Ministry of Agriculture, 1997). In Australia, this pathogenic virus is also named RCV indicating the potential for nomenclature confusion with the non-pathogenic strain.

1.2b) European brown hare syndrome virus.

European brown hare syndrome virus (EBHSV) was first reported in Sweden in 1980 (Gavier-Widen & Morner, 1991), and has spread throughout several European countries including the U.K (Chasey & Duff, 1990). Like RHDV, the disease causes severe necrotic hepatitis and has a high mortality rate (Moussa et al., 1992). Although there are many similarities between EBHSV and RHDV (Capucci et al., 1991, Chasey et al., 1992, Moussa et al., 1992, Wirblich et al., 1994), each virus is host specific as EBHSV can only cause disease in hares and RHDV only in rabbits.

1.3 Human caliciviruses.

Human caliciviruses (HuCVs) were first reported in the early 1970s and are associated with outbreaks of diarrhoea (Cubitt et al., 1987, Kapikian, 1996, Kaplan et al., 1982). Due to the inability to propagate these viruses, earlier classification was based on morphological differences. This resulted in two groups: small round-structured

viruses (SRSVs) and 'classic human caliciviruses' (classic HuCVs) (Caul & Appleton, 1982). They were later genetically reclassified into the two genera: norovirus, which tend to have typical SRSV morphology, and sapovirus, which have typical HuCV morphology (Berke & Matson, 2000, Green et al., 2000, Pringle, 1998). Recent advances in molecular cloning, sequencing and antigenic characteristics of these viruses has enabled clearer relationships to be determined within these groups and confirms considerable differences between noroviruses and sapoviruses (Jiang, 1996, Lambden et al., 1994, Liu et al., 1995, Numata et al., 1997).

1.3a) Genus norovirus.

Noroviruses are an important cause of epidemic gastroenteritis in adults (Kapikian, 1996) (Fankhauser et al., 1998, Parashar et al., 1998, Vinje et al., 1997). The prototype virus, Norwalk virus (NV) was first discovered in 1972 from a patient involved in an outbreak of winter vomiting disease (Kapikian et al., 1972). Initially this virus was thought to be a parvovirus but molecular studies demonstrated that a single major capsid protein was present (Greenberg et al., 1981) and the virus was reclassified as a member of the *Caliciviridae.*

Phylogenetic analysis of capsid sequence has enabled noroviruses to be subdivided into three genogroups (Ando et al., 2000) (Fig. 1). Genogroup I contains at least seven genotypes, and there are at least five in genogroup II. The third genogroup contains the bovine noroviruses (Ando et al., 2000, Bridger & Dastjerdi, 1997, Oliver et al., 2003). Enteric caliciviruses have also been isolated from pigs and have been found to be genetically related to genogroup II noroviruses (Sugieda et al., 1998, van Der Poel

et al., 2000). It has been suggested in a recent study that the number of norovirus genotypes may increase as more isolates are characterised (Ando et al., 2000, Green et al., 2000). The use of sensitive molecular techniques such as RT-PCR is increasingly leading to a fuller understanding of the epidemiology of noroviruses (Glass et al., 2000). Most recently, a norovirus has been identified in mice that appears to represent a new genogroup (Karst et al., 2003).

1.3b) Genus sapovirus.

Sapoviruses were first described in 1976 from an outbreak of human disease in Glasgow (Madeley & Cosgrove, 1976) and are associated mainly with acute gastroenteritis in infants, young children or the elderly. The sapoviruses possess typical surface calicivirus morphology distinct from that of noroviruses (Jiang et al., 1997, Liu et al., 1995, Numata et al., 1997).

Sapoviruses are divided into at least three genotypes, which are based on sequence analysis and also partly based on the antigenic properties of the viruses (Berke et al., 1997, Noel et al., 1997) (Jiang et al., 1997). Phylogenetically, sapoviruses are shown to be more closely related to some animal caliciviruses than to noroviruses (Matson et al., 1995). In recent studies, caliciviruses detected in mink with diarrhoea (Guo et al., 2001) (see section 1.1d) and in pigs (Guo et al., 1999) were shown by phylogenetic analysis to be related to sapoviruses.

1.4 Other caliciviruses.

Caliciviruses have been isolated from a number of species other than domestic animals and humans (Table 1). In many cases classification has not been confirmed and therefore these viruses are known as potential caliciviruses.

VIRUS	REFERENCE
Primate calicivirus	(Smith et al., 1985a, Smith et al., 1985b, Smith et al., 1983a)
Cetacean calicivirus	(Smith et al., 1983b)
Skunk calicivirus	(Seal et al., 1995)
Amphibian calicivirus	(Smith et al., 1986)
Insect calicivirus	(Hillman et al., 1982)
Avian caliciviruses	(Gough et al., 1997, Gough et al., 1992, Gough & Spackman, 1981, Sironi, 1994, Wyeth et al., 1981)
Reptile calicivirus	(Smith et al., 1986)

Table 1. Additional putative caliciviruses isolated from some other species.

Section 2.

2. Properties of the *Caliciviridae.*

2.1 Capsid structure and morphology of caliciviruses.

Caliciviruses typically have a distinctive morphology of the capsid surface which led to the suggestion that they should be classified as a separate family (Burki, 1965, Zwillenberg, 1966). Negative staining electron microscopy (EM) has shown 32 characteristic cup-like depressions on the surface of caliciviruses. Following this observation the name of the virus was determined (calyx is the Latin word meaning cup or chalice).

Studies on calicivirus capsid structure were performed on a primate calicivius (Prasad & Matson, 1994) and recombinant Norwalk virus-like particles (rNVs) (Prasad et al., 1994) by cryo-electron microscopy. The capsid of both caliciviruses was composed of 90 capsomeres arranged in a T=3 icosahedral symmetry, as previously proposed based on the molecular weight of the capsid protein (Almeida, 1968, Burroughs & Brown, 1978). This structure resulted in the formation of the 32 depressions at the 5- and 3-fold axes of the icosahedron (Prasad & Matson, 1994). Recently, using X-ray crystallography, a computerised model for the 3D structure of Norwalk virus has been generated (Prasad et al., 1999) (Fig. 2).

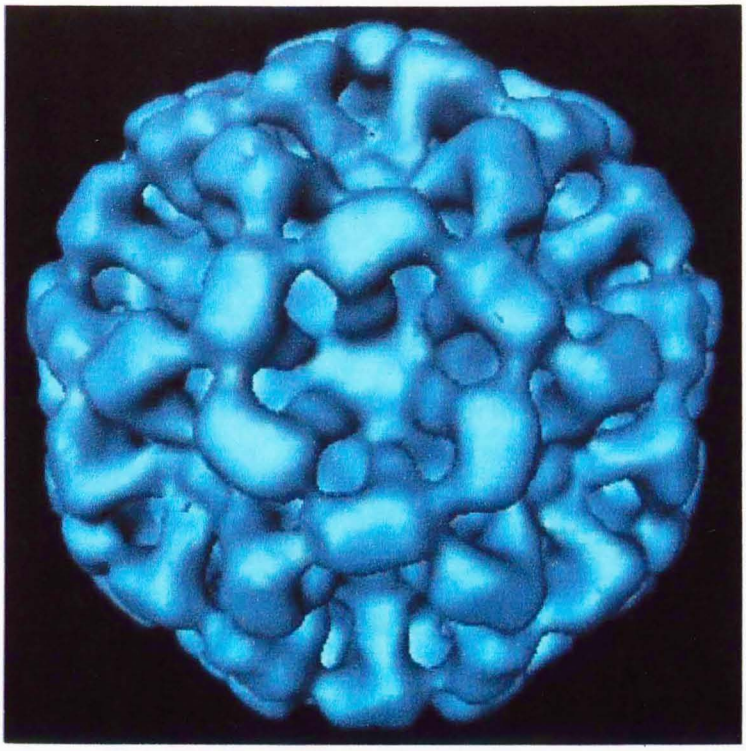


Fig.2

Fig. 2. The three-dimensional structure of Norwalk Virus Capsid taken by X-ray crystallography (Prasad et al., 1999).

2.2 Genome structure and organisation .

Calicivirus genomes are usually between 7.2 and 7.7 kilobases in length (Carter et al., 1992a, Meyers et al., 1991b). They are single stranded positive sense RNA genomes (Bachrach & Hess, 1973), which are polyadenylated at the 3`end (Black et al., 1978, Ehresmann & Schaffer, 1977). The genomes of RHDV, SMSV, VESV, Pan-1 (a primate calicivirus) and FCV have been shown to be covalently bound to a small, 10-15kDa protein termed VPg, at the 5`end (Burroughs & Brown, 1978, Dunham et al., 1998, Herbert et al., 1997, Meyers et al., 1991a, Schaffer et al., 1980, Wimmer, 1982) (Fig. 3). The VPg is required for efficient translation, as its removal by protease digestion eliminates infectivity (Burroughs & Brown, 1978). Further studies have demonstrated that when FCV genome is transcribed *in vitro*, infectivity is dependent on the presence of a 5` end cap structure, which apparently serves as a substitute for the VPg (Sosnovtsev & Green, 1995, Thumfart & Meyers, 2002). These results suggest that the VPg reflects an important function with regard to translation initiation. In addition, further indications for such a function have also been demonstrated by *in vitro* translation studies which showed decreased translatability of FCV RNA after removal of VPg (Herbert et al., 1997).

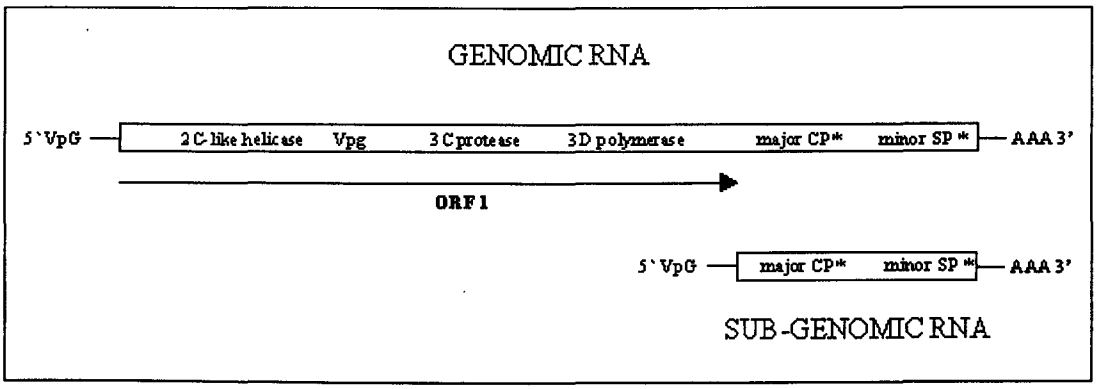


Fig. 3

Fig. 3. Diagrammatic representation of the *Caliciviridae* genome showing genomic and sub-genomic RNAs which are both 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 picornaviruses (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. *SP =structural protein.

There are two main types of genome organisation in the family *Caliciviridae.* The four calicivirus genera can be divided into those which contain three ORFs (genera vesivirus and norovirus) (Fig. 4a), or those which contain two ORFs (genera sapoviruses and lagoviruses) (Fig. 4b). For vesiviruses including FCV and SMSV, which have three separate ORFs (Fig. 4a), ORF 1 encodes the non-structural proteins, ORF 2 the capsid precursor and ORF 3 a small minor structural protein. In FCV, ORFs 1 and 2 are separated by 2 nucleotides (Carter et al., 1992b, Glenn et al., 1997, Neill et al., 1991, Tohya et al., 1991b), in SMSV they are separated by 5 nucleotides (Neill et al., 1995), and in CaCV by 3 nucleotides (Roerink et al., 1999b). In FCV, SMSV, and CaCV the stop codon of ORF 2 and initiation codon of ORF 3 overlap by 1 nucleotide, resulting in a –1 frameshift (Carter et al., 1992b, Glenn et al., 1999, Neill et al., 1991, Neill & Seal, 1995, Roerink et al., 1999b, Tohya et al., 1991b).

Noroviruses also have a genome organisation with three ORFs that are encoded like the vesiviruses (Fig. 4a). In Norwalk and Southampton viruses from genogroup I, ORFs 1 and 2 overlap by 14 nucleotides whereas Lordsdale and Camberwell viruses in genogroup II overlap by 17 nucleotides, resulting in a –2 frameshift (Cauchi et al., 1996, Dingle et al., 1995, Jiang et al., 1993, Lambden et al., 1993). As in vesiviruses, ORFs 2 and 3 overlap by 1 nucleotide and there is also a –1 frameshift.

The second main genome arrangement is found in the sapoviruses and lagoviruses, which encode 2 ORFs (Fig. 4b). ORF 1 of the sapoviruses encodes both the non-structural and major capsid proteins, equivalent to ORFs 1 and 2 in the vesiviruses

Fig.4a; Basic Genome Type 1 with three separate ORFs. Genera vesivirus e.g. (FCV and SMSV), and norovirus e.g. (NV and Lordsdale virus).

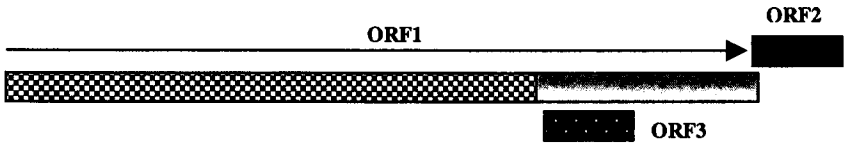
FCV & SMSV

ORF1 and ORF2 are separated by 2 (FCV) and 5 (SMSV) nucleotides.
ORF3 overlaps ORF2 by 1 nucleotide in both FCV and SMSV.

NV & Lordsdale virus

ORF1 and ORF2 overlap by 14 (NV) and 17 (Lordsdale) nucleotides.
ORF2 overlaps ORF3 by 1 nucleotide in both NV and Lordsdale.

Fig. 4b; Basic Genome Type 2 with two separate open reading frames. Genera sapovirus e.g. (HuCV) and lagovirus e.g (RHDV & EBHSV).



classic HuCV

ORF1 contains both the non-structural and major capsid proteins. ORF2 overlaps ORF1 by 1 nucleotide. A third potential ORF3 overlaps the capsid gene region of ORF1.

RHDV & EBHSV

ORF1 contains both the non-structural and major capsid proteins.
ORF2 overlaps ORF1 by 5 (RHDV) and 8 (EBHSV) nucleotides.

Figs. 4a and **4b**. Genome organisation amongst the caliciviruses. The different regions of the genome are;

- Non-structural proteins
- Major capsid protein
- Minor structural
- Potential ORF3

and noroviruses, whilst ORF 2 encodes a small basic protein which probably has a similar function to ORF 3 in noroviruses and vesiviruses (Liu et al., 1995). There is no stop codon between the non-structural and major capsid protein regions as in the other genera. The ORF 2 start codon overlaps ORF 1 stop codon by 1 nucleotide and is frameshifted –1. In addition, sequence analysis has suggested that there may be a third potential ORF overlapping the capsid region of ORF 1 in the strains Manchester virus, Houston/86 and Houston/90 viruses. This ORF has been predicted to encode a small basic protein of unknown function, but to date no confirmation of this has been demonstrated (Jiang et al., 1997, Liu et al., 1995).

As mentioned above, lagoviruses also have 2 ORFs, with ORF 1 encoding the non-structural and major capsid proteins (Capucci et al., 1996, Gould et al., 1997, Le Gall et al., 1996, Meyers et al., 1991b, Rasschaert et al., 1994) (Fig. 4b). The second ORF, ORF 2, encodes a minor structural protein that overlaps ORF 1 by 5 nucleotides in RHDV (Gould et al., 1997, Meyers et al., 1991b, Rasschaert et al., 1994) and 8 nucleotides in EBHSV (Le Gall et al., 1996) creating a –2 frameshift

2.3 RNA.

Earlier studies on caliciviruses suggested that several different RNA molecules were expressed in infected cells, and that these included a full length RNA together with a small abundant sub-genomic RNA (Black et al., 1978, Ehresmann & Schaffer, 1977). However, other studies gave conflicting data for the number and size of FCV-specific transcripts (Carter, 1990, Neill & Mengeling, 1988). It has now been demonstrated that only two populations of RNA molecules exist, one corresponding to the genome

and one to a sub-genomic RNA (Herbert et al., 1996, Meyers et al., 1991a). Sequence comparisons of the sub-genomic mRNA for FCV strain F9 (Herbert et al., 1996) and another FCV strain CFI 68 (Neill et al., 1991) have shown that the 5`end of the sub-genomic mRNA maps to nucleotides 5296-5297 of the full length genomic RNA (Carter et al., 1992a).

The sub-genomic RNA has a genome-linked protein (VPg) at its 5`end like the full length RNA molecule (Fig.3) (Herbert et al., 1997, Meyers et al., 1991a). It has been shown for FCV that the major capsid protein is initially translated from the sub-genomic RNA (Herbert et al., 1996, Neill et al., 1991) along with the small basic protein encoded in ORF 3 (Herbert et al., 1996).

Apart from the sapoviruses, the 5`end of most published full-length calicivirus genomes and sub-genomic mRNA all share similar conserved regions (Carter et al., 1992a, Clarke & Lambden, 1997, Glenn et al., 1999, Hardy & Estes, 1996, Meyers et al., 1991b). It has been suggested that these regions may have a regulatory role such as packaging, replication or translation of the viral genome and the sub-genomic mRNA (Lambden & Clarke, 1995). These regions are predicted to form secondary structures and are discussed further in manuscript 3.

2.4 Proteins encoded by caliciviruses.

2.4 a) The non- structural proteins.

Caliciviruses encode conserved non-structural proteins in the large ORF at the 5`end of the genome including a 2C-like helicase, 3C-like protease and 3D-like RNA-dependent RNA polymerase (Fig. 3). These non-structural proteins are similar to those of picornaviruses based on conserved amino acid domains (Neill, 1990), and are released by specific viral protease activity during a proteolytic cascade (Spall et al., 1997). The location of the protein coding regions is conserved in ORF 1 with the 2C region positioned towards the N terminus, followed by the VPg, protease and the 3D polymerase region at the 3`end (Dingle et al., 1995, Dunham et al., 1998, Guo et al., 1999, Liu et al., 1995, Meyers et al., 1991b, Neill, 1990, Seah et al., 1999, Seal et al., 1995, Sosnovtsev & Green, 2000, Wirblich et al., 1996). A number of studies on the functions of these proteins have been performed and are described in the following sections.

2.4 a)(i) 2C like helicase protein.

Sequences from ORF 1 suggests a protein with similar function to the 2C helicase of the picornaviruses is encoded in most caliciviruses (Liu et al., 1995, Meyers et al., 1991b, Neill, 1990, Neill et al., 1995). The 2C protein of picornaviruses is believed to be involved in RNA synthesis (Mirzayan & Wimmer, 1994), and is a putative helicase (Mirzayan & Wimmer, 1994, Paul et al., 1994). In the 2C protein of polioviruses, ATPase and GTPase activities have also been observed (Rodriguez & Carrasco,

1993). The identity of a motif thought to be responsible for ATP binding has also been detected in the 2C-like helicase of caliciviruses (Dingle et al., 1995, Meyers et al., 1991a, Neill, 1990, Pfister & Wimmer, 2001).

2.4 a)(ii) 3C-like protease.

The 3C-like protease encoded in ORF 1 is responsible for cleavage of the polyprotein to release the VPg, 3C and 3D non-structural proteins. The 3C-like protease of RHDV has been studied in some detail by expression in E-coli, and by *in vitro* translation in rabbit reticulocyte cell-free lysates and is perhaps the best characterised calicivirus protease (Boniotti et al., 1994, Martín Alonso et al., 1996, Wirblich et al., 1995). The protease specifically cleaves the major capsid protein from the polyprotein encoded by the long ORF 1 (Wirblich et al., 1995, Wirblich et al., 1996). In vesiviruses such as FCV and SMSV, the protease also cleaves the major capsid precursor molecule to release the mature capsid protein (Carter, 1989, Carter et al., 1992b, Fretz & Schaffer, 1978, Komolafe et al., 1980, Shin et al., 1993, Sosnovtsev et al., 1998). A summary of polyprotein cleavage for FCV, RHDV and NLV Southhampton virus is shown in (Fig. 5).

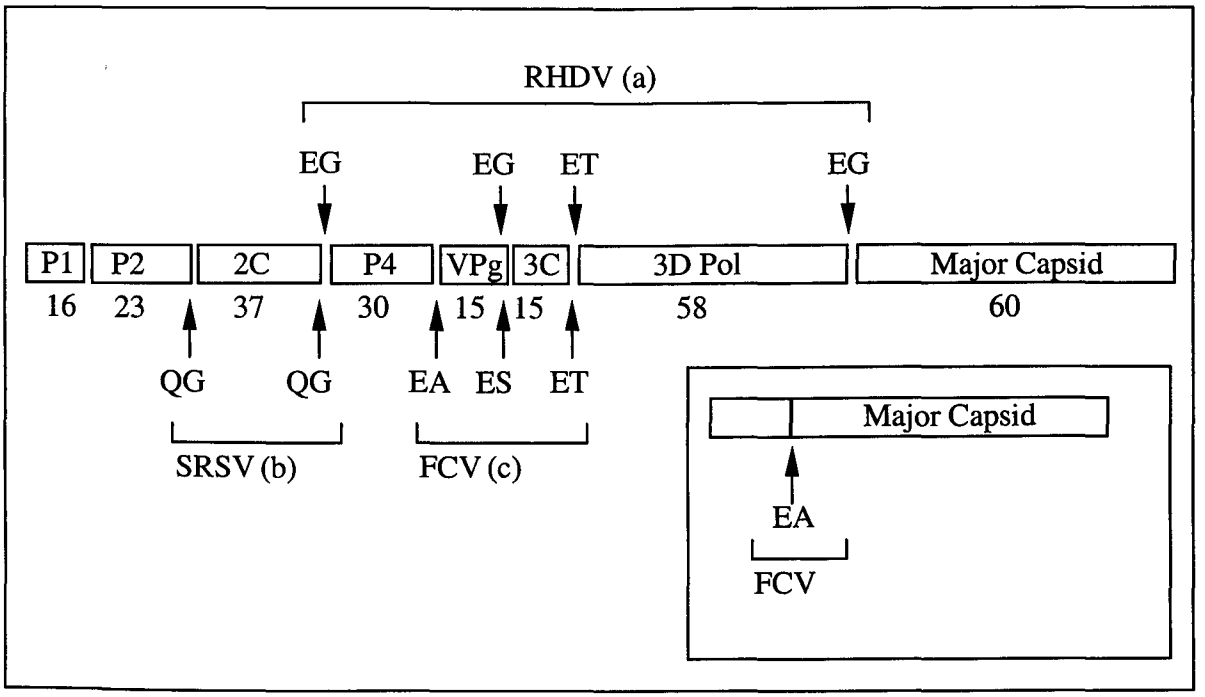


Fig. 5

Fig.5. Summary of polyprotein cleavage in the caliciviruses Adapted from (Clarke & Lambden, 1997).

The primary cleavage products are released by 3C protease activity during polyprotein processing of (a) RHDV polyprotein. The known cleavage sites are shown as dipeptides above the protein products with the estimated molecular masses (kDa) below.

The known cleavage sites for (b), Southampton SRSV, and (c), FCV are shown for comparison at the bottom of the figure.

Inset; cleavage site of the major capsid protein precursor in FCV.

2.4 a)(iii) 3D RNA-dependent RNA polymerase.

There are several conserved motifs present in the putative 3D polymerase of caliciviruses including KDEL, GLPSG, YGDD and FLKR (Green et al., 1994, Liu et al., 1995, Meyers et al., 1991b, Neill, 1990, Neill et al., 1995, Noel et al., 1997, Oshikamo et al., 1994, Tohya et al., 1991b). These motifs are also conserved in RNA polymerases of other plant, animal and bacterial viruses (Kamer & Argos, 1984). Another study has demonstrated that the YGDD motif is required for RNA polymerase function in RHDV (Lopez Vazquez et al., 2001, Vázquez et al., 1998).

2.4 b)(i) Major structural protein.

Caliciviruses possess a single major capsid protein ranging in size from 58-76kD (Clarke and Lambden 1997). In vesiviruses such as FCV and SMSV the capsid protein is produced as a precursor protein (Carter, 1989, Carter et al., 1992b, Fretz & Schaffer, 1978, Komolafe et al., 1980, Shin et al., 1993, Sosnovtsev et al., 1998). In contrast, in lagoviruses, the capsid seems to be expressed as a mature protein (Martín Alonso et al., 1996, Wirblich et al., 1995, Wirblich et al., 1996). For FCV and other vesiviruses such as SMSV, the mature capsid protein can be divided into six regions (A-F) based on sequence variability and conservation, demonstrated both between and within calicivirus species (Neill, 1992, Seal & Neill, 1994, Seal et al., 1993) (Fig. 6).

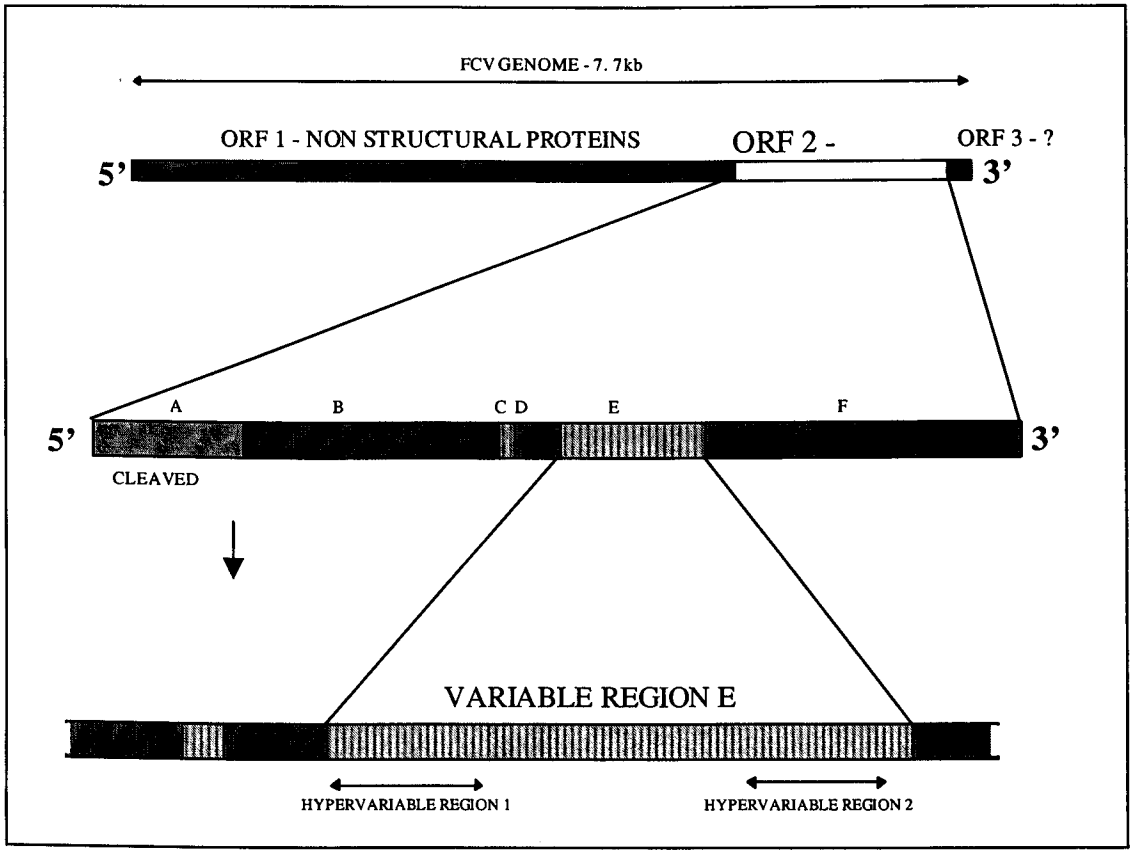


Fig. 6

Fig.6. Capsid variability of FCV. ORF 2 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 (Glenn, 1997, Neill, 1992, Seal, 1994, Seal et al., 1993).

Region A of FCV, and of SMSV, is the part of the capsid precursor that is cleaved to generate the mature capsid (Carter et al., 1992b, Fretz & Schaffer, 1978, Rinehart-Kim et al., 1999, Sosnovtsev et al., 1998). In FCV this region is cleaved by the 3C-like protease during viral replication at the conserved E-124 A-125 cleavage site (Carter et al., 1992b, Shin et al., 1993, Sosnovtsev et al., 1998) (Fig.5). Sequence comparisons describe region A as having intermediate sequence variability between different FCV isolates (Glenn et al., 1997).

Region B of the capsid is conserved between different members of the *Caliciviridae*. In addition there is also considerable homology between this region and that found in the picornavirus VP3 structural protein (Tohya et al., 1991a), suggesting possible similarities in structure and function (Neill, 1992, Tohya et al., 1991a). This VP3 protein in picornavirus folds into a ß-barrel structure (Acharya et al., 1989, Arnold & Rossman, 1990, Hogle et al., 1985, Luo et al., 1997). A putative ATP/GTP binding site and a myristylation site have also been predicted to occur in this region although the significance is not known (Seal et al., 1993).

Region C shows considerable variation between all caliciviruses, whereas regions D and F are regions of sequence conservation within the FCVs and all other caliciviruses.

Region E exhibits a high degree of variability between all caliciviruses. In FCV this region is divided into a 5` and a 3` hypervariable (HV) sequence with a relatively conserved central region (Glenn, 1997, Radford et al., 1997, Radford et al., 1998, Radford et al., 1999, Seal & Neill, 1994, Seal et al., 1993). It has been suggested that

region E is the immunodominant region, as it has been shown that it contains both linear and conformational B-cell epitopes (Milton et al., 1992, Poulet et al., 2000b, Radford et al., 1999, Shin et al., 1993, Tohya et al., 1997).

Sequence analysis of the variable region E has been proved to be of considerable use for the molecular typing of FCV isolates of different clinical origin, and in epidemiological studies (Radford et al., 1997, Radford et al., 2003, Radford et al., 2000, Radford et al., 2001b, Radford et al., 1998, Sykes et al., 1998).

Capsid variability has also been demonstrated in noroviruses (such as Norwalk, Hawaii, Southampton, and Toronto viruses) and in RHDV. Sequence alignments from isolates of the noroviruses divide the capsid into three regions (Lew et al., 1994a, Lew et al., 1994b). Regions 1 and 3 show some degree of sequence conservation and are equivalent to FCV capsid regions B and F respectively. In contrast, region 2 is variable and in FCV is equivalent to the regions C D and E of the FCV capsid.

To date, the sequence of the capsid gene of RHDV has been shown to be highly conserved between different isolates, with only limited variability present in its central region and towards the N-terminus (Gould et al., 1997).

2.4 b)(ii) Minor structural protein.

The third open reading frame (ORF 3) of vesiviruses and noroviruses (analogous to ORF 2 of lagoviruses and sapoviruses), encodes a minor structural protein. The size, and amino acid composition of this protein is extensively variable between different members of the *Caliciviridae* (Clarke & Lambden, 2000). This protein has been detected in FCV infected cells (Herbert et al., 1996), and more recently in purified virions of FCV (Sosnovtsev & Green, 2000). It has also been detected in purified virions of RHDV (Wirblich et al., 1996), in RHDV-infected primary hepatocytes (Konig et al., 1998), and in purified Norwalk virus virions (Glass et al., 2000). It has been predicted that this protein may interact with both the capsid and the viral RNA to encapsidate the viral genome (Wirblich et al., 1996). This view was further supported during an X-ray crystallographic study of the capsid structure (Prasad et al., 1999). Although the ORF 3 protein was not detected, the inner surface of the capsid was predicted to be acidic, complementing the basic properties of the ORF 3 encoding protein. A recent study has shown for Norwalk virus that the ORF 3 encoded protein is a minor structural protein associated with NV virions (Glass et al., 2000).

Although one study has demonstrated that the sub-genomic mRNA of FCV can direct synthesis of both ORF 2 and ORF 3 gene products *in vitro*, the translation mechanism of the minor structural protein has yet to be determined (Herbert et al., 1996). Four possible mechanisms have been suggested Neil *et al* (1992) proposed that the ORF 3 protein of FCV was expressed by a –1 frameshift in a way similar to that observed for retroviruses and coronaviruses (Brierley, 1995). This would result in a fusion protein containing the ORF 2 and ORF 3 gene products, but such a protein has not been

detected in infected cells (Herbert et al., 1996). In addition, this mechanism requires a region of RNA secondary structure, which is predicted to be present in FCV (Neill et al., 1991), but not in Norwalk virus RNA (Jiang, 1990).

A second proposed mechanism for ORF 3 translation was 'leaky scanning' (Kozak, 1989), in which the ORF 3 gene product would be translated by a ribosome if its start codon was in a more ideal context relative to those upstream from it. However this is unlikely to occur in the caliciviruses as the ORF 3 initiation codon of FCV is considered to be in a poor context relative to upstream start codons in more favourable contexts (Herbert et al., 1996).

The third mechanism suggests that ORF 3 may be translated by means of an internal ribosome entry site (IRES), as observed in other RNA viruses including the picornaviruses (Jackson et al., 1990). The IRES is associated with complex secondary structures that have not been predicted in the caliciviruses.

Finally the fourth suggested mechanism is due to the close context of the ORF 2 termination codon and the ORF 3 initiation codon, which is conserved amongst all the caliciviruses. This may lead to re-initiation and translation of ORF 3. This has been demonstrated in other bicistronic mRNAs (Kozak, 1987, Kozak, 1992).

Section 3.

3. Feline calicivirus (FCV).

As mentioned briefly in section 1, FCV is a major cause of upper respiratory tract disease of domestic cats (Gaskell & Dawson, 1998). This section describes the important clinical and epidemiological features of the disease, including the antigenic and genetic relationships of the virus, and control measures undertaken by vaccination.

3.1 Clinical disease.

FCV mainly infects the oral mucosa and upper respiratory tract, (Gaskell & Dawson, 1998), with ulcer formation, particularly on the tongue, being a prominent feature. Other clinical signs associated with infection include pyrexia, nasal and ocular discharges, sneezing, conjunctivitis and anorexia (Gaskell & Dawson, 1998, Hoover & Kahn, 1975, Kahn & Gillespie, 1971, Knowles et al., 1991, Ormerod et al., 1979, Povey, 1974, Reubel et al., 1992, Wardley & Povey, 1977b).

Although the disease is usually mild, a wide spectrum of severity has been observed from asymptomatic infection (Fastier, 1957, Povey, 1974, Radford et al., 2001a) to fatal pneumonia in kittens (Kahn & Gillespie, 1971, Poulet et al., 2000a, Povey, 1974). However, it is possible that the use of aerosol challenge in experimental infections may have over-emphasised the importance of severe diseases such as pneumonia, in that natural infection tends to be by direct contact with infectious

secretions (Kahn & Walton, 1971, Ormerod et al., 1979). Indeed an early study demonstrated that aerosols are not likely to be important in natural FCV transmission probably because the tidal volume of cats is too low to generate a significant aerosol (Wardley & Povey, 1977a).

Some isolates of FCV can cause an acute febrile lameness syndrome (Bennett et al., 1989, Church, 1989, Crandell & Madin, 1960, Dawson et al., 1994, Pedersen et al., 1983, Studdert et al., 1970, TerWee et al., 1997), often accompanied by pyrexia, and with or without respiratory disease. This lameness has been associated with certain field isolates (Dawson et al., 1994) and in some cases with the use of live attenuated vaccines, typically in young kittens following a first vaccination (Dawson et al., 1993a). Sequence analysis of the hypervariable region of the capsid gene of isolates obtained from such animals suggests that the lameness may be due to the vaccine virus in some cases, although the majority are due to coincidental infection with field viruses (Dawson et al., 1993a, Dawson et al., 1993b, Radford et al., 1997, Radford et al., 2000).

Other signs of FCV infection have been reported and include an association with chronic stomatitis and gingivitis (Gaskell & Dawson, 1998, Harbour et al., 1991, Knowles et al., 1989, Thompson et al., 1984). Studies have shown that 70-85 % of cats with chronic stomatitis were shedding FCV, compared to 0-19% of control cats. However, other studies have not found such a clear cut association (Tenorio et al., 1991). Attempts to induce chronic stomatitis in cats using FCV isolates from clinical cases of chronic stomatitis have been unsuccessful (Dawson, 1991, Knowles et al., 1991, Poulet et al., 2000a). It is likely that other factors, such as infection with feline

immunodeficiency, and genetics of the feline host are involved in the aetiology of this disease (Hosie et al., 1989, Knowles et al., 1989, Tenorio et al., 1991, Waters et al., 1993).

FCV has also been isolated from cats with other syndromes including jaundice and abortion (Ellis, 1981), and sudden death in kittens (Love & Baker, 1972). In addition, an association between FCV and feline urological syndrome has also been reported (Fabricant & Rich, 1971, Kruger & Osborne, 1993, Rice et al., 2002), but this has not been supported by other studies (Gaskell et al., 1979). Recently, FCV has also been associated with a severe systemic haemorrhagic disease in cats in California (Pedersen et al., 2000). The disease appeared to target blood vessels, as evidenced by the severe oedema in subcutaneous tissues and lungs, and local necrosis of skin and adipose tissues. This new isolate of calicivirus from infected cats, named FCV-Ari, has weak to negligible cross-reactivity to current vaccine-induced immunity and would therefore pose a considerable risk to cats if it were to spread widely among either vaccinated or unvaccinated animals (Pedersen et al., 2000).

The mechanisms by which FCV induces disease are explored using *in vitro* cell culture systems in manuscript 2.

3.2 FCV Epidemiology.

FCV is highly infectious in the domestic cat population, and is usually transmitted in the acute phase of the disease, by direct contact with infected cats mainly from oropharyngeal, nasal and conjunctival secretions. Infection can also be spread indirectly by fomites as FCV can remain viable in the environment for up to ten days if wet, and eight days if dry (Povey & Johnson, 1970). In addition, macrodroplets produced by sneezing can spread virus over a distance of approximately four feet. Virus has also been isolated from urine and faeces, but the epidemiological importance of this is unclear (Povey & Hale, 1974, Povey & Johnson, 1970).

Despite its infectiousness, careful hygiene standards can prevent the spread of FCV (Gaskell & Dawson, 1998, Wardley & Povey, 1977a). Results of a recent study in a cattery where hygiene measures were being implemented showed that despite 25-30% of cats being FCV positive, only one potential transmission event was detected (Radford et al., 2001a).

Following recovery from the acute disease, a proportion of cats become carriers and continue to shed FCV from their oropharynx (Povey et al., 1973, Wardley, 1976). Such carriers have been defined as those cats that shed FCV for a minimum period of 30 days post infection. Although the majority of cats eventually eliminate the virus, some cats have been shown to shed virus for up to two years (Povey et al., 1973), and it is likely that such animals remain carriers for life. FCV carriers have been described as high, medium or low level shedders depending on the viral titre isolated from their oropharynx (Wardley, 1976). Carriers are widespread within the cat

population (Coutts et al., 1994, Wardley, 1974). Figures for the prevalence of FCV before and following vaccination in the UK in various studies are shown in Table 2. Essentially FCV prevalence is still high despite vaccination, ranging from 8-42% depending on the management situation.

Population	% FCV positive	References
Household cats	8*	(Wardley et al., 1974)
Show cats	24* 25	(Wardley et al., 1974) (Coutts et al., 1994)
Colony cats	42* 26-31	(Wardley et al., 1974) (Radford et al., 2001a)
General practice/hospital	19	(Knowles et al., 1989) (Harbour et al., 1991)

Table 2. The prevalence of FCV in the UK.

*Figures taken before the use of FCV vaccines.

The tonsil is considered to be the main site of FCV persistence (Dick et al., 1989, Povey & Hale, 1974, Povey et al., 1973, Wardley, 1974). However, tonsillectomy of carrier cats does not eliminate viral shedding (Wardley, 1974). In addition, the virus has been detected in other non-tonsillar tissues (Povey et al., 1973, Truyen et al., 1999, Wardley & Povey, 1977c). Taken together these studies suggest the tonsil is not necessary for viral persistence, and it is likely that FCV also persists in other as yet unidentified tissues in carrier cats.

The mechanism by which FCV persists within carrier cats is uncertain. It has been suggested that antigenic variation may allow FCV to escape from a neutralising humoral immune response during persistence (Johnson, 1992, Kreutz et al., 1998, Pedersen & Hawkins, 1995, Radford, 1998, Wardley, 1974). Antigenic differences have been detected between infecting and re-isolated virus during acute and persistent stages of infection (Poulet et al., 2000a, Radford, 1998). Sequence analysis of the immunogenic 5` hypervariable region of the FCV capsid has shown that FCV exists as a 'quasispecies' (Radford, 1998), and that the virus evolves at the nucleotide and amino acid level during persistence infection (Kreutz et al., 1998, Radford, 1998). This has led to the suggestion that during persistent infection, sequence changes in the immunogenic regions of the capsid are selected by the immune response and that these changes ultimately allow the virus to escape neutralisation (Radford, 1998).

3.3 FCV Phylogeny: antigenic and phylogenetic relationships.

There are a large number of different strains of FCV with varying antigenicity and pathogenicity (Dawson et al., 1993b, Kahn et al., 1975, Povey, 1974). All strains of FCV are considered to be part of a closely related group constituting a single serotype (Kalunda et al., 1975, Povey, 1974). Differences between strains allow differentiation between strains to varying degrees using either conventional virus neutralisation assays (Dawson et al., 1993a, Dawson et al., 1993b, Knowles et al., 1990, Lauritzen et al., 1997, Povey, 1974), or monoclonal antibody analysis (Geissler et al., 1997, McArdle et al., 1996). However, varying levels of cross-protection do occur between strains, and on this basis strains have been selected for vaccine use.

Phylogenetic studies of FCV show that their genetic relationship mirrors the serological findings. Sequence comparisons suggest that FCVs belong to a single but diverse genogroup, with no evidence for the existence of distinct subgroups or genotypes (Geissler et al., 1997, Glenn et al., 1999, Glenn, 1997, Horimoto et al., 2001) (Fig.7), although a potential Japanese specific genogroup has been reported (Sato et al., 2002). Such findings are largely based on capsid gene analysis, although limited data on ORF 1 and ORF 2 also supports these observations (Glenn, 1997, Sommerville, 2001). Capsid nucleotide and amino acid similarities were compared between epidemiologically unrelated FCV isolates, and ranged from 75-82% and 81-91% respectively (Glenn, 1997). The phylogenetic analysis of these sequences clearly show that FCVs isolated over a number of years group in a "star-like" cluster, despite their different geographical locations and clinical origins (Fig. 7). All isolates appear to be equally distant from one another, with the genetic diversity restricted to

within specific limits. This suggests that all FCV isolates that have been sequenced to date are of equal ecological fitness. Such an observation is consistent with rapid geographical movement of the virus through a globally mobile host population. The rate of virus movement would need to be quicker than any adaptive evolution to a given ecological location, and may be facilitated by clinically normal carriers that are responsible for maintaining a high prevalence of infection. However, it is also possible that FCV has not been in the cat population long enough for distinct clades to evolve (Glenn, 1997).

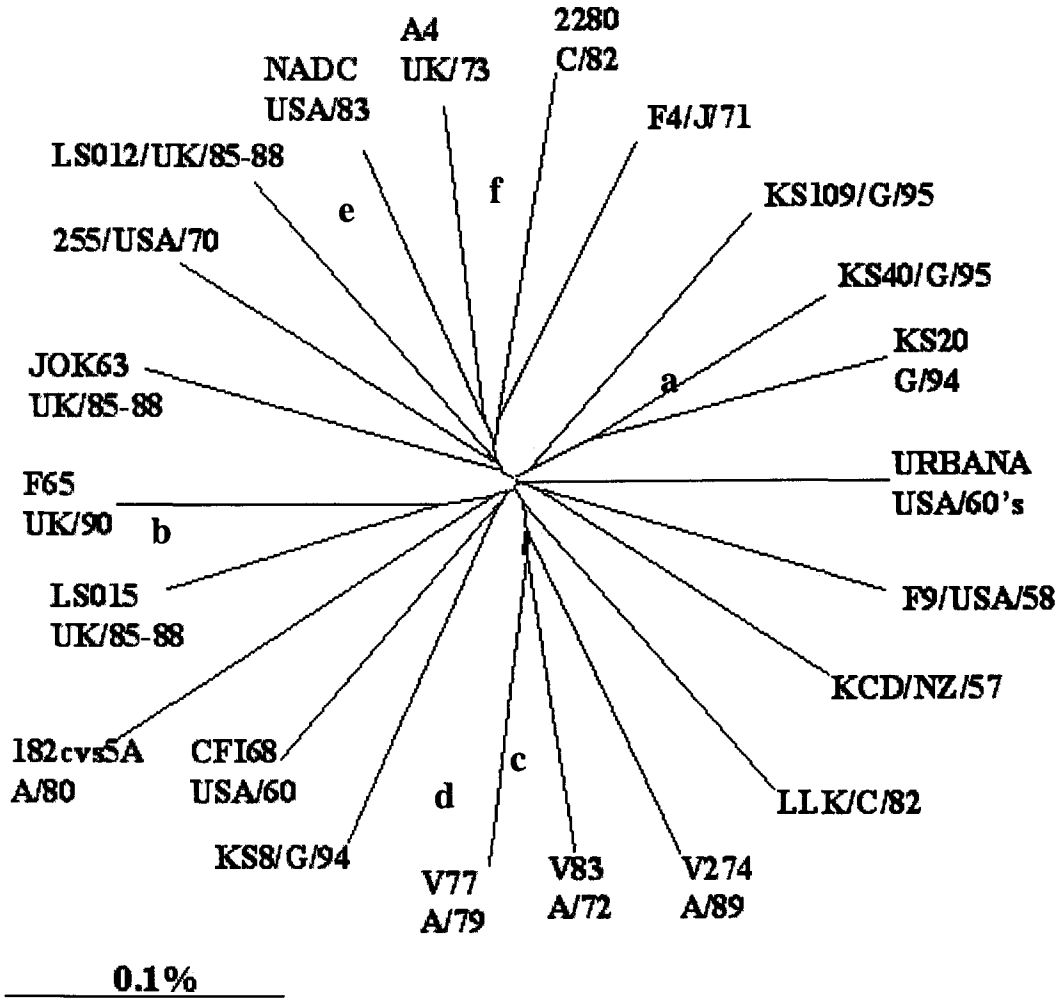


Fig.7. Phylogenetic construction of FCV using conserved capsid nucleotide sequences (Glenn 1997). The origins of each isolate are also indicated by a country letter and year, (A) Australia, (C) Canada, (G) Germany, (J) Japan. For clarity, significant bootstrap values (>70) are included at the relevant node, a=100, b=95, c=88, d=80, e=73, f=76. Evolutionary distances are to scale.

3.4 FCV Immunity.

Serological responses to FCV are usually measured using virus neutralisation assays, and it is thought that humoral immunity is the major route for virus neutralisation *in vivo*. However, cell-mediated immunity may also play a role in FCV neutralisation *in vivo* (Knowles et al., 1991, Povey & Ingersoll, 1975, Povey & Wilson, 1978). Lymphoblast proliferation and MHC-restricted cytotoxic T-cells have been detected in cats when given an inactivated FCV vaccine (Tham & Studdert, 1987), with sensitised lymphocytes persisting for up to six months. In addition FCV antigen has been demonstrated in macrophages in the joints and in alveoli *in vivo* (Bennett et al., 1989, Kahn & Gillespie, 1971). However, the significance of cell-mediated immunity to protection against disease and infection remains uncertain. Antibody classes IgA, IgG and IgM have also been detected in the saliva of FCV-infected cats (Knowles et al., 1991), suggesting local immunity may also have a role in protection.

3.5 FCV Vaccination.

Vaccination against FCV is widespread amongst the domestic cat population (Gaskell & Dawson, 1994). Several types of vaccines have been developed, including live attenuated intranasal and systemic vaccines, and inactivated systemic vaccines. Historically, FCV vaccines have been given as part of a multivalent vaccine with feline herpesvirus, and in many cases, parvovirus. More recently feline leukaemia virus and *Chlamydophila felis* have also been added to the same vaccines (Gaskell & Dawson, 1998).

FCV vaccines are generally effective in preventing FCV-associated diseases (Bittle & Rubic, 1976, Kahn & Hoover, 1976, Povey et al., 1980, Povey & Wilson, 1978, Radford et al., 2001a). However, there are several disadvantages in terms of the safety and efficacy of these vaccines. Firstly, in terms of safety, viral shedding may occur due to the incorrect administration of live attenuated vaccines for example, if the cat licks the injection site or if a subcutaneous vaccine is accidentally aerosolised at the time of administration (Orr et al., 1980, Povey, 1977). It has also been shown that even correctly administered vaccines can sometimes be shed from the oropharynx (Bennett et al., 1989, Pedersen & Hawkins, 1995). A recent study has suggested that vaccine virus may persist within a colony, possibly causing endemic disease (Radford et al., 2001a).

Secondly, in terms of efficacy, vaccinated cats are not protected against infection. Several studies have shown that cats may still become infected with FCV and shed virus after challenge (Dawson, 1991, Gaskell et al., 1982). Furthermore, most live attenuated vaccines have been based on a single FCV isolate designated F9 (Bittle & Rubic, 1976, Pedersen & Hawkins, 1995), although more recently, some vaccines have incorporated other FCV isolates (R.M.Gaskell, personal communication). Although FCV F9 strain and F9-like strains are considered to be widely cross protective, these vaccines do not clinically protect equally well against all field isolates (Dawson et al., 1993b, Harbour et al., 1991, Pedersen & Hawkins, 1995, Radford et al., 2001a). In addition, there is increasing evidence that the proportion of isolates that current vaccines protect against may be decreasing (Lauritzen et al., 1997). These results emphasise the importance of strain selection for FCV vaccines, and the monitoring of field isolates over time.

Due to the problems associated with these conventional vaccines, a number of alternative experimental vaccines have been developed. These include protein subunit vaccines (Komolafe & Jarrett, 1985), virus-like particles (DeSilver et al., 1997), recombinant feline herpesvirus (FHV) expressing an FCV capsid protein (Yokoyama, 1998, Yokoyama et al., 1996a, Yokoyama et al., 1996b), and nucleic acid vaccines (Sommerville et al., 2002). However, most of these vaccines showed only partial protection and none is yet available commercially.

It is possible that some of these vaccination strategies together with further research may improve the control of FCV. However, modelling studies have suggested that eradication of infection in most cases seems unlikely (Reade et al., 1998), unless improved vaccines are introduced which protect against infection.

The possible utility of a peptide vaccine against a conserved linear epitope in the FCV capsid protein is discussed further in manuscript 1.

Aims of this thesis.

It is clear from the introduction that there is only limited knowledge on the more fundamental aspects of FCV replication and host-cell virus interactions with respect to how such aspects relate to pathogenesis. Therefore the aims of this thesis were to increase our understanding in some of these areas. More specifically the aims were:

- to determine the antigenic cross-reactivity of antibodies raised to a conserved region of the capsid gene, and to determine their neutralising ability with a view to the development of improved vaccines.

- to investigate whether the major mechanism of cell death in infection with FCV *in vitro* is associated with the induction of apoptosis.

- to analyse conserved sequences of secondary structure partially shared by the 5` end of both genomic and sub-genomic RNA that may be important for the replication of the viral RNA.

- to amplify the full length FCV genome by one-step long PCR and attempt to generate an infectious clone.

- to attempt to investigate the specific interaction between FCV and its cellular receptor using Western blotting. As FCV replicates almost exclusively in feline cells, this suggests that the FCV receptor is a cell surface molecule specific to this host species.

MANUSCRIPT 1

The capsid gene of feline calicivirus (FCV) contains a linear B-Cell epitope in a conserved region that induces an antibody response in rabbits, and is broadly cross-reactive amongst FCV isolates.

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ABSTRACT.

Linear B-cell epitopes have been previously reported in regions of the FCV capsid that are considered to be conserved on the basis of sequence analysis. One of these epitopes, termed antigenic site 4 (ags 4), is located between amino acids 475-479. This study has investigated the antigenic cross-reactivity of this region, using an antiserum obtained from rabbits with a synthetic peptide corresponding to ags 4 conjugated to a carrier protein. The rabbit antiserum was found to be broadly cross-reactive with a number of FCV isolates in dot immunoblotting, Western blotting, and immunofluoresence. The neutralising ability of the antiserum raised was more equivocal, although one of the two antisera appeared to have a low (mean 1 in 15), but specific, virus neutralisation titre. It is suggested that this conserved epitope, ags 4, may be useful in vaccine development.

INTRODUCTION.

Feline calicivirus (FCV) is an important oral and respiratory pathogen of domestic cats (Gaskell & Dawson, 1998) and belongs to the family *Caliciviridae* (Cubitt et al., 1995). It contains a single-stranded, positive-sense RNA genome of approximately 7.7 kb with three open reading frames (ORFs) (Carter et al., 1992a, Glenn, 1997) (Neill, 1990, Neill et al., 1991, Oshikamo et al., 1994, Sosnovtsev & Green, 1995, Tohya et al., 1991). ORF 1 is located at the 5`end of the genome and codes for the non-structural proteins, which include a 2C-like helicase, a 3C-like protease and a 3D RNA-dependent RNA polymerase (Boniotti et al., 1994, Vázquez et al., 1998). ORF 2 encodes the major capsid protein, which varies between 60- 70 kilodaltons in size between different members (Schaffer, 1979). The FCV capsid protein contains both conserved and variable stretches of sequences and is divided into six regions (A-F) (Neill, 1992) (Fig. 1a). Region A, a variable region is cleaved during capsid processing to generate the mature capsid protein (Carter et al., 1992b, Sosnovtsev et al., 1998). Regions B, D, and F are relatively conserved and C and E are more variable (Neill, 1992, Seal et al., 1993). Region E in FCV contains a central conserved domain of 28 amino acids separating the 5` and 3` hypervariable regions (HVRs) (Seal et al., 1993) (Fig. 1). ORF 3 at the 3`end of the genome codes for a minor structural protein (Glass et al., 2000, Wirblich et al., 1996).

Antigenically, most FCV isolates show some degree of cross-reactivity and therefore are currently considered to belong to a single serotype (Povey & Ingersoll, 1975, Povey, 1974). However, important differences between strains means the degree of cross-reactivity is variable and most viruses can be distinguished from each other at

the antigenic level. This antigenic dichotomy of antigenic cross-reactivity coupled with antigenic variability has important implications for vaccine design and efficacy. Antigenic cross-reactivity has allowed the design of vaccines based on single viral strains that have been shown to induce broadly cross-reactive antisera. Classically, such vaccines were based on one isolate designated FCV-F9, although other strains are now also being used (Bittle & Rubic, 1975, Kalunda et al., 1975, Lauritzen et al., 1997) (R.M.Gaskell, personal communication). Antigenic variability means that whilst current vaccines are likely to offer some degree of clinical protection against the majority of strains, the level of this protection appears to be variable. In some cases where the degree of antigenic cross-reactivity between vaccine and field virus is low, clinical disease may occur (Geissler et al., 1997, Lauritzen et al., 1997). It also follows that continued widespread use of such vaccines may lead to the selection of vaccine resistant strains in the population. Indeed there is some evidence for reduced efficacy of F9-based vaccines in the USA over time (Lauritzen et al., 1997).

Because of the interest in designing more cross-reactive vaccines, several studies have attempted to characterise the antigenically important regions in the FCV major capsid protein. One of the initial studies showed that a recombinant peptide corresponding to amino acids 408 to 517 of the 5`HVR of FCV F9 induced the formation of neutralising polyclonal antisera in rabbits (Guiver et al., 1992) (Fig. 1b). In the same study, cats vaccinated with F9 produced a polyclonal antisera that reacted to this

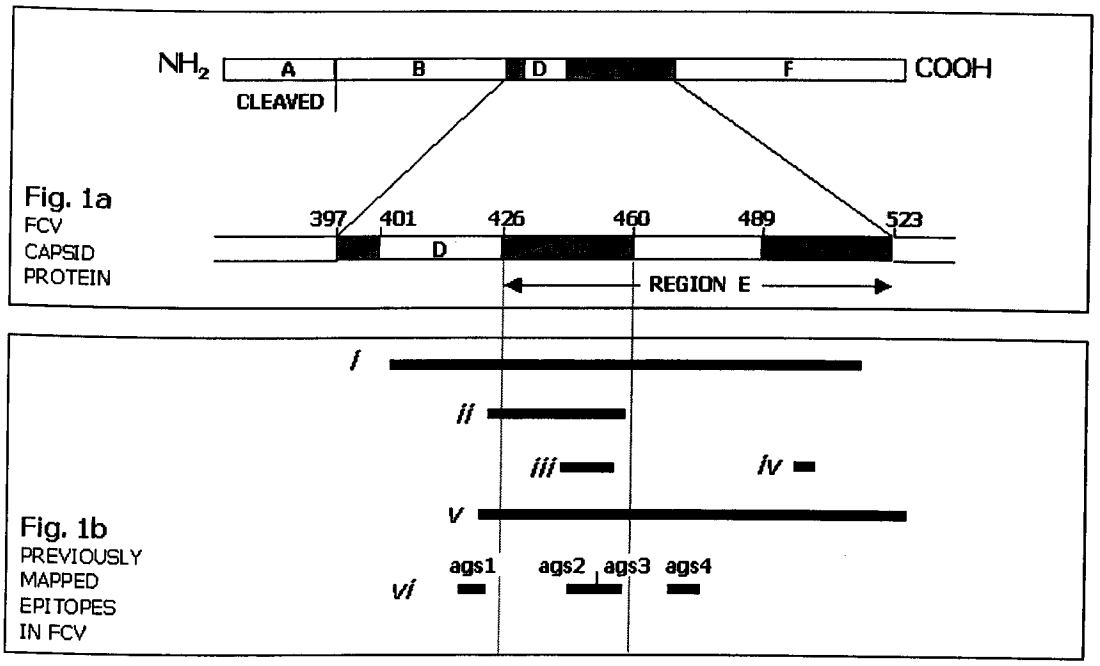


Fig. 1a. Summary of FCV major capsid protein, which is divided into conserved regions (regions B, D, and F) and variable regions (regions C and E, the latter including 5` HRV and 3` HVR). **Fig. 1b** Antigenic regions previously identified: *i*, aa 408 to 517 (Guiver et al., 1992); *ii*, aa 422 to 458 (Milton et al., 1992); *iii*, aa 441 to 455 (Tohya et al., 1997); *iv*, aa 493 to 494 (Tohya et al., 1997); *v*, aa 420 to 529 (Neill et al., 1997); *vi*, ags 1(aa 415 to 421), ags2 (aa 445 to 451), ags3 (aa 451 to 457), ags 4 (aa 475 to 479) (Radford et al., 1999).

Based on a figure in Radford *et al* (1999).

peptide. Subsequently, the epitopes for two neutralising monoclonal antibodies (mAbs) to F9, designated IG9 and 4E7, were located and were found to be present between amino acids 422 and 458 of the 5`HVR (Milton et al., 1992) (Fig. 1b).

In addition, epitopes for other neutralising mAbs have been mapped within the 5`and 3`HVR of a Japanese strain F4, and these have been found to represent both linear and conformational epitopes (Tohya et al., 1997). In another study, facilitated by the availability of an FCV infectious clone, Neill et al (1997) showed that the exchange of variable region E between two antigenically distinct strains of FCV conferred the neutralisation profile of the region E donor to the hybrid virus (Neill et al., 1997). Most recently, Radford et al (1999), using overlapping peptides, has shown that cats infected with FCV F9 produced antibodies to four linear epitopes. Two of these, named antigenic sites (ags) 2 and 3, were present in the 5`HVR of region E (Radford et al., 1999) (Fig. 1b). Collectively these studies all point to the antigenic importance of the variable capsid domains and provide the mechanism for antigenic differences between most FCV strains.

In the same study, Radford et al (1999) also located two linear epitopes in the conserved regions on either side of the 5`HVR of FCV strain F9 (Radford et al., 1999). The epitope in region D (ags 1) only reacted with antisera to a minority of FCV strains. However, the epitope in the conserved central domain of region E was recognised by antisera from 11 of 12 cats. This region, termed (ags 4), was located between amino acids 475 to 479 and consisted of the amino acid sequence AWGDK (Radford et al., 1999). Because ags 4 is highly conserved on the basis of sequence

analysis, it may partly explain the serological cross-reactivity between the majorities of FCV strains. In addition, since most cats were shown in the same study to produce a detectable antibody response to ags 4, this region also has many of the features that might be useful for a potential vaccine. However, crucially, because of the (ELISA) methodology used in this earlier study, it was not possible to determine whether antibodies to ags 4 were able to neutralise the virus.

The purpose of this study was therefore to determine the cross-reactivity of antibodies raised to ags 4, and to determine their neutralising capacity. Antibodies were raised in rabbits injected with a synthetic peptide corresponding to ags 4. The ability of this antisera to react with capsid proteins from distinct FCV strains was then determined by dot immunoblotting, Western blotting, immunofluoresence (IF) and virus neutralisation (VN) assays.

MATERIALS AND METHODS

Antisera production and antibodies.

Polyclonal antiserum to ags 4 was raised in rabbits by a commercial laboratory using a standard technique (Genospheres Biotechnologies Paris France). Briefly, the peptide (C)SLQRAWGDKKI was firstly manufactured using an automated solid-phase peptide synthesiser. Then the peptide was purified by mass spectrum and HPLC analysis to ensure sequence integrity and purity. This peptide consists of the conserved FCV capsid region, containing the ags 4 sequence AWGDK at its C-terminus with an additional cysteine at the N-terminus. The peptide was subsequently

conjugated to keyhole limpet hemocyanine (KLH), an activated carrier molecule, through the thiol group via the N-terminal cysteine. This KLH-ags 4 complex was used as an antigen to immunise two adult rabbits twice, 14 days apart. Seventy days later 100ml of antiserum was obtained from each rabbit and each designated Post-A ags 4 and Post-B ags 4. Pre-immune control serum was also supplied from each rabbit designated Pre-A and Pre-B as negative controls. In addition, a rabbit polyclonal serum produced by the same method but to an unrelated peptide not from an FCV sequence was supplied and used as another negative control designated Post-X. These controls were used in all subsequent experiments. The antibodies were aliquoted into 1ml cryovials and stored at -20°C until required. In addition, monoclonal antibody IG9, whose linear epitope is located in the 5`HVR of FCV strain F9, was used as a positive control (Nova Castra).

Viruses and cells.

Viruses used as antigenic targets in this study are shown in table 1, and consisted of previously reported strains of FCV (F9, LSO15, 255, F65, LSO27) and a panel of FCV isolates randomly collected by this laboratory from throughout the U.K in 2001. This method of collection aimed to ensure that the vast majority of these isolates were likely to be genetically distinct. Viruses were grown in either feline embryo cell line A (FEAs) (European collection of cell cultures) (ECACC) (Jarrett et al., 1973), or Crandell-Rees Feline Kidney Cells (CRFK) (ECACC) (Crandell et al., 1973). Infection was allowed to proceed overnight until cytopathic effect (CPE) was complete. Monolayers were frozen at -80°C and thawed to produce a virus stock that was used for all subsequent experiments.

FCV isolate	Year isolated	Reference	Dot immuno-blotting	Western blotting	Immuno-flouresence
Laboratory F9	1958	(Bittle et al., 1960)	+ve	+ve	+ve
Intervet standard F9	1980	(Bittle et al., 1960)	+ve	+ve	+ve
LSO15	1995-98	(Knowles, 1988)l	nt	+ve	+ve
255	1970	(Kahn & Gillespie, 1970)	+ve	+ve	+ve
F65	1990	(Dawson, 1991)	+ve	+ve	+ve
LSO27	1988	(Knowles, 1988)	nt	+ve	+ve
0507	2001	unpublished*	+ve	nt	+ve
1709	2001	unpublished	+ve	nt	+ve
2004	2001	unpublished	+ve	nt	+ve
2101	2001	unpublished	+ve	nt	+ve
2702	2001	unpublished	+ve	nt	+ve
4720	2001	unpublished	+ve	nt	+ve
5601	2001	unpublished	+ve	nt	+ve
6114	2001	unpublished	+ve	nt	nt
6406	2001	unpublished	+ve	nt	+ve
6603	2001	unpublished	+ve	nt	nt
6810	2001	unpublished	+ve	nt	+ve
7107	2001	unpublished	+ve	nt	+ve
7206	2001	unpublished	+ve	nt	+ve
7402	2001	unpublished	+ve	nt	+ve

Table 1. All isolates used in this study.

nt = not tested

* collected by diagnostic team, (S.A.V.G), University of Liverpool, Leahurst.

such stocks of virus were not routinely titrated but a titre of approximately 10^5-10^7 tissue culture infectious doses (TCID) per 50μl is generally obtained.

Dot immunoblotting.

Ten microlitres of each of the 18 different strains of FCV from table 1 were dotted onto nitrocellulose membranes (Sigma), and air-dried at room temperature. In addition, a sample of tissue culture fluid from uninfected cells was included as a negative control.

Non-specific membrane antibody binding was blocked for 1 hour in 10% (wt/vol) skimmed milk (Marvel) in phosphate buffered saline (PBS; 154mM NaCl, 3 mM KCl, 9mM Na_2HPO_4 65mMKH_2PO_4). The membranes were washed twice in 0.05% Tween 20 (Sigma) in PBS (PBS-T), for 10 minutes per wash, and incubated with rabbit antisera diluted 1:100, or with IG9 as a positive control which resides within the capsid protein of FCV F9 (Carter et al., 1989) diluted 1:200, in 0.5% (wt/vol) skimmed milk in PBS for 1 hour at 37°C. After a further three washes in PBS-T, each for 5 minutes, the membranes were then incubated at 37°C for a further hour in either peroxidase-conjugated anti mouse IgG for IG9 or anti-rabbit IgG for rabbit antisera (Sigma) diluted 1:2,000 in 0.5% (wt/vol) skimmed milk in PBS. All membranes were washed twice in PBS-T for 10 minutes per wash, and then two last washes in PBS for 5 minutes per wash. Finally, membranes were developed at room temperature with 3,3`-diaminobenzadine tetrahydrochloride (DAB; Sigma) according to the manufacturer`s instructions. Briefly, a positive reaction produced a brown insoluble

precipitate that is visible upon development. Washing the membranes in tap water terminated the reaction and the membranes were air dried.

Western blotting.

Western blotting was carried out essentially according to standard protocols and manufacturer's instructions (E-C Apparatus Corporation, NY). Briefly, 10μl of five different FCV isolates (F9, LS015, 255, F65 and LS027) were lysed in 10μl of 2X western sample buffer (2X WSB :0. 5 M Tris-HCl pH 6.8, 5% w/v sodium dodecyl sulphate SDS, 50% v/v glycerol and 0.1% bromophenol blue dye), with 2μl of neat 2-mercaptoethanol (Sigma). The sample was mixed gently, boiled at 100°C for 5 minutes, chilled on ice and spun in a microcentrifuge at 10,000g for 2 minutes before loading on to a 10% SDS polyacrylamide gel in Laemmli electrophoresis buffer (1X Laemmli buffer: 0.025M Tris-base and 0.192M glycine, and 0.1% SDS made to a final volume of 1 litre in distilled water). The gel was run at constant voltage (120V) until the dye front reached the bottom of the gel (approx 45 minutes). Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad), by electroblotting at 100V for 1 hour in ice-cold transfer buffer (0.025M Tris-base, 0.192M glycine, and 200ml methanol made to a final volume of 1 litre) in distilled water. After blocking overnight at 4°C using 10% (wt/vol) skimmed milk in PBS, the membranes were washed twice in PBS-T for 10 minutes per wash. Primary and secondary antibodies were added and incubated as for the dot immunoblotting protocol described above, including all the subsequent washing steps. Finally, peroxidase detection using DAB was also performed as described above for dot immunoblotting.

Immunofluorescence Assay.

A number of different FCV isolates were used to infect CRFK monolayers in 24 well plates until CPE was observed (table 1). In addition, negative controls consisted of an uninfected well and a well infected with a feline herpesvirus (FHV). Growth medium was carefully removed and the plates were washed in PBS and fixed in 70% ethanol for 2 minutes at room temperature. The plates were washed again three times by gentle agitation in PBS and then overlaid with 50μl of either Post-A ags 4 rabbit primary antibody at a dilution 1:40, or Post-B ags 4 a dilution of 1:20. As a positive control, FCV F9 infected cells were also incubated with IG9 at a dilution of 1:100. After incubation at 37°C for 1 hour, the plates were washed three times in PBS and 50μl of goat fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG or anti-mouse IgG (Sigma) diluted 1:40 with PBS was added to each well and incubated for a further hour at 37°C. The conjugated antibody was washed off with three more washes in PBS, and 50% glycerol was added to each well. The plates were then individually viewed under a UV microscope (Zeiss Axiovert 200m) to detect specific immunoflouresence. Images were captured using a Axiocam hrc camera and Axiovision 3.1 software.

Virus neutralisation assays.

Virus neutralisation (VN) assays were performed using a constant virus varying antibody method as previously described (Dawson, 1991, Dawson et al., 1994), except all antisera were preadsorbed with FEA cells to reduce toxicity. Briefly, a $75cm^2$ flask of FEA cells was incubated in 1% trypsin until all the cells were

dislodged from the flask, followed by the addition of 10ml of PBS. The cells were then gently pelleted at 3000g in a MSE benchtop centrifuge for 7 minutes, the supernatant discarded, and the cell pellet resuspended in 1ml of rabbit antiserum and incubated at 37°C for 1 hour. This was followed by a further centrifugation step at 10,000g for 10 minutes to pellet cells and any bound toxins, and the supernatant which contained the serum was transferred to a clean microcentrifuge tube. Duplicate serial two-fold dilutions of this preadsorbed rabbit antiserum were incubated with 30-300 $TCID_{50}$ of FCV F9 virus. This virus/serum mixture was then added to the wells containing confluent FEA cells, and the plates were incubated at 37°C and monitored daily for CPE until day 3. Antibody neutralising titres were recorded as the mean reciprocal of the 50% end-point (Reed & Muench, 1938) Table 2. All tests were repeated between 4-8 times.

RESULTS.

The summary of the results for dot immunoblotting, Western blots, and immunofluoresence are shown in Table 1.

Dot immunoblotting.

The FCV isolates that were applied to the nitrocellulose membrane all produced a strong positive response when probed with Post-A ags 4 rabbit antibody raised to the conserved sequence of ags 4 (fig. 2). A similar although less intense reaction was observed with Post- B ags 4 (fig. 3). This in contrast to antibody IG9 which was used as a F9 positive control on all the same isolates, where only the strain F9 produced a

positive response (figs. 2 and 3). Negative controls consisting of Pre-A, Pre-B or Post-X gave no response with any of the FCV isolates. (figs. 2 and 3). Since immunoblots were performed on separate days, a full set of controls was run on each occasion.

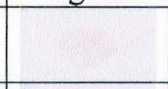
virus	Post-A ags 4	IG9	Pre-A	Pre-B	Post-X
Laboratory F9					
Intervet standard F9					
LSO15					
255	nt	nt	nt	nt	nt
F65					
LSO27	nt	nt	nt	nt	nt
0507					
1709					
2004					
2101					
2702					
4720					
5601					
6114					
6406					
6603					
6810					
7107					
7206					
7402					

Fig. 2. Dot immunoblotting results with Post-A ags 4, showing a strong positive response with all FCV isolates. The positive control IG9 only produced a response with FCV F9, and no response was observed for negative controls Pre-A, Pre-B and Post-X . Φ nt = not tested.

virus	Post-B ags 4	IG9	Pre-A	Pre-B	Post-X
Laboratory F9					
Intervet standard F9					
LSO15					
255	nt	nt	nt	nt	nt
F65					
LSO27	nt	nt	nt	nt	nt
0507					
1709					
2004					
2101					
2702					
4720					
5601					
6114					
6406					
6603					
6810					
7107					
7206					
7402					

Fig. 3. Dot immunoblotting results with Post-B ags 4, showing a weak positive response with all FCV isolates. The positive control IG9 only produced a response with FCV F9, and no response was observed for negative controls Pre-A, Pre-B and Post-X . Φ nt = not tested.

Western Blotting.

All five FCV isolates (F9, F65, LSO15, 255 and LSO27) used for Western blotting produced a positive response with Post-A ags 4 reacting with a band of approximately 70 kDa consistent with the size of the FCV capsid (Carter et al., 1989) (fig. 4a). No positive reaction was seen with Post-B ags 4 (data not presented). The positive control consisting of F9 virus, and monoclonal antibody IG9 also reacted with a band of 70 kDa (fig. 4b). All negative control antiserum consisting of Pre-A, Pre-B and Post-X (figs. 4c, 4d, and 4e) did not react with any of the FCV isolates tested.

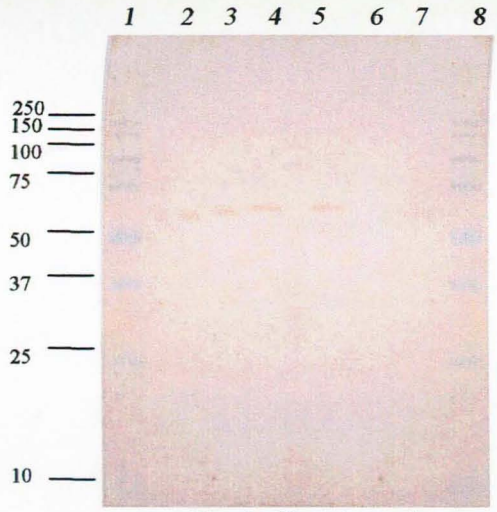


Fig. 4a. Post-A ags 4

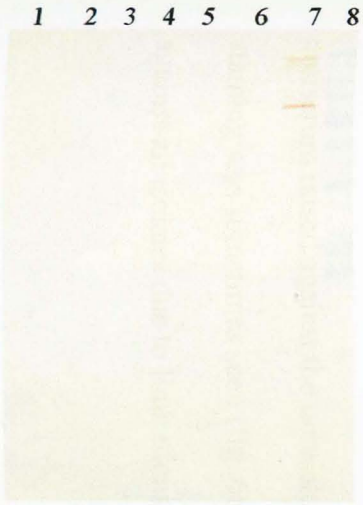


Fig. 4b. IG9 +ve control

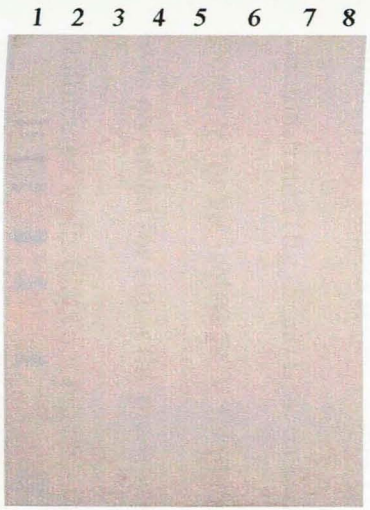


Fig. 4c. Pre-A –ve control

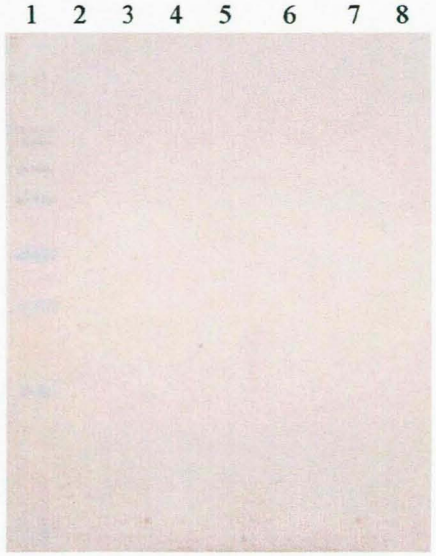


Fig. 4d. Pre-B –ve control

Fig. 4e. Post-X negative control

Figs. 2. Western blotting results. In all Figs. 4a.b, c, d, and e Lanes 2 to 5 represents FCV isolates LSO27, F65, 255, LSO15. Lane 7 represents FCV F9. Lane 6 is negative control consisting of tissue culture fluid without virus. Lanes 1 or 8 are molecular weight markers (Biorad) (with values in kDa indicated on Fig. 4a). As observed in Fig.4a Post-A ags 4 all five FCVisolates produced a band of approximately 70 kDa. Fig. 4b. The +ve control IG9 only reacted with F9 isolate. Figs. 4c, d and e all negative controls did not react with any isolates.

Immunofluoresence.

Indirect immunofluoresent staining was used to examine for the presence of the FCV capsid protein in cells. Using the rabbit antibodies Post-A ags 4 and Post-B ags 4, for all the FCV isolates tested (Table 1) specific cytoplasmic fluoresence was clearly demonstrated (fig. 5 and figs. 6a and 6b). The monoclonal antibody IG9 was only positive for the isolate FCV F9 (data not presented). No specific immunofluoresence was observed for either the pre-immune antisera Pre-A and Pre-B (figs. 6c and 6d), uninfected wells (data not presented), or wells infected with FHV (data not presented). In addition, no specific staining was observed for the negative control antiserum raised against the unrelated peptide Post-X, although some mild nuclear staining was sometimes seen (fig. 6e). This negative control Post-X was not tested against all isolates due to limitations on the quantity of this antiserum.

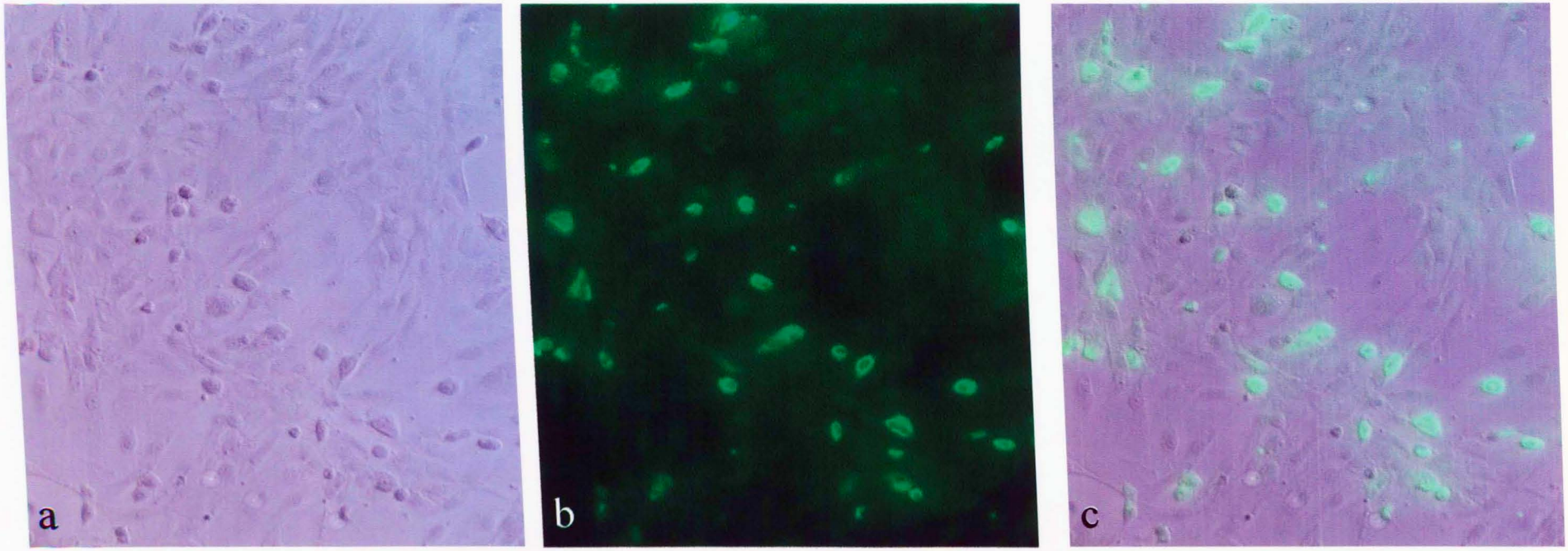


Figure 5; Results of immunofluorescence using FCV F9 infected cells (magnification x20). (a) white light, (b) UV light, (c) merged.

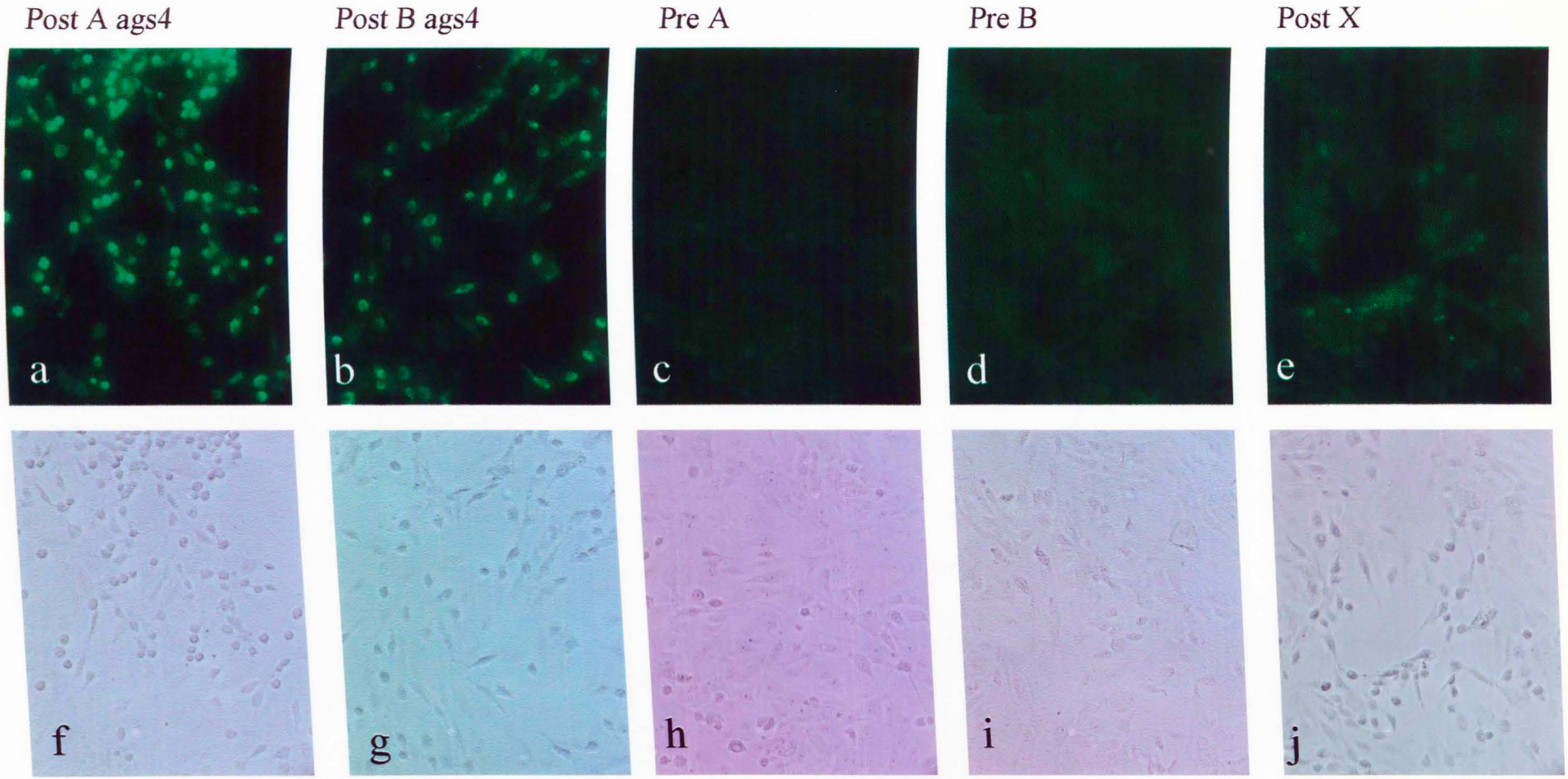


Figure 6; Results of immunofluorescence using FCV F9 infected cells (magnification x20). a-e taken under uv light. f-j taken under white light

Virus neutralisation assays.

The mean result for the virus neutralisation (VN) assays with each of the rabbit antiserum are shown in Table 2. Initial tests were unreadable due to toxicity in Post-A ags 4 and Post-B ags 4 rabbit antiserum, but this was resolved by pretreating the antiserum with FEA cells. The antibody titres of Pre-A, Pre-B, and Post-X were generally less than 2, although on 6 of 18 occasions titres of less than or equal to 6 were seen. In all tests, the titre of Post-B ags 4 antiserum was less than or equal to 2. However, Post-A ags 4 antiserum consistently had titres, which ranged from 6-32, with a mean of 15, suggesting it neutralised FCV F9 to some extent. In view of this relatively low VN titre, the titre of the virus used in each test was also kept low (31-100 $TCID_{50}$) but was within the accepted range (30-300 $TCID_{50}$).

Sera	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8
Pre-A	nt	4	<2	<2	<2	<2	<4	4
Pre-B	nt	6	<2	<2	<2	<2	<4	<2
Post-A ags 4	16	12	6	12	16	12	16	32
Post-B ags 4	<2	2	<2	<2	<2	<2	<2	<2
Post-X	nt	6	<2	<2	<2	nt	nt	nt
$TCID_{50}$ †	62	62	37	100	57	57	31	39

Table 2. Neutralisation titres of Pre-A, Pre-B, Post-A ags 4, Post-B ags 4 and Post-X serum raised against FCV F9. The titre of virus in each test is shown in †.

nt = not tested.

DISCUSSION.

The regions of viruses that closely interact with the host are important and may determine the pathogenesis and the clearance of virus. For many caliciviruses, such regions have not yet been identified, but in FCV it appears that variable region E of the capsid protein contains the immunodominant regions which interact with the host immune response (Guiver et al., 1992, Milton et al., 1992, Shin et al., 1993, Tohya et al., 1997, Viaplana et al., 1997). A number of studies have identified both linear and conformational B-cell epitopes in region E (Radford et al., 1999, Tohya et al., 1997), some of which have been shown to be neutralising. However, identification of such epitopes in a variable region of the capsid is not likely to facilitate the development of a cross-reactive vaccine. In contrast, the two epitopes in conserved regions of the capsid identified by (Radford et al., 1999), may prove useful in vaccine development, especially as one, ags 4, appeared to be detected by the majority of FCV infected cats.

The aim of this study was to explore further the cross-reactivity of antibodies raised to ags 4, and to determine whether it could induce a neutralising antibody response. We have demonstrated that this epitope induced an antibody response in rabbits which reacted with all FCV isolates tested in dot immunoblotting, Western blotting, immunofluoresence and (VN assays). This confirmed the ability of this epitope to induce an antibody response which is broadly cross-reactive amongst FCV strains. Such antiserum could prove highly useful as a diagnostic and research tool for the specific detection of a wide range of FCV isolates.

Whether or not a neutralising antibody response was induced by this epitope was, however, more difficult to evaluate. The rabbit antiserum Post-A ags 4 had a low but relatively consistent titre with a mean of 15, suggesting a specific neutralising response had been produced. However, rabbit antiserum Post-B ags 4 had no detectable titre, and on some occasions, Pre-A and Pre-B also had an apparent low titre of less than or equal to six, which suggests that non-specific responses may have been present. The difference in post-immune responses between rabbits is probably due to individual variation between the rabbits. Future studies to enhance and confirm the specificity of the neutralising response seen would require the synthesis of higher titre antisera. This may be achieved by the use of other carrier proteins such as tetanus toxoid, *Pseudomonas aeruginosa* toxin A, beta-galactosidase, *brucella abortus* (killed bacteria), hepatitis B core and surface antigens, all of which have been previously used to improve the immunogenicity of peptides (Baker et al., 2000, Beekman et al., 2001, Lapham et al., 1996). Recently, the use of T helper recognition sites have also been used to increase the immunogenicity of injected peptides (Wang et al., 2002).

Clearly it is important in terms of potential vaccine development to confirm whether or not the peptide of ags 4 is able to neutralise F9, and indeed other FCVs.

The potential use of a conserved synthetic peptide as an FCV vaccine would have several major advantages (Jackson et al., 2002). These would include:

- the absence of infectious material that may compromise live vaccines. Such disease caused by live vaccines is a rare but well characterised potential

complication of live FCV vaccines in cats (Dawson et al., 1993, Radford et al., 1997, Radford et al., 2000).

- the production of a more cross-reactive immune response. This should reduce the incidence of vaccine breakdowns, as sometimes occurs with conventional FCV vaccination (Dawson et al., 1993).
- a reduced risk of selecting neutralising resistant escape mutants. The high degree of sequence conservation of ags 4 between strains suggests this region of the FCV capsid is subject to strict structural constraints that would limit its ability to mutate.
- the ability to serologically distinguish vaccinated from infected animals through detection of FCV-specific antibodies to sites other than ags4 in FCV-infected cats.
- the ability to immunise with a minimal structure, consisting of a well-defined antigen.
- lipid, protein and carbohydrate groups can be readily introduced in a controlled manner to improve immunogenicity, stability and solubility.
- peptides can be analysed for purity and fidelity of sequence using well-established techniques such as high performance liquid chromatography (HPLC), and mass spectrometry.
- the production of peptides can be carried out economically on a large scale.
- there is no risk of reversion to, or formation of genetic reassortment that may lead to virulence.

- peptide-based vaccines can be designed to include multiple antigenic determinants from a number of pathogens, or multiple antigenic epitopes from the same pathogen.

A number of synthetic peptide vaccines have now been developed for veterinary use, including protection against the DNA parvovirus infection of dogs (Langeveld et al., 1994). Synthetic peptide vaccines have also been developed against positive sense single-stranded RNA viruses, more similar to FCV including foot-and mouth-disease-virus (FMDV). Most of them are able to protect laboratory animals such as mice, rabbits, and guinea pigs, and field trials are still being performed (Vazquez et al., 2002, Villen et al., 2001). A recent study has also reported a peptide-based vaccine for foot-and-mouth disease (FMD) in swine (Wang et al., 2002).

In conclusion, we have demonstrated that antibodies raised to the region ags 4 were immuologically cross reactive with a number of FCV strains: such antisera may prove useful in the development of diagnostic tests for FCV. The ability of ags 4 to induce a neutralising antibody response in rabbits was more equivocal. However, a specific VN antibody response appeared to be present in one of the two antisera, indicating that the ags 4 peptide could be useful in the development of safer and more efficacious synthetic FCV vacccines.

MANUSCRIPT 2

The effect of feline calicivirus infection on cell-cycle progression.

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ABSTRACT.

Apoptosis is an essential, highly conserved and highly regulated mode of cell death that progresses through a series of morphological and biochemical features, including membrane blebbing, nuclear and cytoplasmic shrinkage and chromatin condensation. Many viruses have been found to either inhibit or promote apoptosis in host cells, or even to do both at different stages in their replicative cycles. The exact mechanisms by which FCV induces cell death are unknown, but some of the observed changes in FCV infected cells in cell culture are suggestive of a cell undergoing apoptosis. Such observations include plasma membrane blebbing on the surface of some infected cells. The aim of this study was to investigate whether the major mechanism of cell death in infection with FCV *in vitro* is associated with the induction of apoptosis. In addition we assessed the regulation of cell death by measuring expression of the anti-apoptotic protein Bcl-2 as well as the levels of cell proliferation as indicated by the proliferation cell nuclear antigen (PCNA).

INTRODUCTION.

Feline calicivirus (FCV), is classified in the genus vesivirus of the family *Caliciviridae*, and is an important oral and respiratory pathogen of domestic cats (Gaskell et al., 1988). The clinical signs associated with acute FCV infection are usually mild but a spectrum of severity has been observed from asymptomatic infection (Fastier, 1957, Povey & Hale, 1974) (Radford et al., 2001) to severe pneumonia in young kittens (Kahn & Gillespie, 1971, Povey & Hale, 1974) (Foley, 2002, Pedersen et al., 2000, Poulet et al., 2000). Some FCV isolates may induce a febrile lameness (Bennett et al., 1989, Church, 1989, Dawson et al., 1994, Pedersen et al., 1983). FCV has also been associated with chronic stomatitis and gingivitis (Knowles et al., 1989, Thompson et al., 1984). In addition, following recovery from clinical disease, cats may become carriers and shed virus in some cases for at least two years (Povey et al., 1973, Wardley, 1976) (Radford et al., 2001).

There is a large number of other members of the *Caliciviridae* family that infect a wide range of host species, and cause a variety of disease syndromes (Cubitt et al., 1995). The family can be broadly divided into four distinct genera: vesivirus, lagovirus, norovirus (previously known as 'Norwalk-like viruses') and sapoviruses (previously known as 'Sapporo-like viruses') (Berke et al., 1997, Noel et al., 1997).

As well as FCV, the genus vesivirus includes vesicular exanthema of swine virus (VESV), San Miguel sea lion virus (SMSV), canine calicivirus (CaCV) and mink calicivirus (MCV). An important feature of this genus is that they cause vesicular lesions in their hosts. Caliciviruses belonging to the genus lagovirus cause fatal haemorrhagic disease in their lagomorph hosts, and include rabbit haemorrhagic

disease virus (RHDV) (Ohlinger et al., 1990, Parra & Prieto, 1990, Rodák et al., 1990) and European brown hare syndrome virus (EBHSV) (Chasey & Duff, 1990, Gavier-Widen & Morner, 1991, Moussa et al., 1992, Wirblich et al., 1996).

Members of the genera norovirus and sapovirus include human caliciviruses which are a major cause of gastrointestinal disease (Cubitt & Barrett, 1984, Kapikian, 1996, Kaplan et al., 1982).

Regarding the *Caliciviridae* as a whole, little is known about how these important viruses cause disease at the cellular level. Progress on understanding the mechanisms of disease has been impaired by the lack of suitable cell culture systems in which to study the interaction between virus and cells. Only members of the vesivirus genus can be grown routinely in cell culture including FCV, SMSV and some isolates of CaCV. Because infections caused by FCV are ubiquitous, and the virus has a narrow host range this is an ideal model system for studying calicivirus replication.

When FCV is replicated in cell culture, a cytopathic effect (CPE) is usually first observed between 6-10 hours post infection (Knowles, 1988, Studdert et al., 1970). Because of this, most routine methods of diagnosing FCV are based on virus isolation in cell culture. Morphological changes in infected cells include rounding and retraction of cells. In addition, plasma membrane blebbing can be observed on the surface of some infected cells (Fig.1) (Knowles, 1988). Whilst the exact mechanisms by which FCV induces this cell death are unknown, some of the observed changes suggestive of cells undergoing apoptosis.

Apoptosis is an essential, highly conserved and highly regulated mode of cell death that is important for normal development, host defence and the suppression of oncogenesis (Kerr et al., 1972). It is characterised by morphological and biochemical features, including membrane blebbing, nuclear and cytoplasmic shrinkage and chromatin condensation. A family of cysteine proteases called caspases effects most of these changes. Caspases are the central players in apoptosis, because they catalyse many steps in the death pathway by cleavage of target proteins at specific sites containing aspartic acid (Thornberry, 1998). The caspase gene family contains 14 mammalian members, of which 11 human enzymes have been identified. They are expressed as proenzymes that contain an N-terminal pro-domain, a large subunit and a C-terminal small subunit (Stennicke & Salvesen, 1998). There are at least two general classes of apoptotic caspases: the upstream initiator caspases (caspases 2, 8, 9, and 10) and the downstream effector caspases (caspases 3, 6, and 7) (Fig. 2). The initiator caspases are present in complexes with other regulatory proteins and are activated in response to apoptotic signals. The effector caspases are then activated in a cascade through cleavage by initiator caspases that cleave a number of target proteins leading to cellular destruction, and disintegration of the entire cell contents into apoptotic bodies (Adrain & Martin, 2001, Bratton & Cohen, 2001, Budihardjo et al., 1999, Hengartner, 2000).

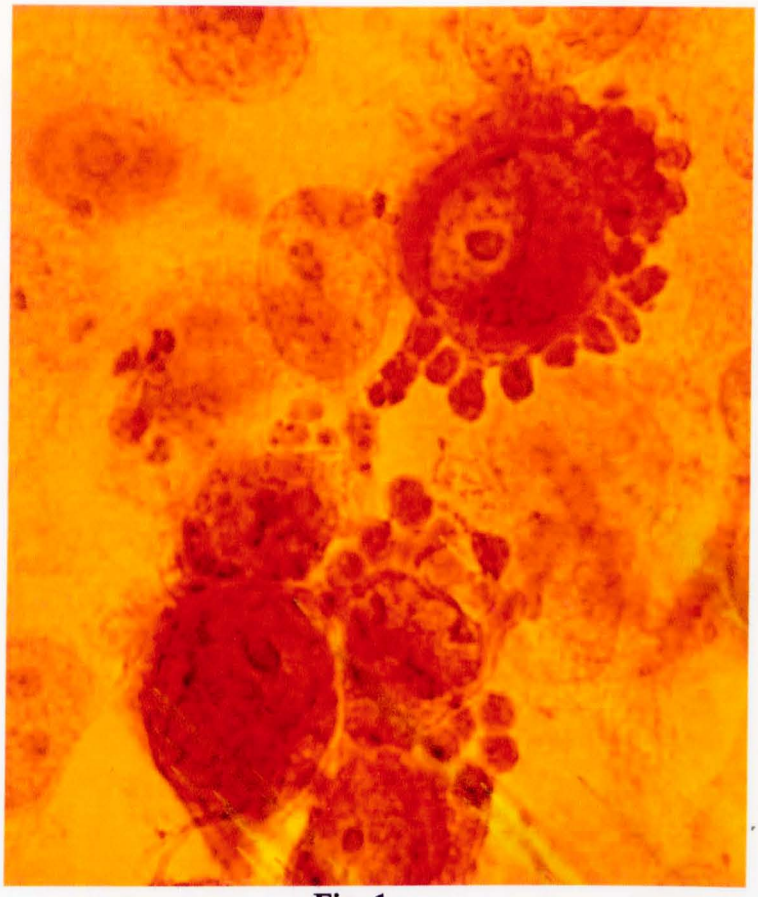

Fig. 1

Fig. 1. Membrane blebbing observed on the surface of FCV-infected cells in cell culture(Knowles, 1988). (PAP stain, AEC chromagen approximately x 7000)

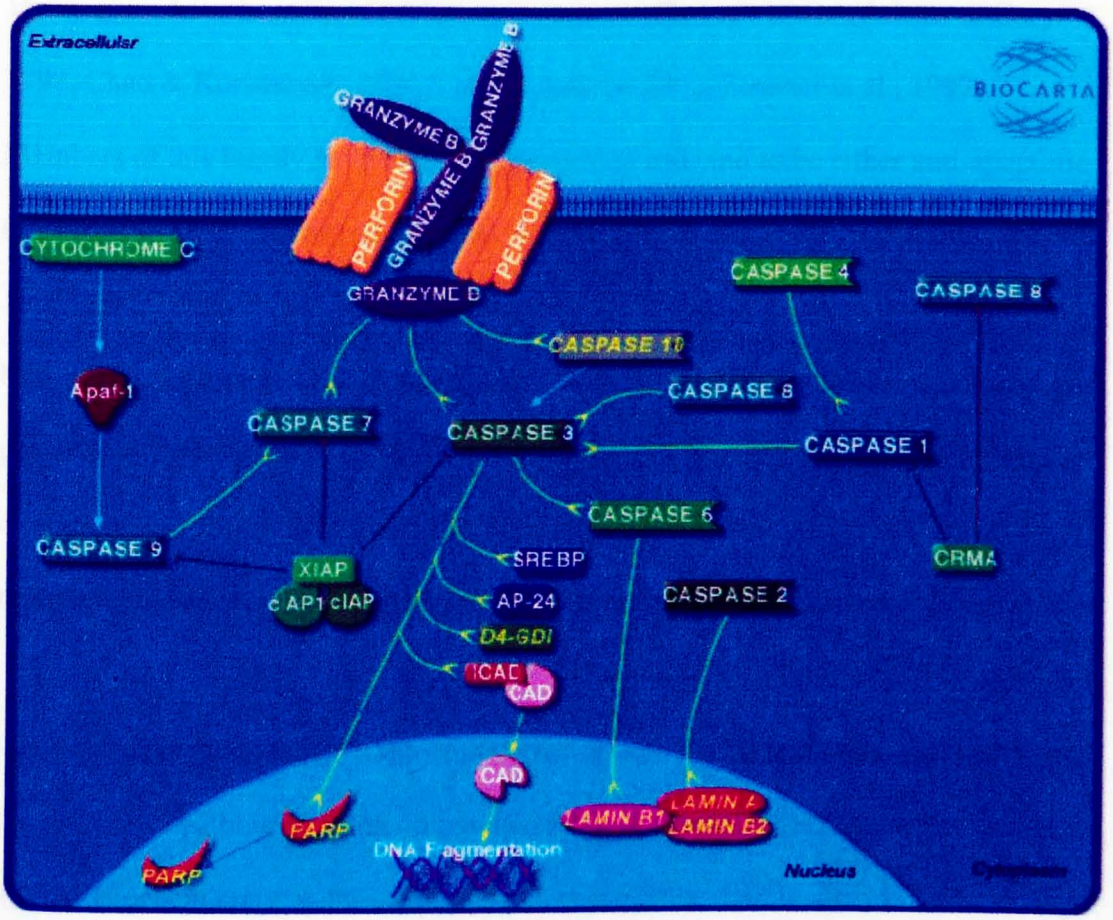

Fig.2

Fig. 2. Caspase cascade in apoptosis. Caspases are a class of cysteine proteases which are involved in apoptosis (see text for details). Diagram from Biocarta. www.biocarta.com/pathfiles/h_caspasePathway.asp (2003)

The regulation of apoptosis is maintained by the Bcl-2 protein family, of which at least 15 members have been identified in mammalian cells and viruses (Adams & Cory, 1998, Chao & Korsmeyer, 1998, Cory & Adams, 2002, Strasser et al., 1997). Members of this family have opposing properties and tend to be either anti-apoptotic (e.g Bcl-2 and Bcl-X_L) or pro-apoptotic (e.g Bax, Bad, Bid and Bim) (Adams & Cory, 1998, Raff, 1998). It has been shown that the Bcl-2 family proteins form homo- and heterodimers (Kurschner & Morgan, 1996) and that the relative levels of the anti- and pro-apoptotic members of the Bcl-2 family appear to be a key determinant of the fate of cells when confronted with an apoptotic stimulus (Korsmeyer, 1999), in part by regulating the activation of caspases, (Grandgirard et al., 1998, Green & Reed, 1998).

Cells undergoing apoptosis fragment into membrane-bound apoptotic bodies that are phagocytosed without eliciting an immune response (Savill et al., 1990, Savill et al., 1993). Biochemically, within apoptotically dying cells, double-strand DNA cleavage occurs leading to nuclear DNA fragmentation. The lengths of these DNA fragments vary by multiples of 180-200 base pairs and can be observed as a ladder on agarose gels (Duvall et al., 1985). In addition, cells containing DNA strand breaks can be used as a marker for apoptosis as detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method (Gavrieli et al., 1992).

Several large DNA viruses, such as poxviruses, herpesviruses and adenoviruses have developed a range of strategies to suppress the induction of apoptosis in infected cells (Koyama et al., 1998, Roulston et al., 1999, Shen & Shenk, 1995). Many use proteins that mimic or counteract host cell functions. The ability to suppress apoptosis enables

these viruses to prevent premature death of the host cell and so to maximise virus progeny from a lytic infection or establish a persistent infection (O'Brien, 1998).

In contrast, there are now many examples of viruses within diverse families that induce cell death by apoptosis during their infection cycles including avian coronavirus, vesicular stomatitis virus and avian leukosis virus (Brojatsch et al., 2000, Gadaleta., 2002, Liu et al., 2001, O'Brien, 1998, Razvi & Welsh, 1995, Roulston et al., 1999). This appears to be an important mechanism for non-enveloped viruses and the function can be ascribed to specific viral proteins. It has been suggested that virus-induced apoptosis may be an important mechanism of efficient dissemination of progeny. Many viruses can demonstrate this both *in vitro* and *in vivo* (Shen & Shenk, 1995, Teodoro & Branton, 1997), but it remains unclear in many of these examples which viral gene products are involved in inducing apoptosis. With some viruses, apoptosis can only be analysed *in vivo,* because of the lack of a suitable cell culture system. This was demonstrated in recent studies with rabbit haemorrhagic disease virus (RHDV), another member of the calicivirus family, (Alonso et al., 1998, Jung et al., 2000) which, unlike FCV, cannot be grown in cell culture. In this work, rabbits were inoculated with RHDV, the results suggesting that apoptosis of hepatocytes was induced by the virus.

In this study, we have used the ability of FCV to replicate in established feline cell lines, to investigate whether the major mechanism of death in cells infected with FCV *in vitro* is through the induction of apoptosis. In addition, the regulation of cell death was evaluated using the anti-apoptotic protein Bcl-2. Finally, levels of cell proliferation were assessed by demonstrating the proliferating cell nuclear antigen

(PCNA), a 36kDa accessory protein that is mainly expressed during the DNA synthesis (S) phase of the cell cycle (McCormick & Hall, 1992).

MATERIALS and METHODS.

Cells and virus.

The continuous feline embryo cell line A (FEAs) (European collection of cell cultures) (ECACC) (Jarrett et al., 1973) was used for this study. Cells were either grown in 75cm^2 flasks or on poly-L-lysine coated slides with Eagles minimum essential medium (EMEM, Sigma), supplemented with 10% foetal calf serum (FCS) as required for growth until monolayers were confluent. All virus infections were prepared using aliquots from the same virus stock using the strain FCV F9 (Bittle et al., 1960). FEA monolayers were inoculated with the virus at a multiplicity of infection (MOI) of approximately 0.5 and incubated for 1 hour at 37°C on slides and flasks. Mock-infected FEA cell controls were prepared concurrently, in which the same procedure was followed in the absence of virus. Virus was allowed to replicate for 0, 4, 8, 10, 12, 16, and 24 hours in flasks and 24 hours on slides. After each time point, cells were collected for further analysis.

Preparation of cell pellets.

Briefly, monolayers of FCV and mock infected cells at all the time points described above were washed three times in maintenance medium (EMEM) without FCS. Then 3ml of trypsin was added and incubated for 5 minutes at 37°C. Uninfected and infected cells were collected, transferred to centrifuge tubes and centrifuged for 5

minutes at 1500g. All supernatants were discarded and the cell pellets resuspended in 1ml of EMEM and centrifuged for 10 minutes at 720g. This step was repeated once more, and all supernatants were discarded. The cell pellets were resuspended in 1ml of 4% paraformaldehyde and used for *in situ* apoptosis detection, Bcl-2 detection, and PCNA detection. All pellets were then routinely embedded in paraffin wax and 3-5μm-thick sections were prepared (kindly provided by Sean Williams, and Anne Griffiths, Department of Veterinary Pathology, University of Liverpool).

In addition, another set of cell pellets were similarly prepared but resuspended and fixed in 0.5% gluteraldehyde for use in transmission electron microscopy. Embedding of cell pellets was performed routinely by Gordon Ross, Department of Veterinary Pathology, University of Liverpool.

Preparation of cell monolayer for scanning electron microscopy.

For scanning electron microscopy, FCV infected cell monolayers at 16 hours post-infection were washed three times with EMEM without FCS then covered and fixed in 0.5% gluteraldehyde.

Scanning and transmission electron microscopy.

Scanning electron microscopy was kindly performed by Dr. Dr. Udo Hetzel, Institut für Veterinär-Pathologie, Justus-Liebig-Universität Gießen, Germany, using standard protocols to study the surface morphology of FCV infected cells.

Transmission electron microscopy was kindly performed by Brian Getty, Department of Medical Microbiology, University of Liverpool, and Dr. Anja Kipar, Department of Veterinary Pathology, University of Liverpool, using standard protocols, to study the morphology of FCV-infected cells

FCV antigen detection on pelleted cell sections.

For immunohistological detection of FCV antigen, 3-5 μm sections were prepared from paraffinwax-embeded cell pellets on APES-coated slides (Aminopropyltrithoxysilane A-3648, Sigma). Sections were deparaffinised in xylene and rehydrated in graded ethanols through to water. Then endogenous peroxidase was blocked by incubating rehydrated tissue sections in 0.5% hydrogen peroxide in methanol for 30 minutes at room temperature. The slides were then washed for 5 minutes in distilled water and incubated at 97°C for 10 minutes in prewarmed Target Unmasking Fluid (TUF) (Dianova GmbH, Hamburg, Germany) diluted 1:3 in distilled water. Then the slides were cooled in TUF for a further 15 minutes at room temperature followed by a 5-minute wash in distilled water. Slides were placed into cover plates and Sequenza racks (Thermolife Sciences, Basingstoke) and washed in Tris-buffered saline (TBS; 0.1M Tris-HCl with 0.9% sodium chloride NaCl, pH 7.6), followed by a further 10 minute incubation with 10% rat serum in TBS to block non-specific binding of antibody.

Slides were then incubated overnight at 4°C with a mouse anti-feline calicivirus monoclonal antibody (FCV2-167A1, Custom Monoclonals International, Sacramento, USA) diluted 1:20 in TBS. A negative control was included using a non-reactive mouse-monoclonal antibody at the same dilution.

This was followed by washing all slides in TBS and incubation with rat anti-mouse IgG diluted 1:100 in TBS (H&L, 415-005-100 Dianova GmbH, Hamburg, Germany) for 30 minutes, followed by a further wash in TBS.

The peroxidase anti-peroxidase (PAP) method was then applied using mouse PAP complex diluted 1:500 in TBS (223-005-025, Dianova GmbH). Following a further wash in TBS all slides were removed from coverplates and incubated with 3`-diaminobenzidine tetrahydrochloride (DAB) (Fluka) with 0.01% of 30% Hydrogen peroxide H_2O_2, (Sigma) in 0.1% imidazole/HCL buffer (pH 7.1) for 10 minutes at room temperature. This was followed by a further three washes in TBS for 5 minutes per wash, then a final wash in distilled water for 5 minutes. The slides were counterstained with Papanicolaou`s haematoxylin diluted 1:20 in distilled water, and dehydrated in ascending ethanol and xylene before mounting.

Immunohistology was performed according to protocols developed by Dr.Anja Kipar, Department of Veterinary Pathology, University of Liverpool.

TUNEL method for detection of apoptosis in FCV infected cells.

The TUNEL assay was performed using a commercial kit (ApopTag^{TM}) according to the manufacturer`s instructions (In Situ Apoptosis Detection Kit; Oncor Heidelberg, Germany). Staining was performed on 3-5 μm sections from paraffinwax-embedded cell pellets on APES-coated slides as previously described (Köhler et al., 2000) (Kipar et al., 2001). Briefly, following deparaffinisation in xylene and rehydration through graded ethanols to water, the cell pellets on all slides were pretreated with proteinase

K (20μg/ml) for 15 minutes at room temperature to permeabilise the cells. This was followed by two washes in distilled water for 2 minutes per wash. Then endogenous peroxidase was inactivated by placing the slides in 2% H_2O_2 in PBS for 5 minutes. After rinsing twice in PBS for a further 5 minutes per wash, equilibration buffer (approximately 15μl/cm^2) was applied immediately to the slides which were then incubated under a plastic cover slip for 1 minute in a humid chamber. Excess liquid was carefully blotted from the slide and approximately 15μl/cm^2 of Terminal deoxynucleotidyl Transferase solution (TdT) was applied to each slide which was then incubated for 1 hour in a humidified chamber at 37°C. For negative controls, the TdT enzyme was replaced by distilled water.

To stop the reaction, all slides were placed into a Coplin jar in stop/wash buffer and incubated for 10 minutes at room temperature. Then approximately 15μl/cm^2 of anti-digoxigenin peroxidase conjugate was applied to each slide followed by incubation in a humidified chamber at room temperature for 30 minutes. All slides were washed in 4 changes of PBS in a Coplin jar for 2 minutes per wash at room temperature. The slides were developed in (DAB), counterstained and mounted as described above.

Dual staining for FCV antigen and TUNEL assay.

Dual staining for FCV antigen and TUNEL assay was carried out using cells cultured on glass slides. Although difficulties were encountered in producing such slides consistently, sufficient were produced for most of this study.

The commercial kit DeadEnd™ Colorimetric Apoptosis Detection System (Promega), was chosen for the TUNEL assay as this is designed for rapid detection of apoptotic cells grown on glass slides. Briefly, FCV and mock infected glass slides were placed in 4% paraformaldehyde in phosphate buffered saline (PBS; 154mM NaCl, 3 mM KCl, 9mMNa_2HPO_4, 1.65mMKH2PO4) to fix the cells for 25 minutes at room temperature. This was followed by two five-minute washes in PBS at room temperature. Then the cells were permeablised by immersing the slides in 0.2% Triton X-100 solution (Sigma) in PBS for 5 minutes at room temperature followed by a further two five-minute washes in PBS. A positive control slide was prepared using mock infected fixed cells was incubated with 100µl of DNase I buffer containing 1 unit/ml of DNase I (Promega) for 10 minutes at room temperature to demonstrate DNA fragmentation. Excess liquid was removed from all slides and the cells covered with 100µl of equilibration buffer for 5-10 minutes at room temperature. After blotting equilibrated areas, 100µl of TdT (Terminal deoxynucleotidyl Transferase) enzyme reaction mix was applied and covered with plastic cover slips. A negative control was prepared for both FCV and mock infected slides without the addition of TdT. The slides were incubated at 37°C for 60 minutes inside a humidified chamber to allow the end labelling reaction to occur. The reaction was then terminated by immersing the slides in 2X stop reaction buffer in a Coplin jar for 15 minutes at room temperature. This was followed by a further 3 washes in PBS for 5 minutes per wash. Streptavidin horseradish peroxidase solution was then applied to the slides at a dilution of 1:500 in PBS and incubated at room temperature for 30 minutes. The slides were washed in PBS three times for 5 minutes per wash. For detection, DAB was used and 100µl was applied to each slide until a light brown background appeared,

approximately after 10 minutes. Slides were then washed several times in deionised water.

Finally, a slide was processed for immunofluoresence detection of FCV antigen. Briefly, the slide was incubated with the rabbit antiserum Post-A ags 4 described in manuscript 1, at a dilution of 1:40. After incubation at 37°C for 1 hour, the slide was washed three times in PBS and 50μl of goat fluorescein isothiocyanate (FITC) conjugated anti- rabbit IgG (Sigma) diluted 1:40 with PBS was added to the slide and incubated for a further hour at 37°C. The conjugated antibody was washed off with three more washes in PBS, and 50% glycerol was added to each well. The slide was then viewed under a UV microscope to detect specific immunofluoresence.

Detection of anti-apoptotic protein Bcl-2.

The anti-apoptotic protein Bcl-2 was detected by immunohistology as previously described (Köhler et al., 2000). Briefly, Bcl-2 was demonstrated using the PAP method described earlier (see FCV antigen on pelleted cell sections), but the slides were pretreated in prewarmed citrate buffer (10 mM, pH 6.0) at 96°C for 30 minutes. The primary antibody used for was mouse anti-human Bcl-2 diluted 1:50 in TBS (clone 124, Dako Diagnostika GmbH, Hamburg, Germany) (Guinee et al., 1997). Secondary antibody and PAP complex were used as described above for FCV antigen detection on pelleted cell sections.

Detection of proliferating cellular nuclear antigen (PCNA).

Levels of cell proliferation were assessed by immunohistological demonstration of PCNA. For the demonstration of PCNA by immunohistology, previously published protocols were applied (Köhler et al., 2000) (Kipar et al., 2001). Briefly, the PAP method was applied as described earlier but the slides were pretreated in prewarmed citrate buffer (10 mM, pH 4.0) at 96°C for 15 minutes. The primary antibody used was mouse anti-PCNA diluted 1:100 in TBS (clone PC10, Dako Diagnostika GmbH, Hamburg, Germany) and secondary antibody diluted 1:100 in TBS was used as described above for FCV antigen detection on pelleted cell sections.

Statistical analyses.

A total of 10 fields were counted using light microscopy x40 for each time point on all slides for FCV antigen detection in FCV infected cells, apoptosis and Bcl-2 detection, and demonstration of PCNA in FCV and mock infected cells. The percentage of positive cells was calculated by dividing the number of positive cells by the total number of cells counted. Error bars on all graphs represent (+/-) 95% confidence intervals. Data was compared using exact binomial +/-ve 95% confidence interval (EPIINFO version 6). As multiple tests were being performed, the critical significance level were corrected by the Bonferroni method ($\alpha=\kappa$) where α= critical significant level, and κ= number of tests within an experiment.

Scanning electron microscopy of FCV infected cells.

Scanning electron microscopy of monolayers of FCV-infected cells 16 hours post-infection demonstrated rounded cells amongst the cell monolayer, some of which showed surface blebbing (Figs. 3a and 3b), but without dual staining for FCV, it is not possible to determine if such cells were infected with FCV.

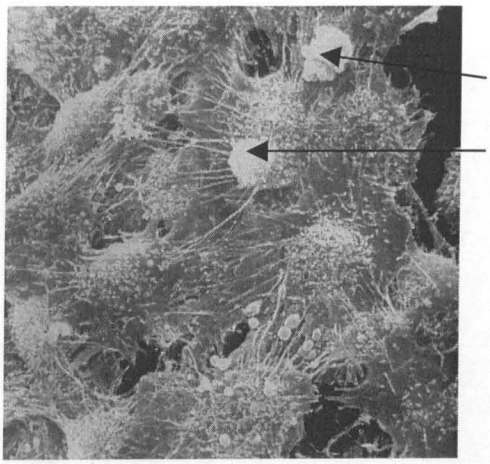

Fig. 3a

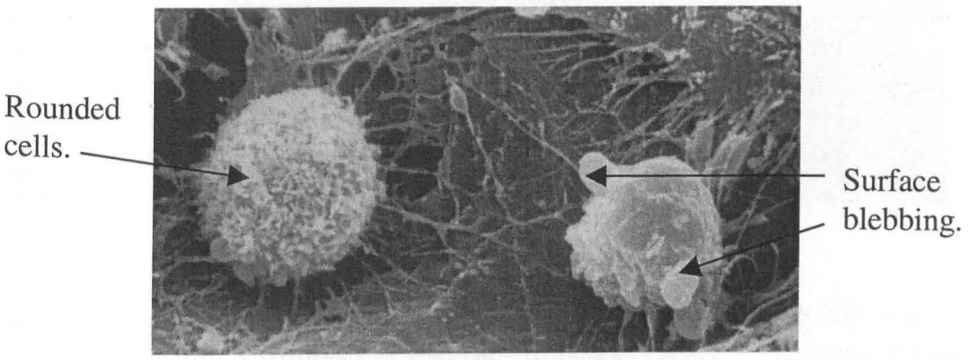

Fig. 3b

Figs. 3a and 3b. Scanning electron microscopy of FCV infected cells 16 hours post-infection. Scattered cells among the cell monolayer loose contact to the plate surface and exhibit a rounded shape (arrows). Some of these cells show surface blebbing. **Fig. 3a** x1000, **Fig. 3b** x2000.

Transmission electron microscopy of FCV infected cells.

Transmission electron microscopy of FCV infected cells (Fig 4a) revealed a spectrum of ultrastructural features. Cells normally exhibited slender cytoplasmic processes. They often contained large empty vacuoles, possibly representing dilated endoplasmic reticulum (Fig. 4a). Some cells were round and occasionally exhibited chromatin condensation, suggestive of cells in the early stages of apoptosis (Fig. 4a). Viral particles were found both single or as aggregates in the cytoplasm (Fig. 4b).

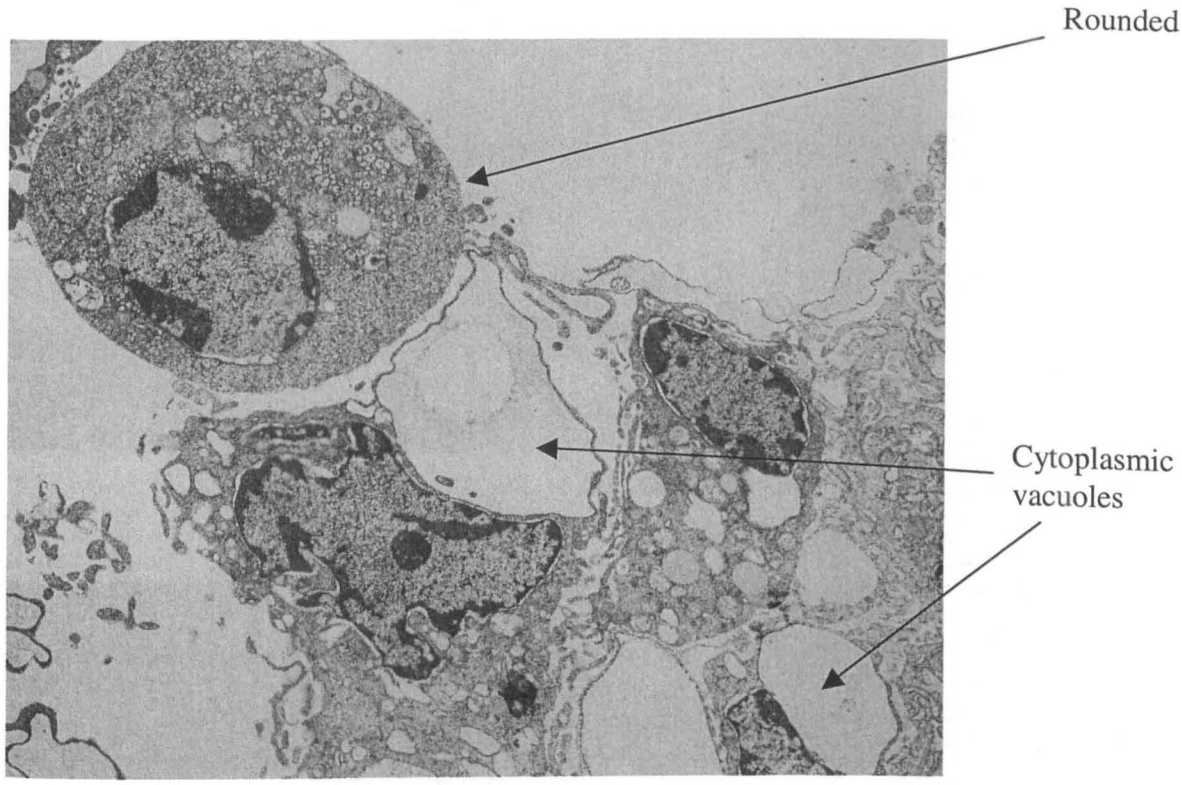

Fig. 4a

Fig. 4a. Transmission electron microscopy of FCV infected cells 24h post-infection. Several cells exhibit cytoplasmic vacuoles (arrows), likely to represent dilated endoplasmic reticulum. One cell has lost the slender cytoplasmic processes and exhibits a rounded shape. x6000.

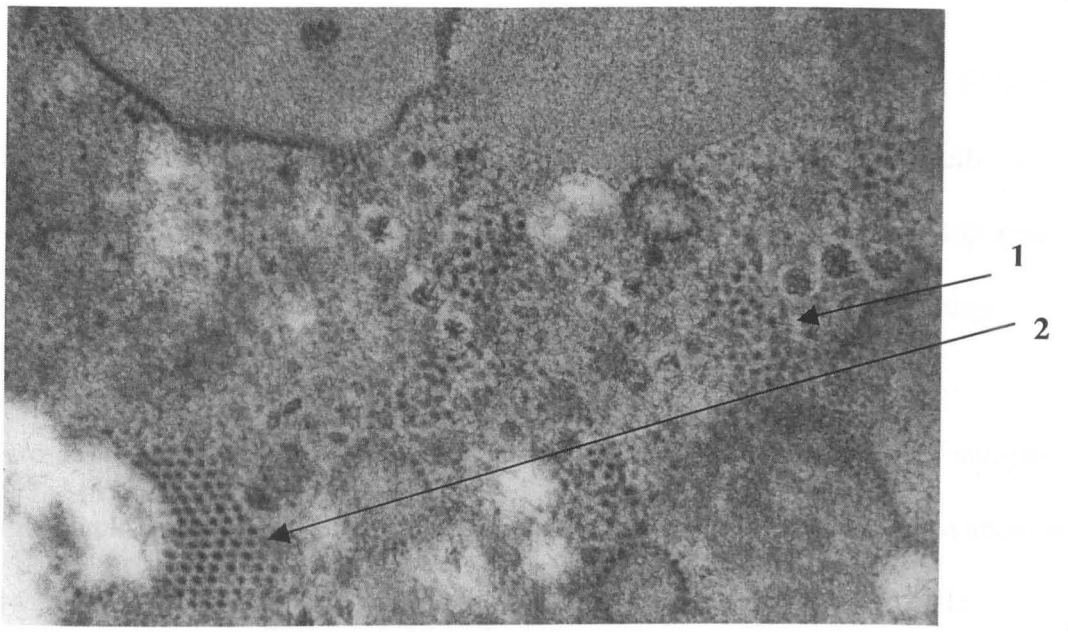

Fig. 4b

Fig. 4b. Transmission electron microscopy of FCV infected cells 17 hours post-infection showing single (**1**) and aggregates (**2**) of FCV particles. x150,000.

Time course of FCV antigen expression in FCV infected cells.

Representative images obtained from the immunohistochemical detection of FCV in infected cells are shown in Figs. 5a and 5b. As expected, antigen positive cells showed a predominantly cytoplasmic staining. Within positive cells, the cytoplasm often contained areas free of staining. These areas were reminiscent of the large vacuoles (dilated endoplasmic reticulum) detected by transmission electron microscopy, and suggestive of cytoplasmic vacuolation. The time course of antigen positive cells is represented in Fig. 5c. FCV antigen positive cells were most abundant between 16 and 24 hours post-infection. Very few FCV antigen positive cells appeared in pellets from infected FEA cell cultures up to 14 hours post-infection.

This time course of the production of antigen positive cells probably reflects the MOI used (0.5) which may have led to a two-step growth curve. Previous studies by Knowles et al (1988) indicated maximum antigen production of FCV F9 in FEA cells at 8 hours post-infection.

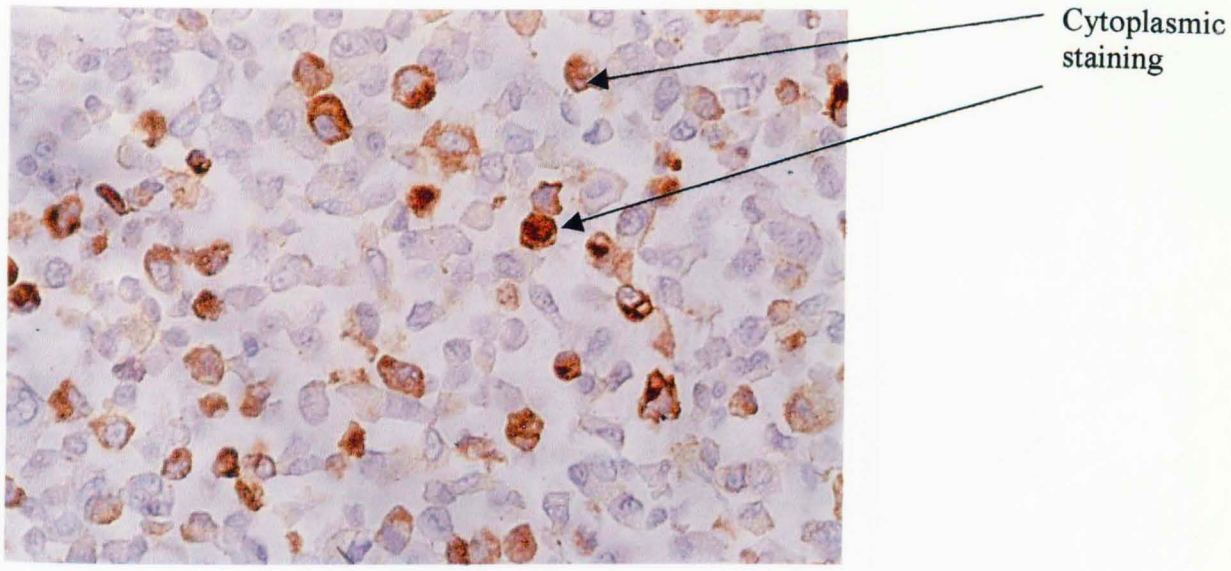

Fig. 5a

Fig. 5a. FCV antigen positive cells 16 hours post-infection. Numerous cells exhibited a moderate to strong cytoplasmic staining for FCV antigen. Papanicolaou`s haemotoxylin counterstain. x40.

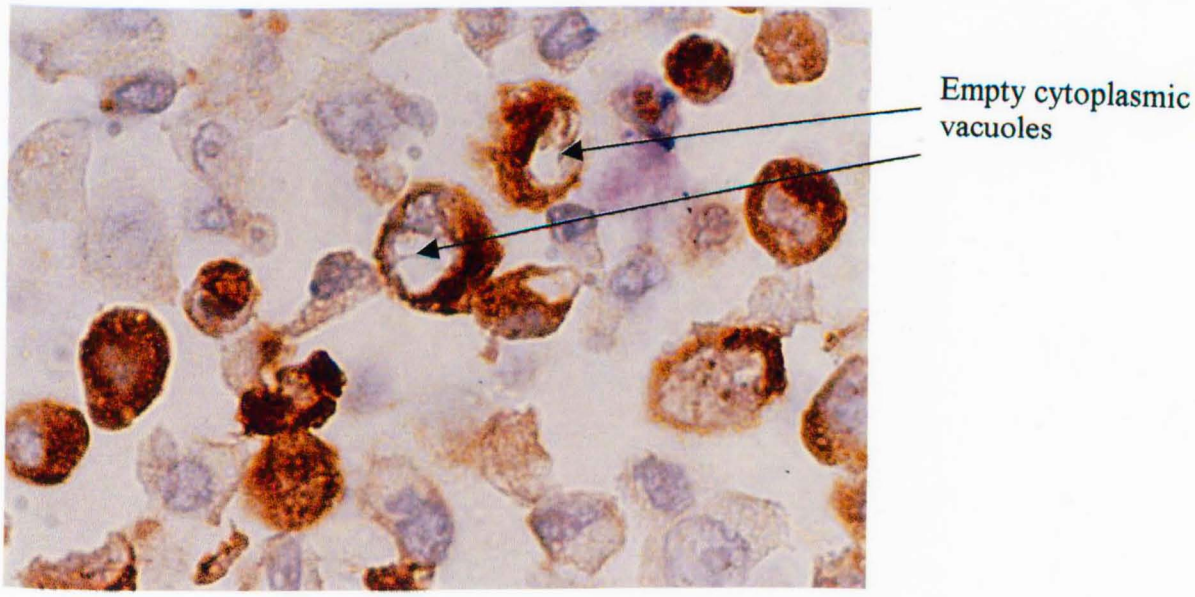

Fig. 5b

Fig. 5b. FCV antigen positive cells 24 hours post infection. FCV antigen-positive cells often exhibited large empty cytoplasmic vesicles. Papanicolaou`s haemotoxylin counterstain. x100.

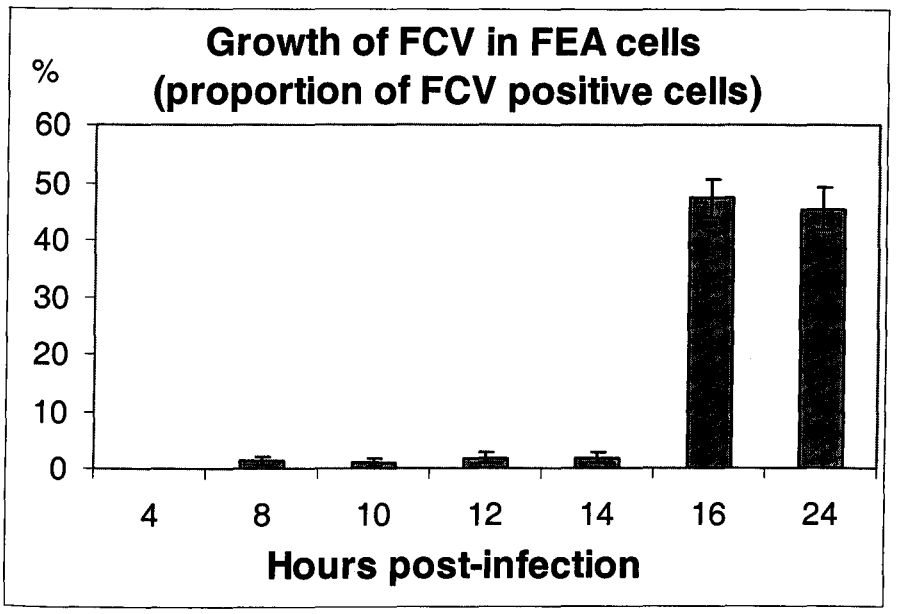

Fig. 5c

Fig. 5c. Proportion of FCV antigen positive cells in infected FEA cells. Error bars represent (+/-) 95% confidence intervals.

Apoptosis detection in FCV and mock infected cells.

(i). Giemsa staining for detection of apoptotic cells.

Giemsa staining of FCV infected and uninfected cells demonstrated the occurrence of occasional apoptotic cells in all samples (Fig 6a).

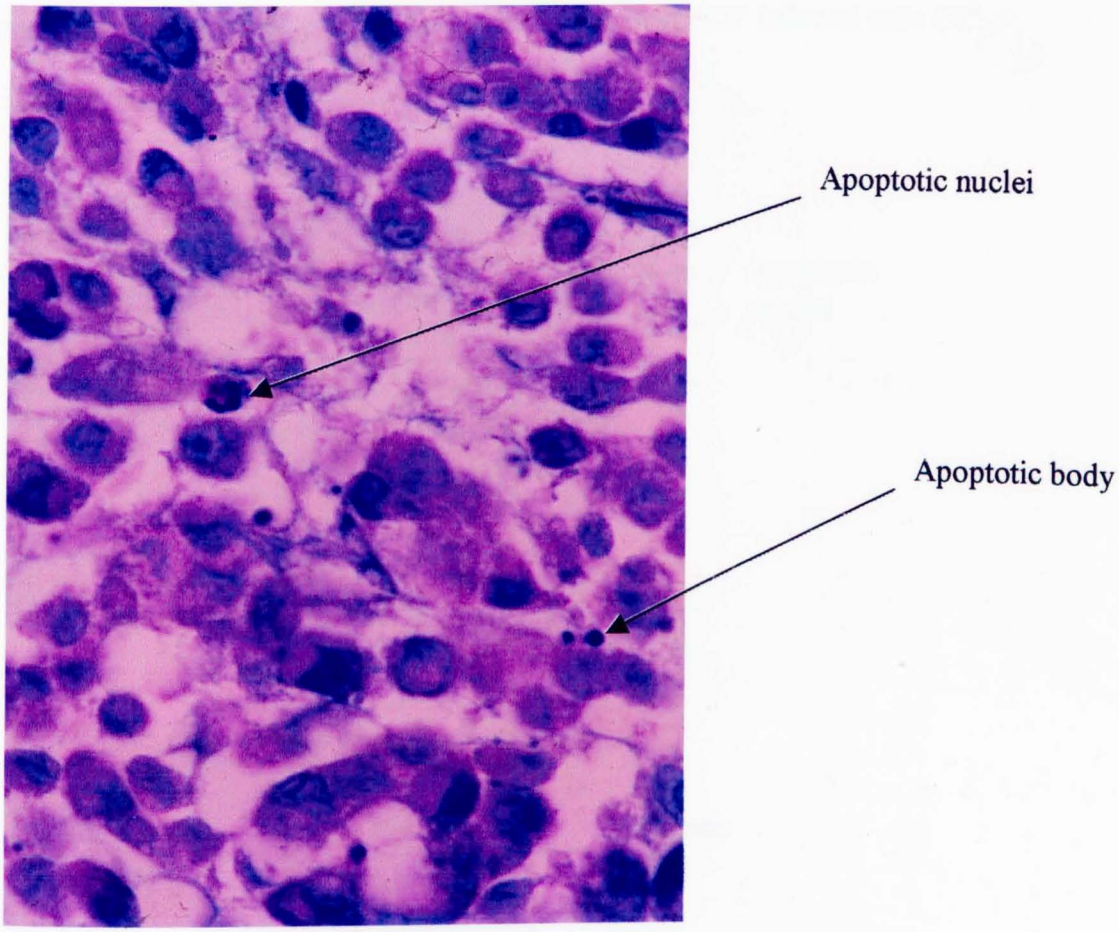

Fig. 6a

Fig. 6a. Giemsa stain of mock infected cells at time point 0 demonstrating occasional apoptotic cells. x40.

(ii). TUNEL method for detection of fragmented DNA in FCV and mock infected cells.

The TUNEL reaction performed on FCV and mock infected cells demonstrated occasional apoptotic cells (Figs. 6b and 6c). There was no significant difference in the proportion of apoptotic cells in FCV infected and mock infected cells at different time points (Fig. 6d), apart from the 8 hour time point when mock infected cells appeared to have a higher proportion of TUNEL-positive cells than FCV infected cells ($X^2_1 = 2.316$, $p = 0.0001$).

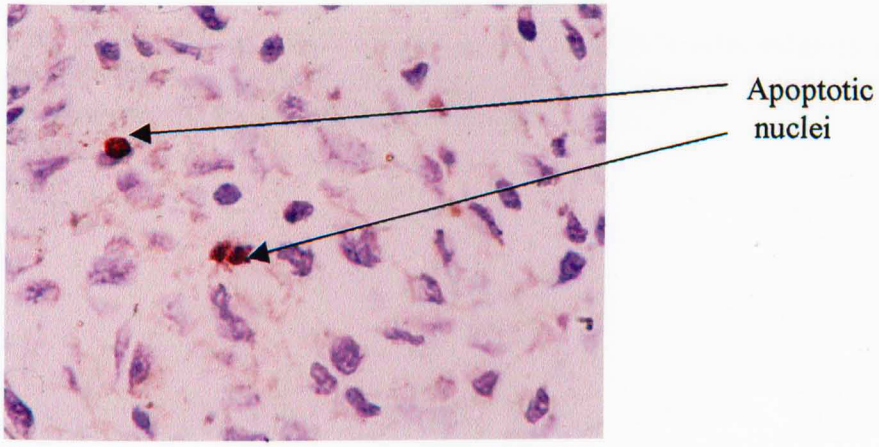

Fig. 6b

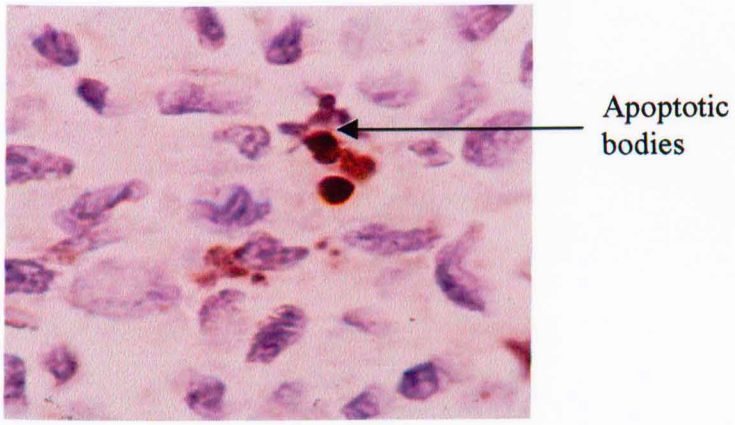

Fig. 6c

Figs. 6b and 6c. Demonstration of apoptotic cells using TUNEL labelling of mock infected cells at time point 0. Both Figs. represent the same sections but different fields. Papanicolaou`s counterstain. Light microscopy x40.

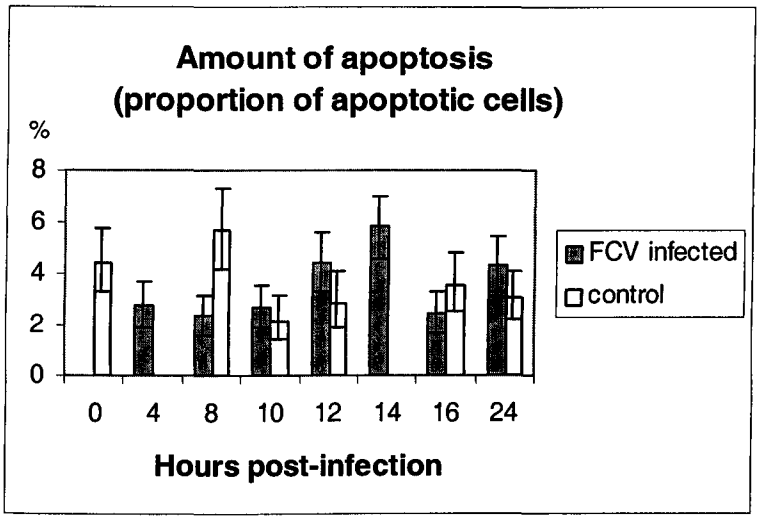

Fig. 6d

Fig. 6d. Percentage of apoptotic cells in mock and FCV infected cells stained for fragmented DNA using the TUNEL method at intervals over a period of 24hrs. Error bars represent (+/-) 95% confidence intervals.

(iii). Dual staining for FCV antigen and DNA fragmentation (TUNEL assay).

Dual staining of FCV infected FEA cell monolayer 24 hours post-infection for both FCV antigen and fragmented DNA using the TUNEL method demonstrated both antigen positive and TUNEL positive cells (Fig.7). The majority of cells were positive either for antigen or DNA fragmentation with only the occasional cell showing dual staining (Fig 7). The positive control for the TUNEL assay using DNase I caused DNA fragmentation in the majority of uninfected FEA cells (data not presented). Negative controls without the TdT enzyme for the TUNEL assay remained unchanged for both FCV and mock infected FEA cells (data not presented).

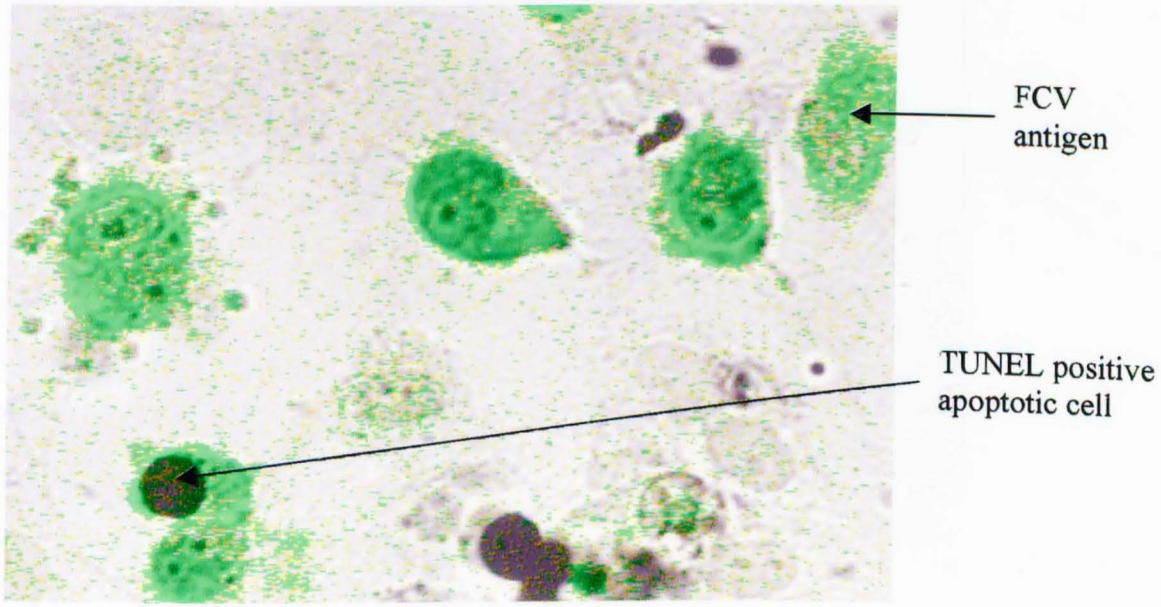

Fig. 7

Fig. 7. Demonstration of dual staining for FCV antigen and TUNEL assay. Positive cells for FCV antigen are shown in green and putative positive apoptotic cells are black.

Detection of anti-apotototic protein Bcl-2.

Both FCV infected and mock infected cells showed positive staining for the anti-apoptotic protein Bcl-2 over a period of 24hrs (Fig. 8a). The proportion of positive cells in both infected and uninfected cultures appeared to rise over a 16 hour period (Fig 8b). At both 8 hours and 16 hours, there was a significant higher proportion of Bcl-2 positive cells in FCV infected cell culture ($X^2_1 = 7.516$, $p= 0.0061$) and ($X^2_1 = 27.949$, $p<0.01$) respectively, compared to uninfected cells, whereas at 12 hours this was reversed ($X^2_1 = 10.424$, $p=0.0012$). However, overall interpretation of the data was difficult due to the absence of a data set at some of the time points.

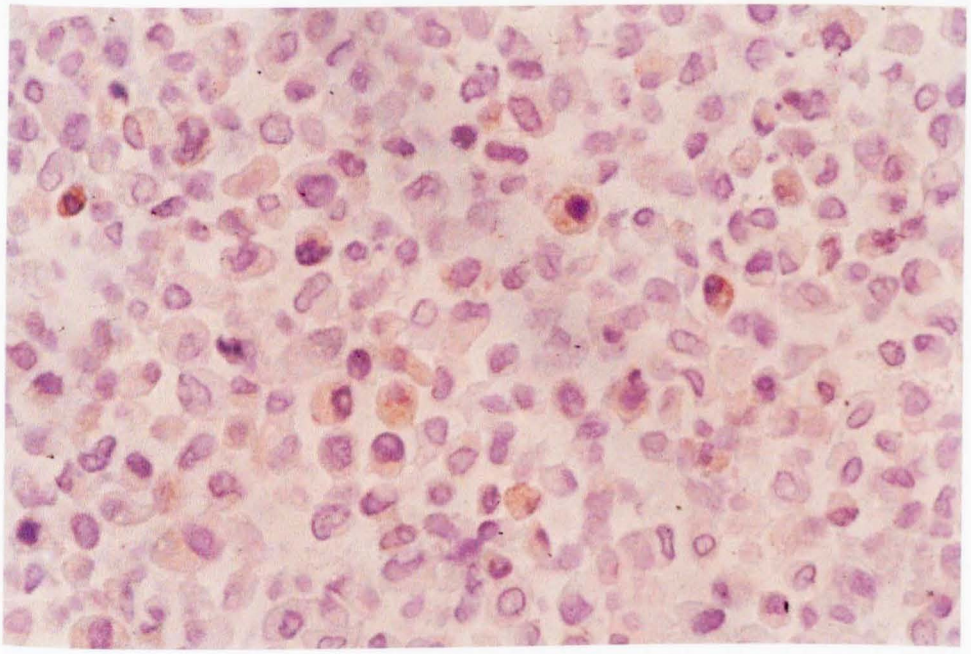

Fig. 8a

Fig. 8a. Staining for the anti-apoptotic marker Bcl-2 16 hours post-infection. Numerous cells are positive for Bcl-2, the staining is cytoplasmic. Papanicolaou`s haemotoxylin counterstain. x40.

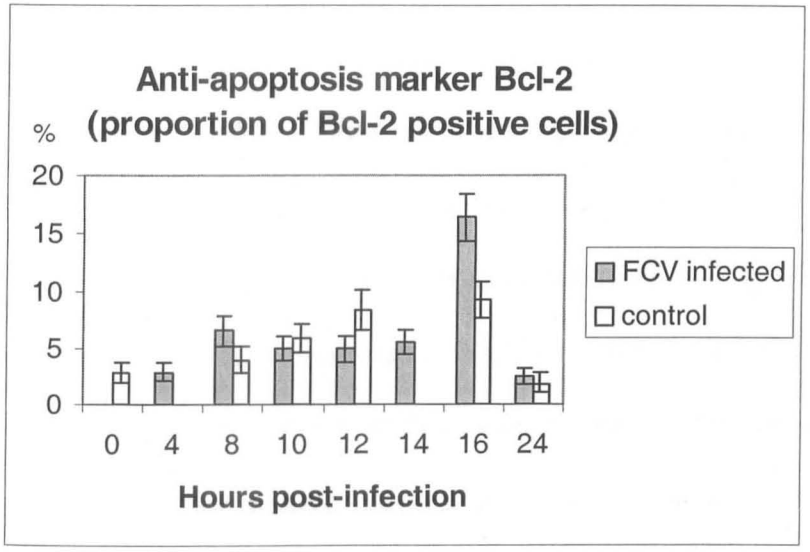

Fig. 8b

Fig 8b. Percentage of FCV infected and mock infected cells stained for the anti-apoptotic protein Bcl-2 at intervals over a period of 24hrs. Error bars represent (+/-) 95% confidence intervals.

Detection of cell proliferation (demonstration of PCNA).

Both FCV infected and mock infected cells demonstrated positive staining for the cellular proliferation marker PCNA (Fig. 9a). The proportion of positive cells in all FCV infected samples at the time points 8, 12 and 24 hours (Fig. 9b) was significantly higher when compared to uninfected cells ($X^2_1 = 206.41$, $p<0.01$) ($X^2_1 = 225.82$, $p<0.01$) ($X^2_1 = 204.43$, $p<0.01$). Only this set of data could be compared due to the absence of FCV mock infected controls for the time points 4, 10, 14 and 16 hours (Fig 9b).

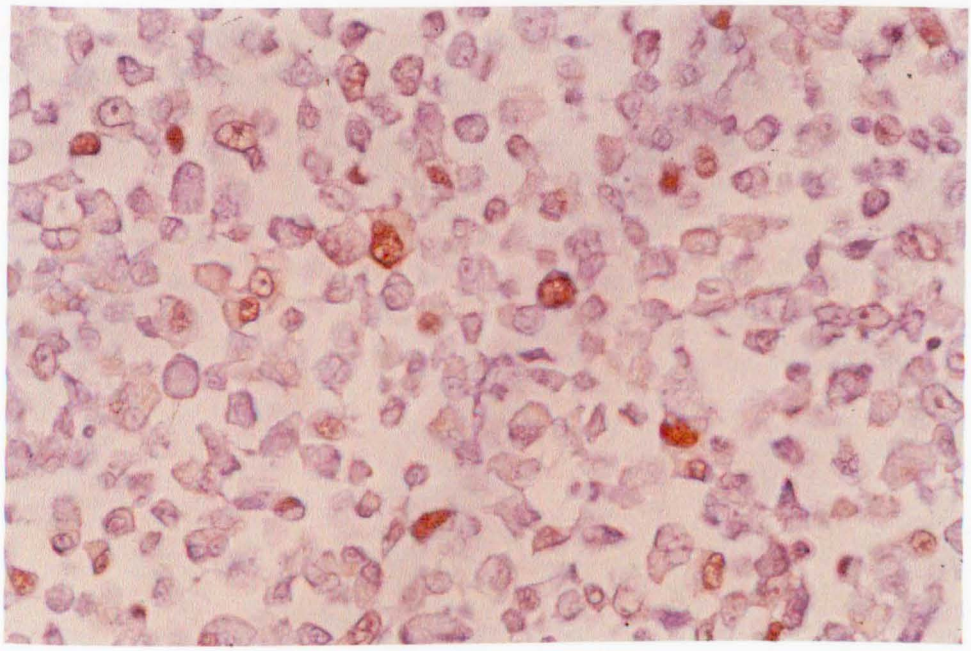

Fig. 9a

Fig. 9a. Demonstration of cell proliferation by immunohistological demonstration of PCNA at 16h post-infection. Numerous cells exhibit a faint to intense nuclear staining for PCNA. Papanicolaou`s haematoxylin counterstain. 40x.

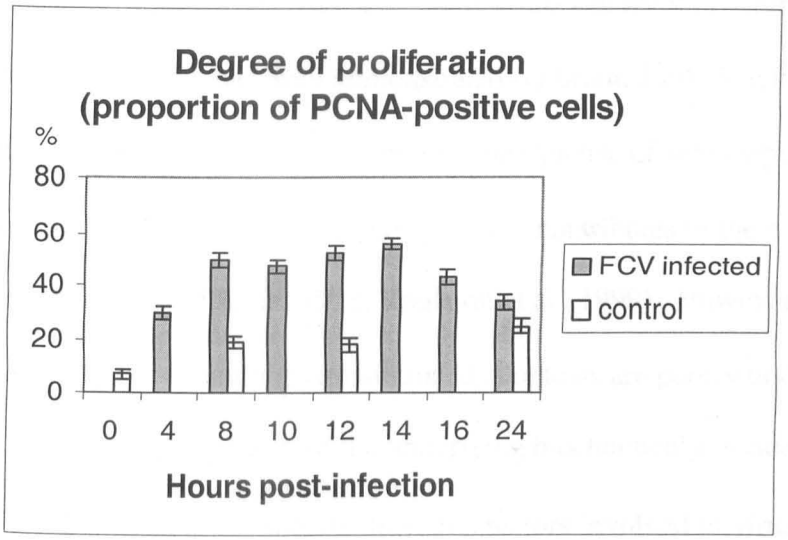

Fig. 9b

Fig. 9b. Percentage of FCV infected and mock infected cells stained for the levels of cell proliferation (PCNA expression) at intervals over a period of 24hours. Error bars represent (+/-) 95% confidence intervals.

DISCUSSION.

The ability of numerous RNA and DNA viruses to elicit or inhibit apoptosis in cell culture and *in vivo* has been demonstrated (O'Brien, 1998, Roulston et al., 1999), signifying that apoptosis is a common consequence of virus replication. It has also been suggested that virus-induced apoptosis contributes to the cytopathic effect (CPE) of these viruses (O'Brien, 1998, Roulston et al., 1999). However, the mechanisms controlling and executing virus-induced apoptosis are poorly understood, partly because of the complexity of the underlying biochemical cascades (Budihardjo et al., 1999). In addition, the specific host cell factors involved in virus-associated apoptosis have not been identified (Teodoro & Branton, 1997).

The role of apoptosis in calicivirus replication is not well understood. In this study, we investigated whether apoptosis contributes to the death of cultured FEA cells infected by FCV. Our results suggest that the CPE that follows FCV infection *in vitro* is not mediated by the induction of apoptosis. In contrast, during completion of this work, two recent reports have proposed that FCV replication does induce apoptosis in cultured cells (Al-Molawi et al., 2003, Sosnovtsev et al., 2003). However, dissimilar approaches were used in these studies compared to those described in this report. In addition, both studies used a different cell line, with a higher multiplicity of infection (MOI), and in the latter study, a different virus strain was used: some of these factors may have accounted for the differences in our findings.

The first study by Al-Molawi et al (2003) proposed that FCV infection triggered an apoptotic response in the host cell that was mediated by caspases. Caspases were implicated in the cleavage of the viral capsid protein in infected cells, and studies *in*

vitro with recombinant caspases identified which caspase cleaves the FCV capsid protein. However, further studies will be necessary to identify the exact cleavage site(s). The study by Sosnovtsev et al (2003) also demonstrated that FCV infection was associated with increases in the activities of some members of the caspase family. In addition, changes characteristic of apoptosis were observed in FCV infected cells including translocation of phosphatidyl serine to the cell outer membrane, chromatin condensation, and DNA fragmentation.

The present work describes different experimental approaches for the detection of apoptosis in FCV infected cells. The results obtained suggest that, in contrast to the above reports, FCV does not influence apoptosis in FEA cells. Firstly, the TUNEL assay was employed for the appearance of cellular DNA fragmentation. Only a small percentage of stained nuclei were observed, and these were in approximately equal proportions in both infected and mock-infected cells. In addition, in dual staining studies, apart from the occasional cell, there was no evidence to support co-localisation of FCV antigen in apoptotic cells, with many cells being apoptosis positive, but FCV antigen negative, and many other cells being apoptosis negative, but FCV antigen positive.

Further supporting evidence that the effect of FCV on the cell is not primarily apoptosis in our virus host system, was obtained when we assessed the expression of the anti-apoptotic protein Bcl-2. This was detected at high levels 16 hrs post infection, coinciding with maximal FCV expression, and in relatively low levels in mock infected cells. Members of the Bcl-2 family are involved in different pathways of induction and inhibition of apoptosis. Usually, Bcl-2 and Bcl-XL inhibit apoptosis

whereas Bax promotes cell death (Adams & Cory, 1998, Raff, 1998). Our results suggest that FCV infection either has no effect on the apoptotic pathway or may inhibit or delay apoptosis by upregulation of anti-apoptotic proteins.

In addition, we evaluated the proportion of cells showing proliferating activity by staining for PCNA. PCNA is essential for the correct functioning of DNA polymerase delta, which plays a role in leading strand DNA synthesis (McCormick & Hall, 1992). It is produced in the late (G1) phase, but is mainly expressed during the (S) phase of the cell cycle (McCormick & Hall, 1992), and it is thought to shield cells from apoptosis. Our studies showed that PCNA positive cells were present in significantly higher proportions in FCV compared to uninfected control cells. Taken together, our results suggest that FCV might retain infected cells from apoptosis and stop the cell cycle in the S phase which will provide the optimal conditions for FCV replication.

In conclusion, this is the first study to suggest that FCV-infected FEA cells may inhibit apoptosis through upregulation of Bcl-2 and PCNA. Other recent studies have suggested that FCV replication does induce apoptosis in infected cells. However, different methodological approaches were used, and also a dissimilar cell line with a higher MOI, and in one study a different virus strain. Therefore further investigations are needed, possibly with different cell lines and virus strains, to confirm whether or not FCV does induce apoptosis in cultured cells.

MANUSCRIPT 3

Identification of putative RNA secondary structures amongst feline calicivirus isolates.

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ABSTRACT.

Many positive-stranded RNA viruses are predicted to contain secondary structures at the extreme ends of the genome that have been shown to be important in genomic replication. To date, there has been no attempt to systematically identify putative secondary structures within these regions of the FCV genome. This purpose of this study was to analyse the 5`ends of all published FCV full-length sequences for the existence of any potential secondary structures. Areas containing putative secondary structures were identified using the RNAdraw programme that uses energy rules to determine optimal secondary structures for RNA molecules. We have obtained evidence for seven putative stem-loop structures that were conserved amongst all FCV isolates analysed. In contrast, only one conserved stem-loop was predicted at the 5`end of the sub-genomic RNA.

INTRODUCTION.

The *Caliciviridae* is a family of positive-sense single stranded RNA viruses that contains a number of important pathogens of man and animals. Viruses within the family can been divided into four genera based on their genomic arrangement (Clarke & Lambden, 1997) and phylogenetic analysis. The vesiviruses (e.g. feline calicivirus – FCV) and noroviruses (e.g. Norwalk virus) contain three open reading frames (ORFs) encoding the non-structural proteins, the major capsid protein and a minor capsid protein (Carter, 1994). In contrast, there are only two ORFs in the lagoviruses (e.g. rabbit haemorrhagic disease virus – RHDV) and sapoviruses, the non-structural and major capsid proteins being fused together into one long ORF (Liu et al., 1995, Meyers et al., 1991b). As well as the genomic RNA, a 3' co-terminal subgenomic mRNA has been demonstrated for both FCV and RHDV (Carter, 1990, Meyers et al., 1991b) and it is likely that this is a common feature of all members of the *Caliciviridae*. The sub-genomic mRNA has been shown to direct translation of the major capsid protein (Herbert et al., 1997). Both the genomic and sub-genomic mRNA are bound at their 5'end to a virally encoded protein (VpG) (Herbert et al., 1997, Meyers et al., 1991a) encoded within ORF1 (Dunham et al., 1998) and are polyadenylated at their 3' end (Black et al., 1978, Ehresmann & Schaffer, 1977). The mechanism by which the sub-genomic RNA is produced in infected cells is unknown (Black et al., 1978).

Despite the importance of this family of viruses as causes of disease, little is known about the key steps in the viral life cycle including viral RNA replication, genome packaging, and initiation of viral protein translation. In many other positive stranded RNA viruses, signals key to these viral processes are found in RNA secondary

structures located at the ends of their genome. This is perhaps best characterised in the picornaviruses, particularly poliovirus, where 3' stem-loops and a 5' 'cloverleaf' structure have been identified in the positive sense genome. These interact with both the viral RNA polymerase and cellular proteins to form ribonucleoprotein complexes that are essential for negative-sense and plus-sense genome synthesis respectively (Xiang et al., 1997). In addition, the 5'-cloverleaf structure is critical to the switch from genome replication to translation and the binding of the VpG to the 5'-end of the genome (Gamarnik & Andino, 1998, Xiang et al., 1997). Other critical elements of secondary structure also located in the 5' non-translated region immediately downstream of the poliovirus cloverleaf are responsible for initiation of translation at internal start codons (internal ribosome entry site – IRES) (Stewart & Semler, 1997). Secondary structures that are involved in the initiation of negative strand RNA replication have also been identified at the 3`end of flavivirus genomes (Blight & Rice, 1997, Kolykhalov et al., 2000, Zeng et al., 1998).

In caliciviruses there are few studies that have sought to identify these potential areas of secondary structure that may act as key signal motifs for viral replication. In a study of the sequence of the prototype norovirus, Norwalk virus, conserved stem-loop structures were identified at the 5' end of the genomic RNA, the 5' end of the putative sub-genomic RNA, and the 3'end of the genome leading the authors to speculate that these structures may play a role in both genomic and sub-genomic RNA replication (Jiang et al., 1993). Recently, the 110 nucleotides at the 5' end of the Norwalk virus genome, which includes these putative secondary structures, has been shown to bind cellular proteins leading to the suggestion that, as in picornaviruses, these viral RNA-host protein interactions were likely to be important for viral genome replication and /

or translation (Gutierrez-Escolano et al., 2000). Secondary loop structures have also been predicted at the 3' end of the genomic RNA of FCV, RHDV and several human caliciviruses (Pletneva et al., 2001, Seal et al., 1994). However, these authors also noted that this region of the genome lacks sequence conservation and the predicted secondary structures were highly variable, thus questioning their significance.

Although there are many similarities between the caliciviruses and picornaviruses, a major difference between these two virus families is the presence of the calicivirus sub-genomic RNA. Interestingly, the 5' end of the genomic and sub-genomic RNA molecules of many caliciviruses shows a variable but significant degree of sequence conservation with one another (Clarke & Lambden, 1997, Clarke & Lambden, 2000) (Hardy & Estes, 1996, Lambden et al., 1995, Rinehart-Kim et al., 1999). This is best characterised in the human caliciviruses and RHDV leading to suggestions that the same sequence motifs required for plus strand genome replication are also necessary for plus strand sub-genome replication.

Despite the likely importance of the 5' end of both the genomic and sub-genomic RNA molecules to key processes in viral replication, there has been no attempt to systematically identify secondary structures within this region of the FCV genome. The aim of this study was therefore to analyse these regions from all FCV isolates that have been fully sequenced to date. Areas containing putative secondary structures were identified using the RNAdraw programme. This uses energy rules to determine optimal secondary structures for RNA molecules (Matzura & Wennborg, 1996). In addition, we also reassessed the sequence of the 5' ends of both the genomic and sub-genomic RNA molecules to identify regions of sequence conservation. Such studies

will be important to identify genomic regions likely to be important in viral replication.

METHODS.

Virus sequences.

Currently available complete genomic sequences of FCV isolates were obtained via the Calicivirus sequence database (http:www.iah.bbsrc.ac.uk/virus/Caliciviridae/). The strains, their accession numbers and cited publications are given in Table 1.

Virus strain	Year of isolation	Country of isolation	Clinical origin	Accession numbers	Reference for full sequence
F65	1990b	UK	Lameness & OD	AF109465	(Glenn et al., 1999)
F4	1971	Japan	URTD	D31836	(Oshikamo et al., 1994)
F9	1958c	USA	URTD & OD	M86379	(Carter et al., 1992)
Urbana	1960`s	USA	URTD & OD	L40021	(Sosnovtsev & Green, 1995)
CFI/68	1960	USA	URTD	U13992	(Neill, 1990)
2024	NA	NA	NA	AF479590	(Thumfart & Meyers, 2002)

Table 1. Origins of FCV sequences used in this study. URTD; Upper respiratory tract disease. OD; Oral disease. NA; not available.

Secondary structure predictions.

Predictions of RNA secondary structure were performed using RNAdraw version V1.1 (Matzura & Wennborg, 1996). This programme uses the energy rules to determine optimal and suboptimal secondary structures for RNA molecules at 37°C in 1M Na^+ and 0M Mg^{++}. The only allowable base pairs are A-T, G-C and G-T. The secondary structures for FCV were predicted using sequence lengths of 60, 150, 250

and 500 nucleotides for the 5' end of the genomic RNA and sequence lengths of 60, 150 and 250 nucleotides for the 5' end of the sub-genomic RNA.

Sequence comparisons.

Sequences alignments were performed using programmes PILEUP and PRETTY available in the Genetics Computer Group software package (Deveraux et al., 1984). For comparisons of the 5' end of the genomic and sub-genomic RNA molecules, these alignments were manually adjusted to improve areas of sequence identity in the alignment.

RESULTS.

The 5' end of the genomic RNA.

Results of secondary structure prediction identified seven putative stem loops in the first 300 nucleotides of the FCV genome (Fig. 1). The position of these stem loops was conserved in all six sequences analysed and was still preserved when the first 500 nucleotides of genomic sequence were analysed (Fig. 2). Other potential stem-loops identified in the sequences between nucleotides 300 and 500 were not conserved in different FCV isolates (Fig. 2). The positions of the seven putative conserved stem-loop forming sequences in the FCV genomic sequence are shown in Fig. 3. The first three stem-loops are predicted to form in areas of 100% sequence identity between the six FCV isolates. Loops four to seven are predicted in areas of lower sequence similarity. In results of less favourable structure predictions, loops 1, 3, 4, 5 and 7 remained largely unaltered whereas loops 2 and 6 became inconsistent (data not presented).

Fig. 1. Predicted secondary structures based on the first 300 ribonucleotides at the 5'-end of the genomic RNA. Free energy values for each prediction were F9 –88.27; 2024 –87.25; Cfi68 –85.7; F4 –72.42; F65 –86.98; Urbana –87.77. Each loop is numbered 1-7 according to the sequence in Figs. 3 and 6.

Fig. 1. *Legend on opposite page.*

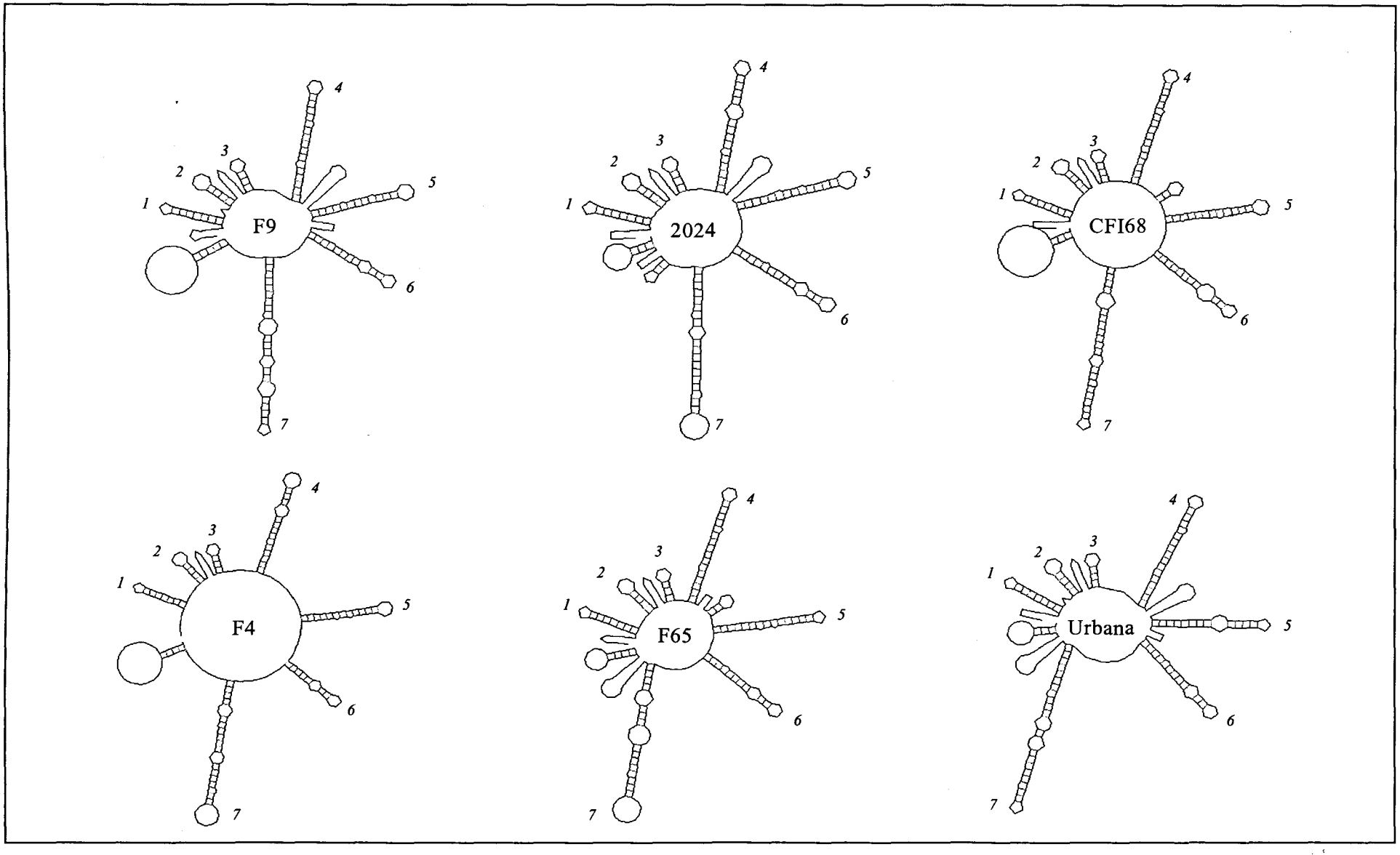


Fig. 2. Predicted secondary structures based on the first 500 ribonucleotides at the 5'-end of the genomic RNA. Free energy values for each prediction were F9 –134.82; 2024 –133.54; Cfi68 136.865; F4 –114.97; F65 –122.95; Urbana –132.003. Each loop is numbered 1-7 according to the sequence in Figs. 3 and 6.

Fig. 2. *Legend on opposite page.*

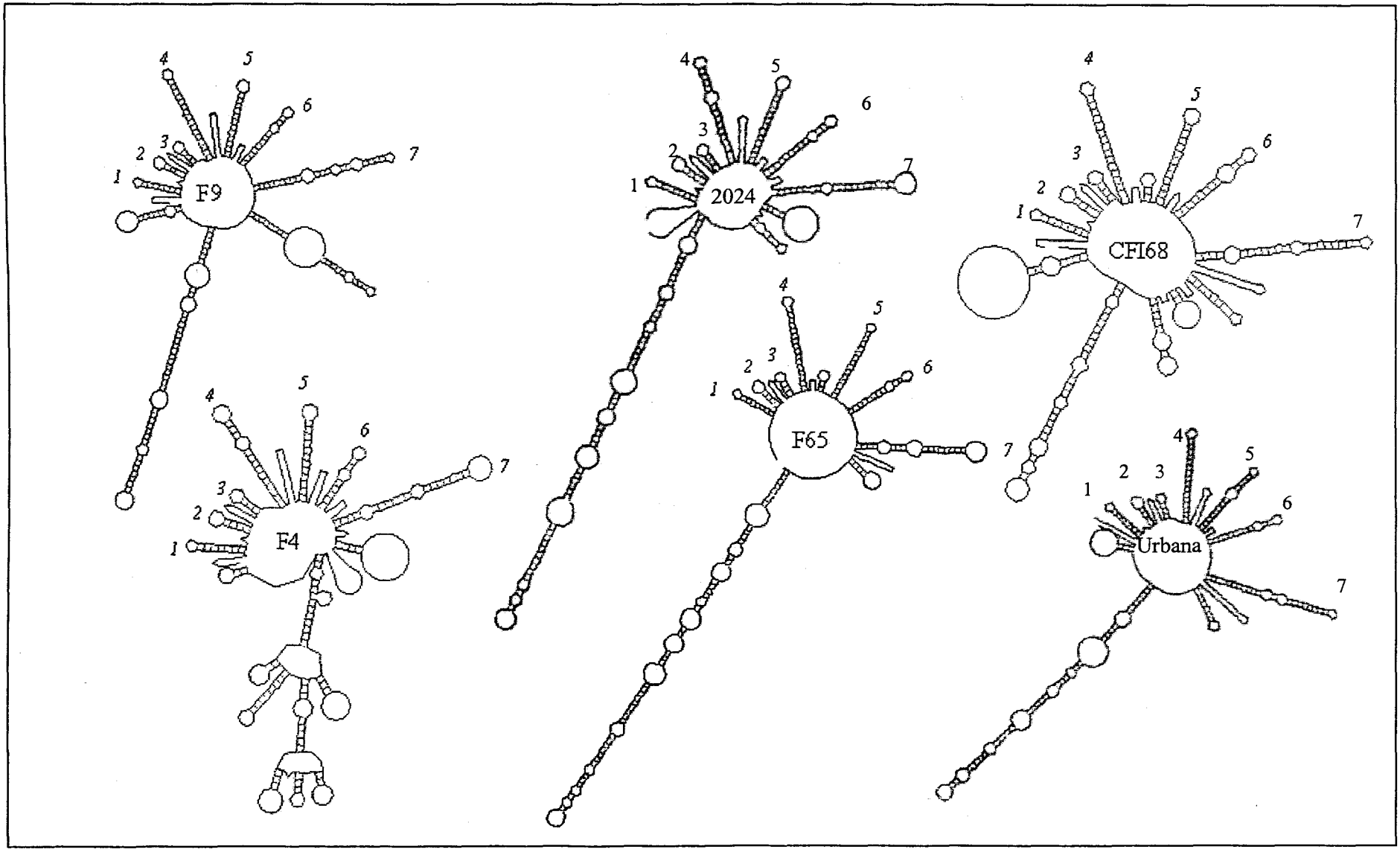
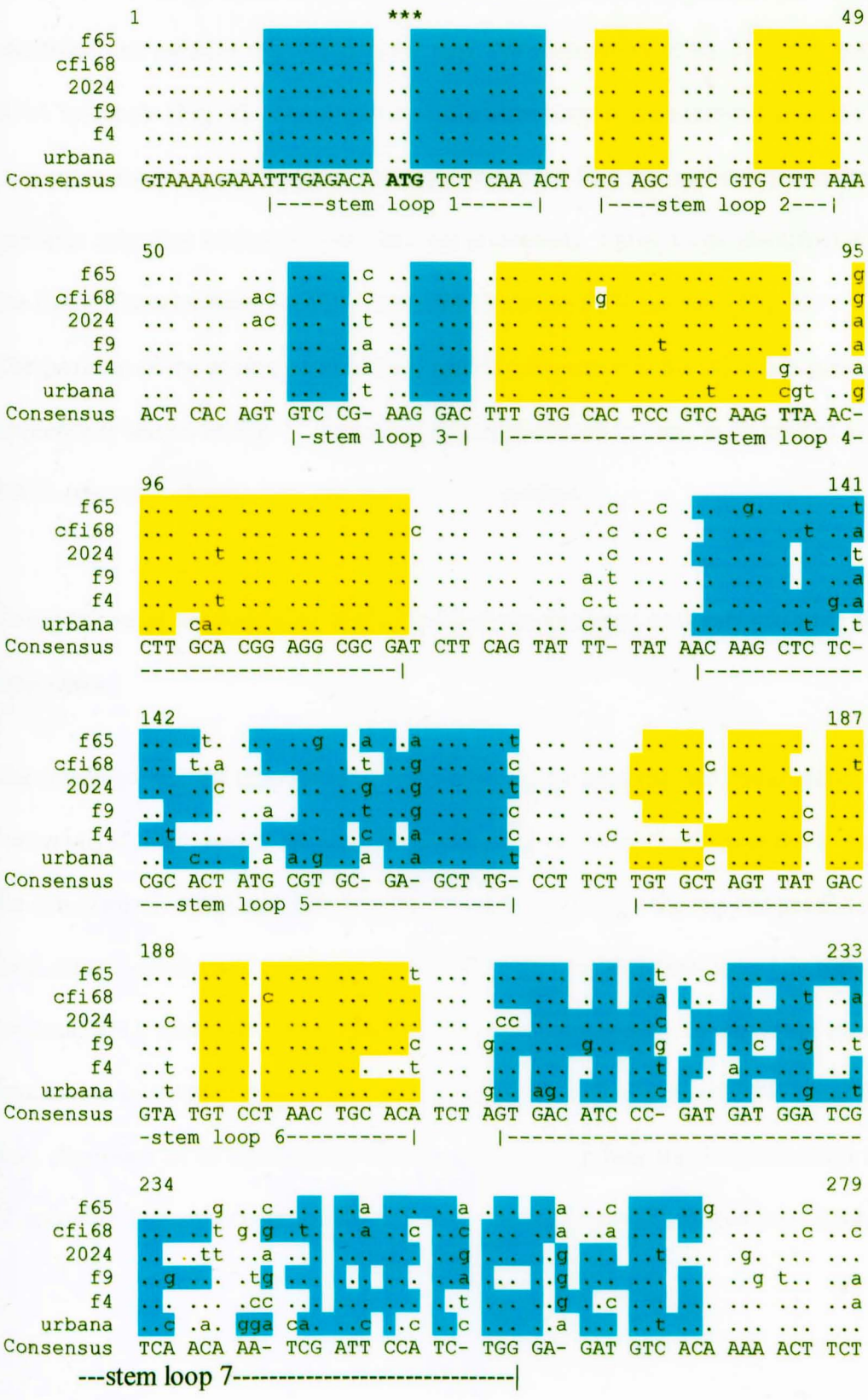


Fig. 3. Sequence alignment of the first 279 nucleotides of the FCV genomic RNA molecule. Each sequence is compared to the consensus sequence, a dot (.) representing identity to the consensus. The regions predicted to form the stems of stem-loops 1 to 7 are shaded and also indicated below the alignment. The first putative start codon of ORF1 is in bold and thereafter the alignment is arranged in triplets according to this ORF.

Fig. 3. Legend on opposite page.



The 5' end of the sub-genomic RNA.

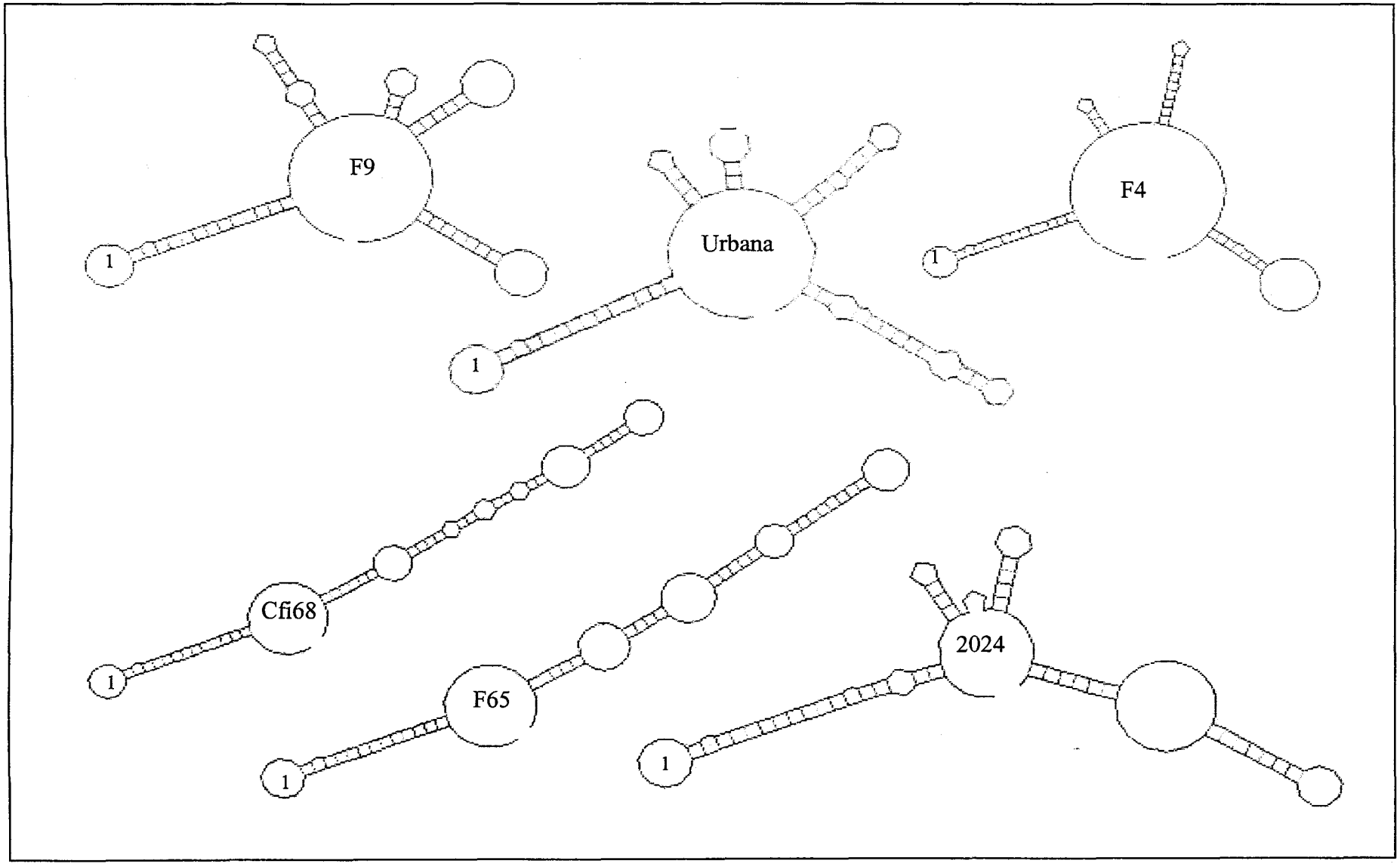
In contrast to the genomic RNA, results of secondary structure prediction only identified one putative stem-loop in the first 150 nucleotides of the FCV sub-genomic RNA molecule (Fig. 4). The position of this stem-loop was conserved in all six sequences analysed and was still preserved when the first 500 nucleotides of the sub-genomic sequence were analysed (data not presented). Other loops identified within the first 150 nucleotides were not conserved between FCV isolates (Fig. 4).
The position of the predicted stem-loop forming sequence in the FCV sub-genomic sequence is shown in Fig. 5. The stem-loop is predicted to form in an area of almost 100% sequence identity between the six FCV isolates.

Comparison of the sequence at the 5'end of the genomic and sub-genomic RNA molecules.

The results of manual alignment of the first 56 nucleotides that are completely conserved at the 5' end of the genomic RNA with the 56 nucleotides at the 5' end of the sub-genomic RNA are shown in Fig. 6. Also shown are the regions predicted to form stem-loops 1 and 2 in the genomic RNA molecule and stem-loop 1 in the sub-genomic RNA molecule. The alignment was created manually to maximise areas of sequence conservation and to align the two putative start codons of ORF 1 and 2. The final alignment of 58 nucleotides contained six gap residues and 36 nucleotides (62%) of complete sequence conservation between the genomic and sub-genomic RNA

Fig. 4. Predicted secondary structures based on the first 150 ribonucleotides at the 5'-end of the sub-genomic RNA. Free energy values for each prediction were F9 –31.05; 2024 –27.39; Cfi68 –27.67; F4 –27.53; F65 –30.04; Urbana –24.6. Loop 1 is numbered according to the sequence in Figs. 5 and 6.

Fig. 4. *Legend on opposite page.*



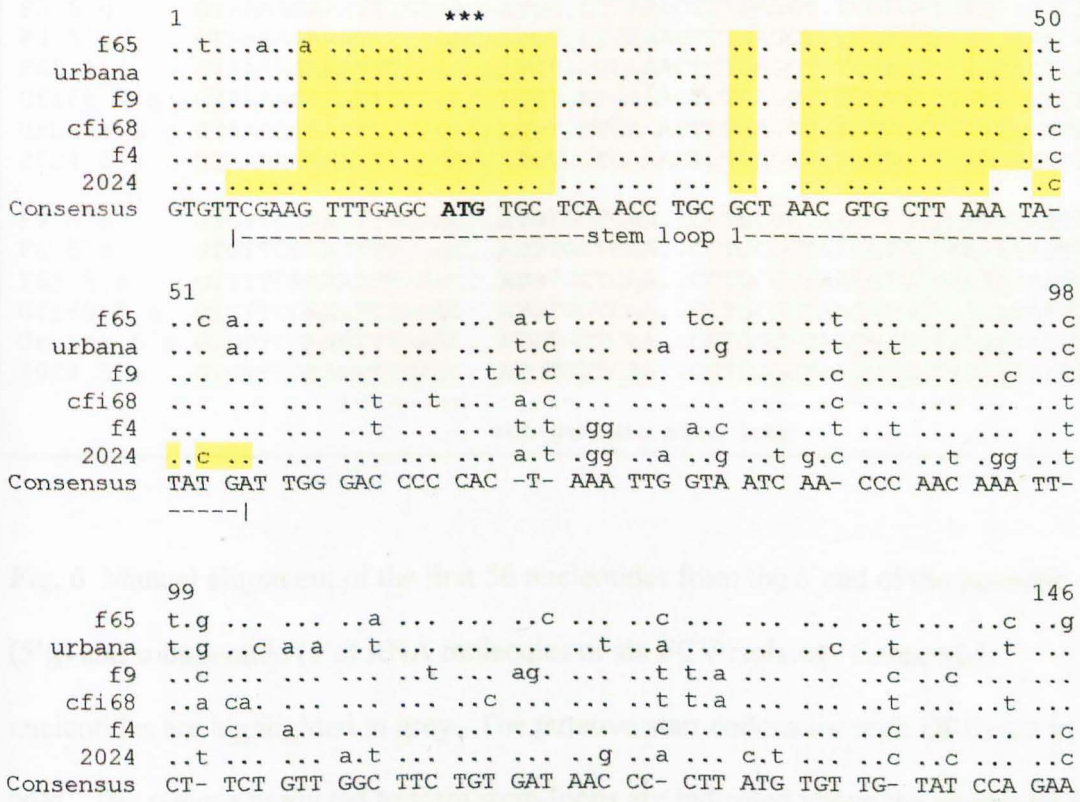


Fig. 5. Sequence alignment of the first 146 nucleotides of the FCV sub-genomic RNA molecule. Each sequence is compared to the consensus sequence, a dot (.) representing identity to the consensus. The region predicted to form the stem of stem-loop 1 is shaded and also indicated below the alignment. The first start codon of ORF2 is in bold and thereafter the alignment is arranged in triplets according to this ORF.

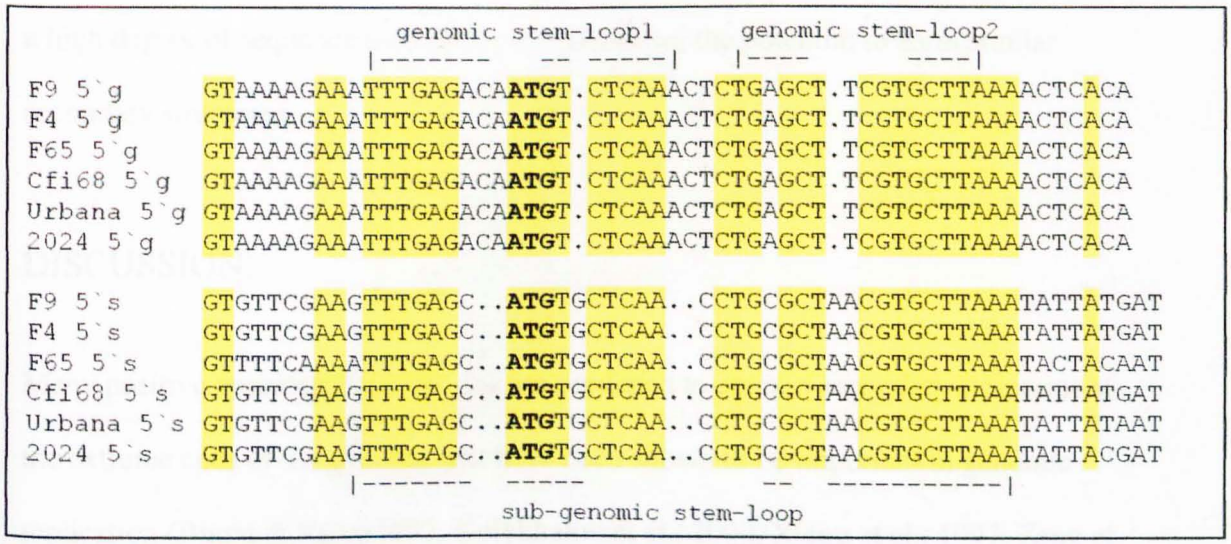


Fig. 6. Manual alignment of the first 56 nucleotides from the 5`end of the genomic (5'g) and subgenomic (5's) RNA molecules of six FCV isolates. Conserved nucleotides are highlighted in grey. The putative start codons for both ORFs are in bold. The regions predicted to form stem-loops are indicated above the alignment for the 5' region of the genome and below the alignment for the 5' region of the sub-genome. Dots (.) represent gaps manually introduced to the alignment to maximise sequence conservation.

molecules. This includes the conserved guanine-thymine nucleotide pair at the 5' end of both molecules that is also conserved in other caliciviruses (Rinehart-Kim et al., 1999).

This alignment apposed the regions predicted to form stem-loops 1 and 2 in the genomic RNA molecule with the single stem-loop predicted in the sub-genomic RNA molecule. In this smaller region that is predicted to be prone to forming secondary

structure, the degree of sequence conservation was particularly high with 31 out of a total of 42 nucleotides (74%) being completely conserved. Since these regions share a high degree of sequence similarity, they also have the potential to form similar secondary structures.

DISCUSSION.

Many positive-stranded RNA viruses are predicted to contain secondary structures at the extreme ends of the genome that have been shown to be important in genomic replication (Blight & Rice, 1997, Kolykhalov et al., 2000, Xiang et al., 1997, Zeng et al., 1998). The presence of a potential stem-loop structure or structures has been previously reported at the 3`end of the genome for several caliciviruses including FCV, RHDV and Norwalk virus (Pletneva et al., 2001, Seal et al., 1994). Although these structures were not conserved within viral species, it was suggested that they might be important as signals for negative strand synthesis. The purpose of the current study was to analyse the 5`ends of all published FCV full-length sequences for the existence of any potential secondary structures. By applying a computational method we obtained evidence for seven putative stem-loop structures that were conserved amongst all FCV isolates analysed. In contrast, only one conserved stem-loop was predicted at the 5`end of the sub-genomic RNA. In addition, an extended area of sequence conservation between the 5'end of the genomic and sub-genomic RNAs provided the potential for both ends of these molecules to form similar structures. Such putative structures are candidate targets for RNA-protein interactions that are critical to the successful replication of the FCV genome.

This is the first report of potential secondary structure in the 5'end of the FCV genome. We have characterised up to seven putative stem-loops within the first 260 nucleotides of the genome. The first three loops are formed by regions of 100% sequence conservation suggesting that both ribonucleotide sequence, as well as RNA conformation, may be necessary for the correct functioning of these putative genomic targets. In contrast, despite the secondary structures of loops four to seven remaining largely conserved between the six isolates studied, there are sequence differences within the stem forming regions of these loops suggesting structure may be more important than primary sequence in these regions. Based on similar regions of secondary structure at the 5'end of other positive sense RNA viruses, these regions in FCV may play a role in one or more of VpG binding, negative strand synthesis, or genomic encapsidation. It seems less likely that these predicted structures in FCV will have an analogous function to the IRES of picornaviruses in directing internal translation initiation since it has been predicted for a human calicivirus that translation is initiated at the first in-frame start codon (Liu et al., 1996). For FCV, the first conserved start codon at the 5'end of ORF 1 is located within putative stem-loop1 between nucleotides 20 and 22 (fig 6). This contrasts to, for example poliovirus translation, where five defined stem-loops spanning approximately 600 ribonucleotides at the 5' end of the genomic RNA initiate translation at a start codon at position 743 (Stewart & Semler, 1997).

The extent of secondary structure predicted at the 5'end of the FCV sub-genomic RNA was much lower than that at the 5'end of the genome. In this study, we found clear evidence of only a single stem-loop conserved among all six genomes analysed. If taken at face value this would suggest the requirements for secondary structure

within this RNA molecule are considerably lower than for the FCV genome. However, many of the functions required at the 5'end of the genomic RNA are also likely to be required in the subgenomic molecule since it is also bound to VpG, is encapsidated (Neill, 2002) and needs to be replicated (Carter, 1990). The mechanism for the generation of the sub-genomic RNA has not been determined for any members of the caliciviruses. Potential mechanisms include either internal initiation of transcription from a promoter region on the negative strand template, or premature termination of replication from the genomic plus strand RNA template to produce a sub-genomic negative strand template (Miller & Koev, 2000). The development of replication assays will be required for caliciviruses to ultimately understand sub-genomic RNA synthesis.

Previous studies have identified areas of sequence conservation shared between the 5' ends of both the genomic and sub-genomic RNA molecules of caliciviruses. In general, the extent of this conservation is highest in RHDV and human caliciviruses. In FCV, previous reports have identified a lower and more limited degree of conserved sequence at the 5'end of these two molecules. Carter et al, (1992) who were the first to sequence an entire FCV genome (F9), highlighted 14 conserved nucleotides shared in a 24-nucleotide alignment of the two sequences which included one gap residue (Carter et al., 1992). Interestingly, in the Carter alignment, the putative start codons were not aligned. In this study, we have manually aligned the two ends of the genomic and sub-genomic RNA molecules and introduced gap positions to force alignment of the two putative start codons. This was performed on the assumption, which remains unproven, that the mechanism of translation from both RNA molecules will be the same. This resulted in an alignment of 56 nucleotides in

length, which included six gap positions and a total of 36 conserved nucleotides and suggests that the extent of shared sequence between these two molecules is higher than previously identified. Interestingly, the conserved areas of this alignment coincide with the areas predicted to form stem-loops 1 and 2 of the genomic RNA and stem loops 1 in the sub-genomic RNA. This co-localisation raises the possibility that both molecules have the potential to form similar secondary structures in this region. Specifically, the large stem loop1 predicted in the sub-genomic RNA may actually form two smaller stem-loops in a similar fashion to that predicted for the genomic RNA. Whilst the reverse is also clearly possible, it is interesting that two stem loops have also been predicted at the 5'end of the genomic and putative sub-genomic RNA molecules of two noroviruses (Jiang et al., 1993, Pletneva et al., 2001), in a region shown to bind several cellular proteins (Gutierrez-Escolano et al., 2000). These proteins had molecular weights similar to those of the HeLa cellular proteins that bind to the IRES of picornavirus RNA, suggesting that these protein-RNA interactions are likely to play a role in Norwalk virus translation and/or replication. Taken together, the conserved areas at the end of both the FCV genomic and sub-genomic RNA molecules and their potential to form similar secondary structures may suggest these shared features play similar roles in the function of these two molecules. However, the role of these predicted structures remains unknown. Ultimately, functional and mutation analysis using infectious clones or genomic fragments will be required to dissect the precise structures and their functions at the end of both molecules.

In conclusion, we have identified extensive regions of potential secondary structure at the 5' end of both RNA molecules produced in FCV infected cells. These structures have been predicted by computational analysis and therefore must be considered

putative. The link between function and predicted structure will only be resolved by functional studies that seek to confirm (or otherwise) the validity of the predicted stem-loops in both RNA molecules and help to determine their role in RNA replication, negative strand synthesis, VpG binding and / or packaging. The availability of an infectious clone would be an invaluable tool to carry out such studies.

MANUSCRIPT 4

Amplification of the full-length genomes of feline calicivirus isolates by long RT-PCR and attempts to generate infectious clones.

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ABSTRACT.

Infectious clones represent important tools for studying the genetics of viral replication and the molecular mechanisms of virulence. However, their construction has often been difficult, involving the cloning and ligation of many subgenomic cDNA fragments. There are now several reports for other positive single stranded RNA viruses that describe a rapid one-step RT-PCR technique, which simplifies and shortens the experimental time to construct infectious clones. In theory this protocol is not dependent on prior knowledge of viral sequences, is applicable to most strains of a given virus, and has been made possible by the availability of improved thermostable DNA polymerases with increased fidelity and yield. We have used this technique to generate full-length 7.7kb amplicons of three different FCV isolates. An amplicon from one of these isolates has been successfully cloned into a commercial vector. However, no infectious virus was recovered from RNA transcripts derived from these clones. Whilst we have not generated infectious virus, we propose that this methodology will represent a valuable tool for generating infectious clones of FCV.

INTRODUCTION.

Feline calicivirus (FCV) belongs to the family *Caliciviridae* (Cubitt et al., 1995) and is an important respiratory pathogen of domestic cats, mainly causing acute oral and upper respiratory tract disease (Gaskell & Dawson, 1998b). The clinical signs associated with infection include pyrexia, nasal and ocular discharges, sneezing, conjunctivitis and anorexia (Gaskell & Dawson, 1998a, Hoover & Kahn, 1975, Kahn & Gillespie, 1971, Knowles et al., 1991, Ormerod et al., 1979, Povey, 1974, Reubel et al., 1992, Wardley & Povey, 1977). Although the disease is usually mild, a wide spectrum of severity has been observed from asymptomatic infection (Fastier, 1957, Povey, 1974, Radford et al., 2001) to fatal pneumonia in kittens (Kahn & Gillespie, 1971, Poulet et al., 2000, Povey, 1974). In addition, some isolates of FCV can cause an acute febrile lameness syndrome (Bennett et al., 1989, Church, 1989, Crandell & Madin, 1960, Dawson et al., 1994, Pedersen et al., 1983, Studdert et al., 1970, TerWee et al., 1997), often accompanied by pyrexia, and with or without respiratory disease. This lameness has been associated with certain isolates (Dawson et al., 1994) and also with the use of live attenuated vaccines, typically in young kittens following a first vaccination (Dawson et al., 1993).

FCV has a positive sense, single stranded RNA genome of approximately 7.7Kb in length, (Carter et al., 1992) which is polyadenylated at the 3`end and covalently bound to a viral encoded protein (VpG) (Herbert et al., 1996) at the 5`end (Burroughs & Brown, 1978, Sosnovtsev & Green, 1995). The genome is organised into three open reading frames (ORFS) (Fig.1). ORF1 is located at the 5`-end of the genome and encodes the non-structural proteins, which based on sequence similarity with

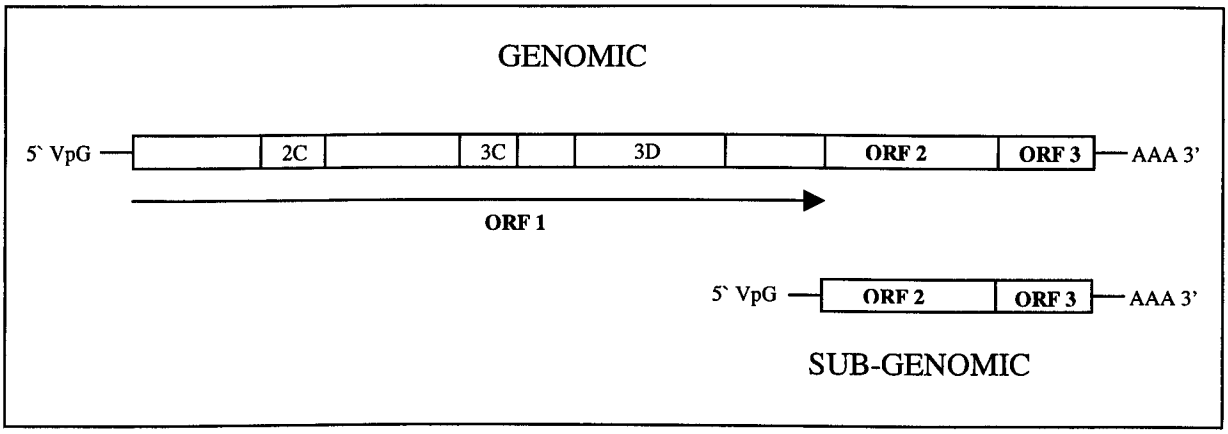


Fig. 1

Fig. 1. Diagrammatic representation of FCV showing genomic and sub-genomic RNAs which are both polyadenylated at the 3' end and linked to protein (VPg) at the 5' end. ORF 1 encodes the non-structural proteins: 2C-like helicase, 3C-like protease and 3D-like RNA-dependent RNA polymerase, which are conserved with the non-structural proteins of picornaviruses. ORF 2 encodes the capsid protein, and ORF 3 encodes a minor structural protein.

picornaviruses and some functional studies (Meyers et al., 1991, Neill, 1990, Oshikamo et al., 1994) includes a VpG, a 2C-like helicase, 3C-protease and a 3D RNA polymerase (Sosnovtseva et al., 1999). This is followed by ORF2 located towards the 3`-end of the genome encoding the capsid precursor that is cleaved to generate the major capsid protein (Neill, 1990, Neill et al., 1991, Sosnovtsev et al., 1998) (Fig.1). ORF3 is located at the 3`-end of the genome and has recently been characterised as a minor structural protein (Sosnovtsev & Green, 2000) (Fig.1). As well as the full-length genome, a sub-genomic mRNA is also produced in infected cells that contains ORF2 and is co-terminal with the 3`end of the genome (Black et al., 1978) (Fig.1).

Whilst some information is known about the function of individual proteins encoded by the FCV genome, in particular the capsid protein, little is understood about genomic replication or the molecular mechanisms of virulence. Such studies of several other positive-stranded RNA viruses including FCV have benefited from the availability of infectious cDNA clones, which involves conversion of purified viral genomic RNA into DNA before insertion into plasmid vectors e.g. encephalomyocarditis virus (EMCV), (Kassimi et al., 2002), Hepatitis C virus (Yanagi et al., 1997), coxsackievirus B6 (Martino et al., 1999), infectious bursal disease virus (IBDV) (Huang et al., 2003). Infectious cDNA clones allow direct manipulation at the molecular level of cloned viral genomes for studying virus replication, virulence or pathogenesis.

Conventional methods of generating an infectious clone from positive-stranded RNA viruses usually involve reconstruction of a full-length genomic sequence from several

cloned sub-genomic cDNA fragments. This methodology has been used to generate infectious clones of the FCV Urbana isolate (Sosnovtsev & Green, 1995), and vaccine strain 2024 (Thumfart & Meyers, 2002). In both cases the sequence of the genome was determined from overlapping clones, which were subsequently religated to reconstruct the full-length genome. The genome was placed under transcriptional control of the phage T7 RNA polymerase promoter to generate full-length positive sense RNA genome copies by *in vitro* transcription. Using these infectious clones it was demonstrated that the genome transcribed *in vitro* and lacking VpG was of either low infectivity or non-infectious, but infectivity could be reinstated by substituting the VpG with a cap structure analog (m7G (5`)ppp(5`)G) (Sosnovtsev & Green, 1995, Thumfart & Meyers, 2002). In addition, infectious virus was also recovered from both strains when the DNA constructs were introduced into cells expressing phage T7 RNA polymerase provided endogenously by prior infection of cells with a recombinant vaccinia expressing T7 RNA polymerase.

However, construction of infectious clones by this method is often difficult, laborious and requires full genomic sequence to identify restriction enzyme sites within the genome suitable to ligate the sub-genomic fragments back together. In addition, the constructed full-length clone may be non-infectious either due to the amplification of defective genome segments present within most RNA viral populations or sequence errors introduced during PCR.

There are now several reports for other positive single stranded RNA viruses that describe a rapid one-step RT-PCR technique, which simplifies and shortens the experimental time to construct infectious clones (Huang et al., 2003, Lindberg et al.,

1997, Martino et al., 1999, Tellier et al., 1996). In theory this protocol is not dependent on prior knowledge of viral sequences and is applicable to most strains of a given virus. This technique has been made possible due to the availability of improved thermostable DNA polymerases with increased fidelity and yield. Using this approach, 5` sense and 3`antisense primers are designed to conserved sequences present at the extreme ends of the genome. Genomic transcription is ensured by placing a T7 promoter sequence within the 5` sense primer, adjacent to the start of the viral sequence, thereby ensuring accurate recreation of the 5` end of the viral genome. Full length cloning of amplicons is facilitated by the addition of rare restriction enzyme sites, such as *Sma I* and *Not I* in both primers.

The purpose of this study was:

- to produce full-length DNA amplicons of different FCV isolates using one step long range RT-PCR.
- to clone putative full-length amplicons into commercial vectors.
- to recover infectious from any cloned genomes by *in vitro* transcription.

Two different types of template were used during the course of these studies in earlier experiments.

1. Initially cDNA was obtained directly from infectious viral RNA of several different FCV isolates.
2. In later experiments a previously cloned full-length genomic copy of FCV F9 was used as template.

METHODS AND RESULTS.

1. Attempts to generate infectious clones using the FCV isolates, F9, F65, LSO15, and LSO27.

Viruses and cells.

The viruses used in this part of the study were F9 (Bittle et al., 1960), F65 (Dawson, 1991), LSO15 (Knowles, 1988), and LSO27 (Knowles, 1988). Viruses were grown in either feline embryo cell line A (FEAs) (European collection of cell cultures) (ECACC) (Jarrett et al., 1973) or Crandell-Rees Feline Kidney Cells (CRFK) (Crandell et al., 1973). Infection was allowed to proceed overnight until cytopathic effect (CPE) was complete. Monolayers were frozen at -80°C and thawed to produce a cellular lysate.

Primer design.

Primers FCVgen 5 and GEN 3 are shown in Figs 2a and 2b and were used for one step RT-PCR. The sense primer FCV gen5 (Fig 2a) begins with a four base clamp to facilitate restriction enzyme digestion. Following this is a *Sma 1* six base restriction enzyme site (underlined), the core sequence of the T7 promoter (dot underlined), and the first 36 nucleotides of FCV F9. This region of the FCV genome has been predicted to be prone to secondary structure formation (Fig 2a) and this thesis (manuscript 3) rendering these primers extremely prone to dimer formation. The 36 nucleotides of FCV genomic sequences incorporated in FCV gen 5 were chosen to minimise the likelihood of this occurring. The antisense primer GEN 3 (Fig 2b)

contains eighteen thymidine residues as Sosnovtsev and Green (1995) have previously produced infectious virus from a similar primer (Sosnovtsev & Green, 1995) and have also shown that synthetic transcripts with less than 10 adenine residues at their 3` end shows greatly reduced infectivity. GEN 3 (Fig 2b) begins with a four base clamp, and then a *Not* 1 eight base restriction site (double underlined) followed by a length of 18 thymidine nucleotides and 16 nucleotides which are complementary to the FCV F9 sequence upstream of the poly (A) tail. This primer was used in both the RT and PCR steps. The GC content of FCV genome is 46% (data not presented), and as both restriction enzymes *Sma 1* and *Not* 1 are GC rich this will therefore minimise the chances of such restriction sites occurring within FCV.

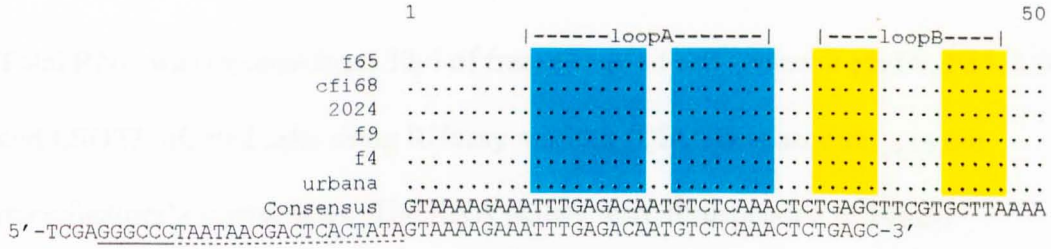


Fig. 2a

Fig. 2a. Sequence of primer FCVgen 5 at the bottom of the figure, begins with a four base clamp, underlined is the *Sma I* restriction enzyme site and dot underlined is the core sequence of the T7 promoter. The first 50 nucleotides of the genome of six sequenced FCV isolates are compared. The shaded areas show the genomic sequences which indicate two areas with the potential to form stem loop structures (loop A and loop B) (this thesis manuscript 3). This primer was used in the one step long RT-PCR reaction.

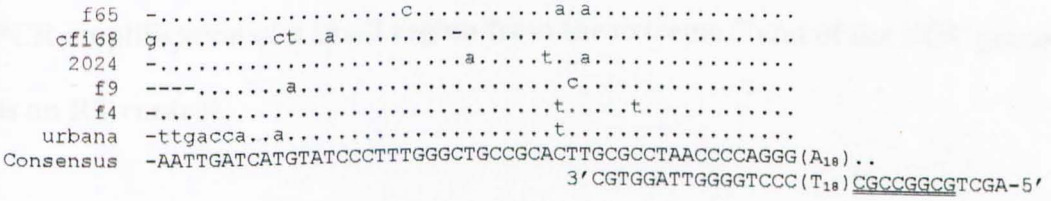


Fig. 2b

Fig. 2b. Primer GEN 3 begins with a four base clamp, double underlined is a *Not I* restriction enzyme, followed by 18 thymidine nucleotides and 16 nucleotides complementary to FCV F9 sequence. This primer was used for the RT reaction and one step long RT-PCR.

RNA Isolation and Reverse transcription reaction.

Total RNA was isolated from 30µl of freeze-thawed cell lysates from F9, F65, LSO15 and LSO27 infected cells using RNeasy mini kit (QIAGEN) according to the manufacturer`s instructions. The RNA pellets were resuspended in 10mM dithiothreitol and 5% (vol/vol) Rnasin (Promega) in 30µl Rnase free water (Sigma) and stored on ice for the reverse transcription (RT) reaction. For this reaction, 10µl of the isolated RNA was heated for 5 min at 65°C and placed on ice. To this was added 4µl of 5X Superscript II reverse transcription buffer (Gibco/BRL), 0.5µl Rnasin (Promega), 1µl of 100mM DTT (Promega), 1µl of 10mM dNTP mixture (ABgene), 2.5µl of 10µM downstream primer GEN 3 (Fig. 2b) and 1µl of Superscriptase II reverse transcriptase (Gibco/BRL) and incubated at 42°C for 50 mins. Subsequently, template RNA was removed by the addition of 1µl of Rnase H (1-4 U/µl) (Gibco/BRL) and 1µl of Rnase T1 (900-3000 U/ml) (Gibco/BRL) and incubation at 37°C for 20 min.

PCR amplification of a small region from the extreme 5`end of the FCV genome as an RT control.

In order to determine if the reverse transcription step had produced full-length negative strand cDNA copies of the genome, a PCR reaction was set up using primers FCVgen5 and cp1 (5`-GCCGGGTGGGACTGAGTGGATAGC-3`). Primer cp1 anneals at nucleotides (385→362) of the FCV genome and should produce a product of 413 base pairs with FCVgen5 corresponding to the extreme 5` end of the genome. The PCR reaction was made up to a final volume of 50µl in 1 X KlenTaq PCR

reaction buffer (Clontech) containing 1µl of 10mM dNTP mixture, 1µl of 10µM of each primer (FCVgen5 and cp1), and 1µl Taq Polymerase (Abgene). Mineral oil (Sigma) was added above the PCR mixture. PCR was performed using a thermal cycler (Omnigene), and the PCR cycling parameters used with FCVgen5 and cp1 are shown in table 1.

Denaturation	95°C	3mins	1 cycle
Denaturation	95°C	1min	40 cycles
Annealing	50°C	1min	
Primer extension	72°C	8mins	
Final extension	72°C	10mins	1 cycle

Table 1

Table 1. PCR cycling parameters for primers FCVgen5 and cp1.

As observed in Fig. 3, the PCR amplification of the extreme 5`end of the FCV genome using the primers FCV gen 5 and cp1 produced an amplicon of the expected size of approximately 413 base pairs. This suggested that the RT stage was successful in producing a negative sense cDNA molecule equivalent to the full length of the FCV genome.

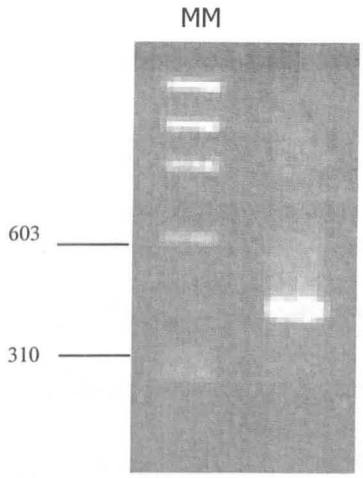


Fig. 3

Fig. 3. Results of the PCR reaction using primers FCV gen 5 and cp1. The molecular weight marker is *HaeIII* digested ϕX174 (Abgene), with relevant sizes shown on the left. The anticipated amplicon size is 413 bp.

Amplification of putative full-length DNA genomic copies from cDNA.

To attempt to obtain putative full-length amplicons, firstly elongase enzyme mix (Gibco BRL Life Technologies) was used which is designated to amplify long PCR products as *Taq* DNA polymerase lacks proofreading (3` to 5` exonuclease) activity and is generally limited to DNA templates less than 5 kb. Elongase consists of a mixture of *Taq* and *Pyrococcus species* GB-D thermostable DNA polymerases. However, it was not possible to obtain putative full-length amplicons despite attempts to optimise template and magnesium concentrations, annealing temperatures and cycle conditions changing (data not presented).

The Extensor long master mix (Abgene) was then used that has a reported fidelity about four times higher than *Taq* DNA polymerase alone. Long one step RT-PCRs for all isolates was set up to a final volume of 50µl in 25µl of 2 X High fidelity PCR reaction enzyme mix (Abgene), containing the *taq* DNA polymerase proofreading enzyme (2.5 units), dNTP`s (350µM), $MgCl_2$ (2.25mM), 1µl of a 10µM solution of each primer (FCVgen 5 and GEN 3 Table 1), and 22µl Rnase free water (Sigma). Then 50µl of mineral oil (Sigma) was added above the PCR mixture and 1µl of the RT reaction was added under the oil. The PCR reaction was performed using a thermal cycler (Omnigene) using the cycling conditions shown in Table 2. Previous studies have recommended that a stepwise increase in the elongation time can be beneficial (Martino et al., 1999, Tellier et al., 1996).

		15cycles	10 cycles	10 cycles
Denaturation	99°C	35secs	35secs	35secs
Annealing	67°C	30secs	30secs	30secs
Elongation	68°C	9mins 45secs	11mins	13mins

Table 2

Table 2. Cycling parameters for one step long PCR.

Representative results of a one step long RT-PCR experiment using Extensor long master mix are shown in Figs 4a and 4b. Products of the anticipated size were obtained for F9, F65 and LS027. As well as these full-length products, other variable and smaller fragments were also observed in each of these PCRs. These were presumed to represent non-specific products resulting from false primer binding. No products of the anticipated size were obtained for the isolate LS015 despite PCRs being repeated several times both from the cDNA and from a fresh RNA isolation.

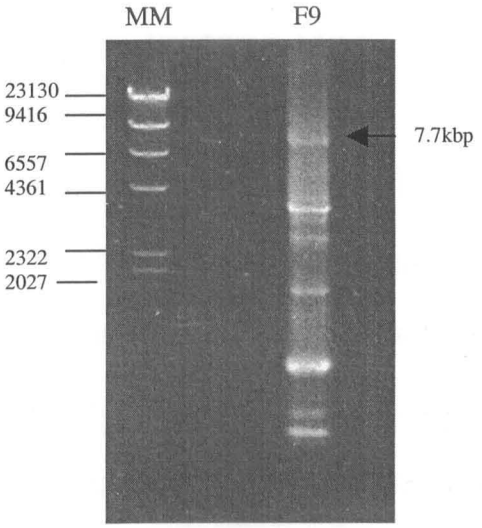


Fig. 4a

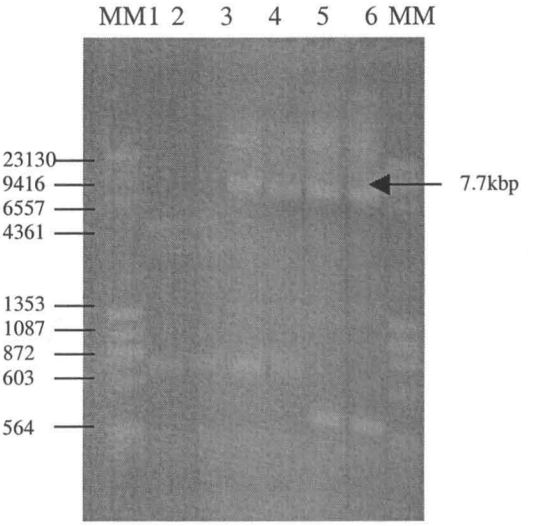


Fig. 4b

Figs. 4a and **4b**. Results of amplification using Extensor long master mix.

Fig. 4a. Putative full-length product using FCV F9 as template.

Fig. 4b. Putative full-length products using F65 as template (lanes 3 and 4), and using LSO27 as template (lanes 5 and 6). When using LSO15 as template in (lanes 1 and 2), a full length PCR product was not detected. In all cases, variably sized smaller products were also obtained.

To increase the yield of the putative genomic amplicon for F9, F65, and LSO27 a further 20 PCR reactions were performed for each isolate, pooled, and ethanol precipitated using standard laboratory protocols to a total volume of 40μl. Long PCR products often yield multiple products, as observed in Figs. 4a and 4b making gel purification a necessary step prior to cloning. The pooled and concentrated PCR products were then loaded onto a crystal violet gel to enable visualisation of DNA bands under ambient light, ensuring gel purification of intact DNA. This eliminates ethidium bromide and exposure to UV light, which can nick and damage DNA. All three putative genomic amplicons were excised and put into sterile microcentrifuge tubes and gel purified using reagents provided with the TOPO XL PCR cloning kit (Invitrogen). Purified amplicons were assessed on an agarose gel and, as observed in Figs 5a and 5b, a clear band of the anticipated size was present for each isolate. An additional band was also present for F9 (Fig. 5a), which is likely to be an artefact of the gel purification protocol (personal communication Dr. Phil Turner). Based on the intensity of the genome sized product in relation to those of the molecular weight marker, it was estimated that only 100ng of each putative full-length product was available for further studies. Attempts to characterise these amplicons by sequencing with the T7 primer were unsuccessful due to the low concentration of DNA (data not presented). Because of the restricted amount of DNA available, it was not possible to further characterise these amplicons.

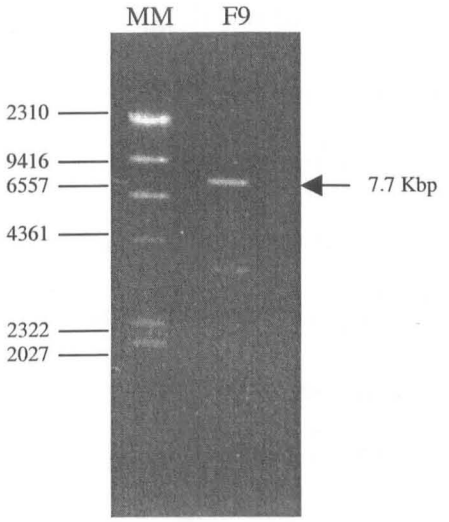


Fig. 5a

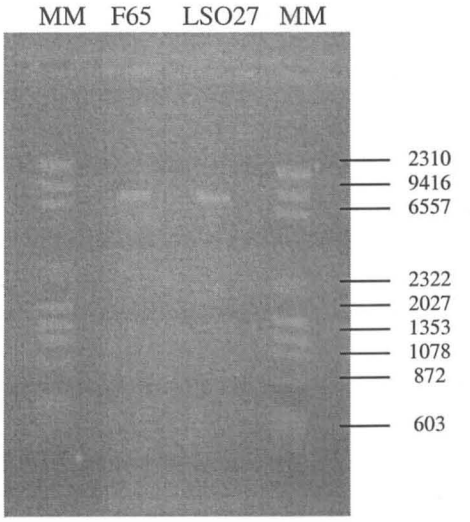


Fig. 5b

Figs. 5a and **5b.** Purification of genome sized products

Fig. 5a. Gel purified PCR product of FCV F9 showing a band of approximately 7.7Kbp with an additional band of approx 3Kbp (see text). Molecular weight marker is lambda *HindIII* digest (Abgene) and the base pairs are shown on the left of the figure. **Fig. 5b**. Gel purified PCR products of F65 and LSO27 with molecular markers lambda *HindIII* digest mixed with ϕX174 *HaeIII* digest shown on the right and left of the figure.

Attempts to Clone purified PCR products.

The limited amount purified F9, F65 and LSO27 PCR product available represented a limitation to the number of DNA manipulation steps that could be performed prior to attempting their cloning. It was therefore decided to abandon the use of the restriction enzyme sites in the primers and to use instead a commercially available vector designed for direct cloning of PCR products (TOPO XL PCR cloning kit; Invitrogen) (Fig.6). This vector was chosen as it is designed for cloning long PCR products up to 3-10 Kbp. In addition, the vector is provided linearized and is toposisomerase I activated to enable efficient 5-minute ligation with over 80% recombinants usually produced (manufacturer`s data). Recombinant plasmids are efficiently selected by insertional inactivation of a lethal gene (*ccdb*) and selected for kanamycin resistance.

Briefly, 4μl of each of the purified PCR products for F9, F65 and LSO27 were added to 1µl of pCR-XL-TOPO vector (Invitrogen) in a sterile eppendorf tube, mixed gently and incubated for 5minutes at room temperature. Immediately following this incubation 1µl of 6X TOPO Cloning Stop Solution was added and mixed for several seconds at room temperature. The tubes were then briefly centrifuged and placed on ice ready to transform. Transformation was performed using One Shot TOP 10 chemically competent cells (Invitrogen). Briefly 2µl, of the TOPO cloning reaction was added to a single vial of One Shot cells, gently mixed, and incubated on ice for 30 minutes. Then the cells were heat-shocked for 30 seconds at 42°C and placed on ice for 2 minutes. This was followed by the addition of 250µl SOC medium (Invitrogen). All tubes were then shaken horizontally in an orbital shaker at 37°C for 1 hour and then placed on ice. Three dilutions of 50µl, 100µl, and 150µl for each

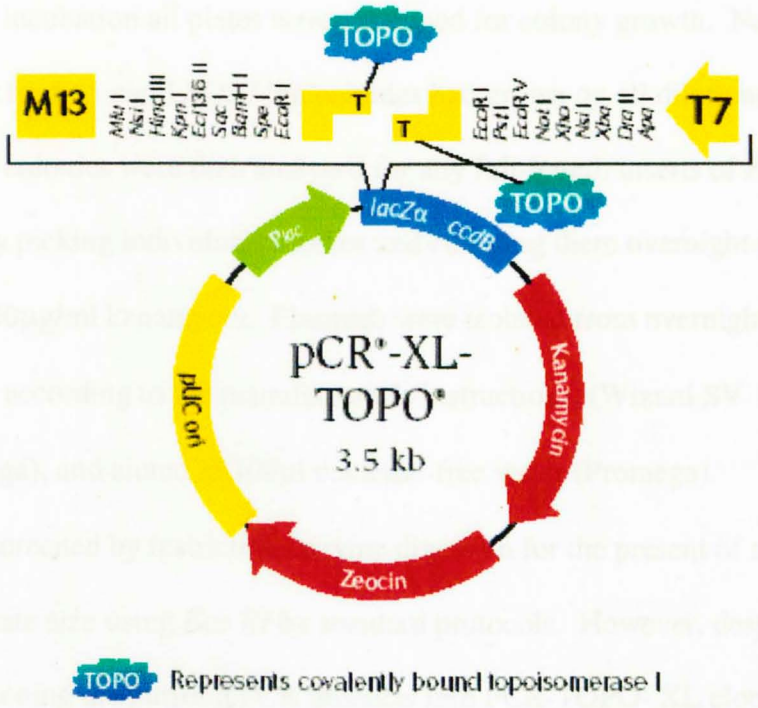


Fig. 6

Fig. 6. PCR- XL-TOPO cloning vector used to attempt to clone amplicons from FCV isolates F9, F65 and LSO27.

transformation were spread onto prepared warmed LB (Luria-Bertani) plates containing 50μg/ml kanamycin and incubated overnight at 37°C.

Following overnight incubation all plates were examined for colony growth. No growth was obtained for F65 and LSO27 but colonies had grown on all dilutions of the F9 plates. These colonies were then analysed for any full-length inserts of F9. This was achieved by picking individual colonies and culturing them overnight in LB medium containing 50μg/ml kanamycin. Plasmids were isolated from overnight cultures and purified according to the manufacturer's instructions, (Wizard SV Miniprep Kit, Promega), and eluted in 100μl nuclease-free water (Promega). Plasmids were then screened by restriction enzyme digestion for the present of an insert of the appropriate size using *Eco RI* by standard protocols. However, despite several attempts at cloning the purified PCR products into PCR-TOPO- XL cloning vector, and analysis of numerous plasmids no full-length inserts were observed.

2. Attempts to generate infectious clones using a cloned full-length genomic copy of FCV F9.

As efforts directed towards amplifying and cloning genomic copies derived from infectious virus were proving unsuccessful, a second approach was instigated based on a previously cloned full-length genomic copy of FCV F9 designated pF9FL2 (Glenn, 1997). This clone had been produced previously in this laboratory by ligating two *SmaI* fragments together. The complete genome was placed downstream of a Rous Sarcoma virus long terminal repeat (LTR) promoter in order to attempt recovery of infectious RNA. However, no infectious RNA was produced. Reasons put

forward to explain this apparent lack of infectivity included base additions at the 5' end of the genome introduced by transcription initiation at the RSV promoter, the presence of cryptic splice signals in the viral genome, and cloning of a defective genome. It was felt that using this template in our studies would provide a readily available source of template DNA from which to optimise amplification and cloning protocols. Since this genome already contained a known *SmaI* site, the FCVgen5 primer was modified by replacing its *SmaI* site with a *SalI* restriction site which from sequence data was known to be absent in the F9 genome (Table 3). In addition, a guanosine residue was placed between the T7 promoter and the 5` FCV genomic sequence. This has been shown to increase T7 transcription efficiency and is rapidly lost from recovered viruses when replicated in cell culture (Personal communication Dr. Raymond Tellier). Amplification from pF9FL2 should remove the RSV promoter and place the F9 genome downstream of the T7 promoter. Amplicons derived from pF9FL2 could then be cloned into the pBluescript™ SK- vector (Stratagene) using *SalI* / *NotI* sites present in that vector.

Primer	Sequence (5' →3')
T7 BEG	TCGAGTCGACTAATAACGACTCACTATA**g**GTAAAAGAAATTTGA GACAATGTCTCAAACTCTGAGC

Table 3

Table 3. The primer T7 BEG with the *Sal I* restriction enzyme underlined, the core sequence of the T7 promotor (dot underlined), a single guanosine nucleotide(lower case in bold) and the first 36 nucleotides of FCV F9.

Long PCR using pF9FL2 as template.

Plasmid F9FL2 in *E.coli* DH5α cells, was streaked onto LB plates containing 50μl/ml ampicillin. After incubation overnight at 37°C, a single colony was picked and resuspended in 500μl of sterile distilled water (Sigma). This colony suspension was boiled for 10 mins prior to using as a template in the long PCR reaction.

Amplification conditions of pF9FL2 using Extensor long master mix was essentially identical to amplification of the cDNA template described above with minor modifications. These were replacement of the cDNA template with 1μl of pF9FL2 and replacement of primer FCVgen 5 with equivalent amounts of the primer T7 BEG. The results of this PCR are shown in Fig. 7 and clearly show a consistent band of the anticipated size in all lanes. Unlike the amplification of cDNA derived from FCV isolates used initially, non-specific products were not observed. All PCRs were repeated multiple times to increase product yield then pooled and concentrated by ethanol precipitation and gel purified to remove unincorporated primers and nucleotides as described previously.

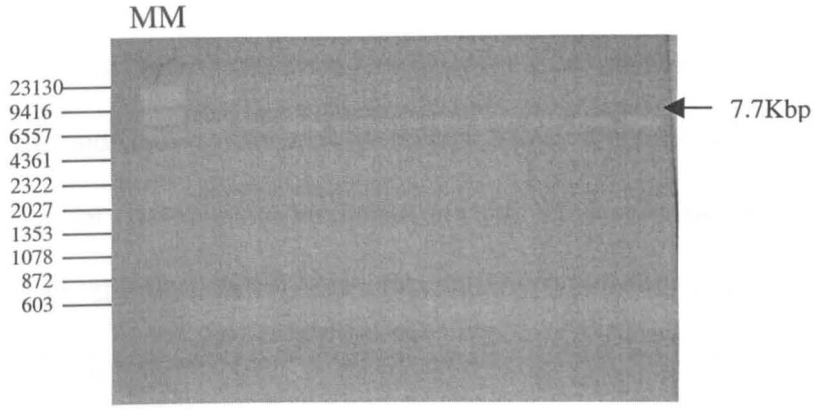


Fig. 6

Fig. 6. Full-length PCR products using pF9FL2 as template. As observed in all lanes a band of approximately 7. 7 Kb is produced. Molecular markers are lambda *Hind III* digest and ϕX174 *Hae III* digest, and base pairs are shown left to the figure.

Following ethanol precipitation and gel purification, the full-length FCV F9 amplicon was cloned into a commercial vector pBluescript™ SK- Stratagene (Fig. 7). This vector contains the restriction enzymes *Sal I* and *Not I* that are also incorporated into the primers T7 BEG and GEN 3 respectively. Vector SK- was grown and purified using standard laboratory protocols to produce a working stock solution of approximately 200ng / µl. Both the PCR product and the vector SK- were digested using standard protocols with *Sal I* and *Not I* (Roche). Digested SK- was purified by agarose gel extraction to remove the intervening multiple cloning site (Qiaquick gel extraction kit, Qiagen), and dissolved in 30µl sterile water (Sigma). Digested pF9FL2 was purified using the Wizard PCR preps DNA purification system (Promega). Then 1µl of both PCR product and vector were run on an agarose gel to confirm that digestion had been achieved and to estimate the concentration of purified DNA (Fig. 8 and Fig 9).

Ligation was performed with T4 DNA ligase (Roche) according to manufacturer`s instructions. Since the ideal ratio of insert to vector DNA is variable, three ligations were set up with insert to vector ratios of 0.2µg:0.1µg, 0.3µg:0.1µg, and 0.2µg:0.2µg. Each ligation was incubated overnight at 12°C. The products of ligation were then transformed into XL1-Blue MRF` supercompetent cells (Stratagene) according to manufacturer`s instructions, and potential recombinant colonies identified by blue-white colour selection. Briefly, LB agar plates were prepared containing 80µg /ml 5-Bromo-4-chloro-3-inolylβ-D-galactoside X-Gal; (Sigma), 20mM IPTG; isopropylthio-β-D-galactoside IPTG; (Sigma) and 50µg/ml ampicillin (Sigma).

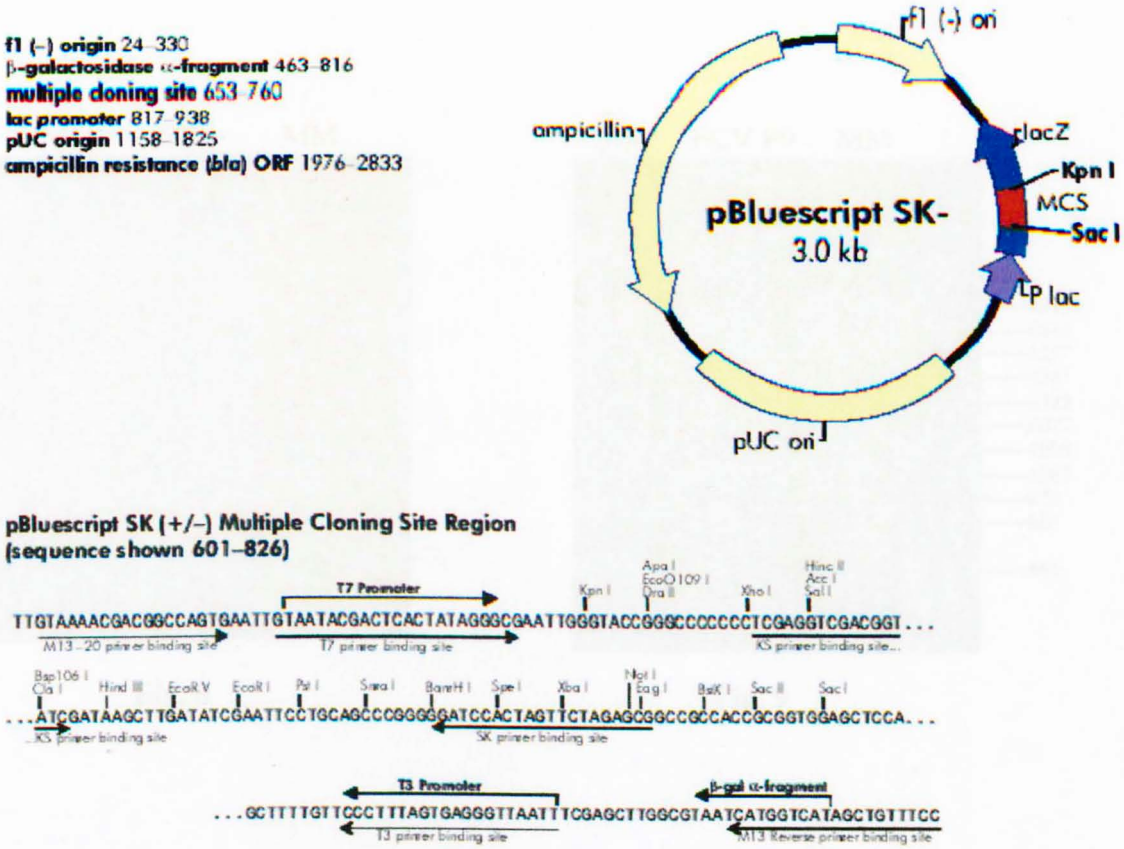


Fig. 7

Fig. 7. The commercial cloning vector pBluescript^{TM} SK- (Stratagene).

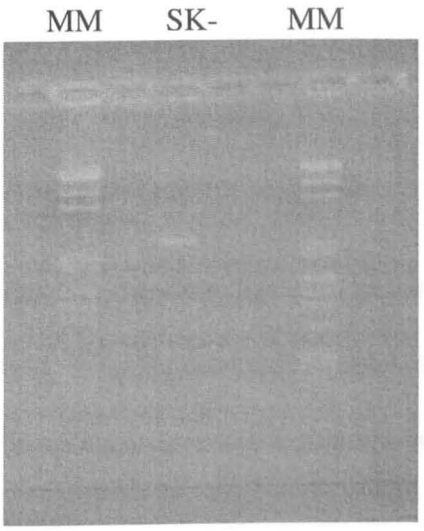


Fig. 8

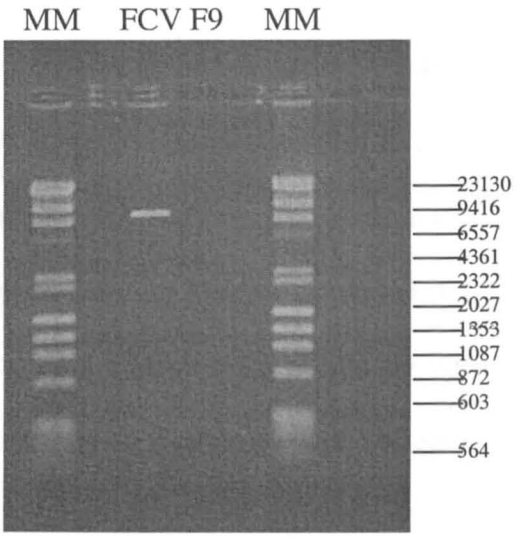


Fig. 9

Fig. 8. Digested and purified vector pBluescript SK- and **Fig. 9**. Full-length PCR product. The vector and PCR product were both digested with the enzymes *Sal I* and *Not I*. Digested vector and PCR product were then purified and 1µl run on an agarose gel to confirm that digestion had been achieved for the vector and that sufficient DNA was available for cloning the PCR product. Molecular markers are lambda *Hind III* digest and ϕX174 *Hae III* digest, and base pairs are shown on **Fig. 9**.

Three different quantities (50µl, 100µl and 150µl) from each transformation reaction were then spread onto the plates and incubated overnight at 37°C. Approximately 30 transformed bacterial white colonies were inoculated into 10ml LB broth containing 50µg/ml ampicillin. The broth was incubated overnight in an orbital shaker at 37°C and centrifuged at 3000 rpm in a benchtop centrifuge for 10mins. The supernatant was discarded and the bacterial pellet washed in phosphate-buffered saline (PBS), and purified using a commercial plasmid purification kit (Wizard SV Miniprep Kit, Promega) according to the manufacturer's instructions, and eluted in 100µl nuclease-free water.

Characterisation of clones containing the full- length insert.

Sixty plasmids were screened for the presence of genomic FCV F9 insert by performing a series of different restriction enzyme digests using *EcoRI, Not I,* and *Sal I* by standard protocols. These results suggested 25 full-length PCR products of FCV F9 had been cloned and were designated pCPFLF9 1-25. An example of restriction enzyme digests obtained for one of these clones (pCPFL9-4) is shown in Fig. 10, together with restriction enzyme digests of the full-length PCR product before cloning, and also pF9FL2 that was used as template in the original PCR reaction.

Partial sequencing of seven potential clones was performed at a commercial laboratory (Kings College London) using M13 forward and reverse primers located either side of multiple cloning site in plasmid SK- (Fig. 7) under the conditions specified by the manufacturer (ABI prism dye terminator cycle sequencing ready

Fig. 10a

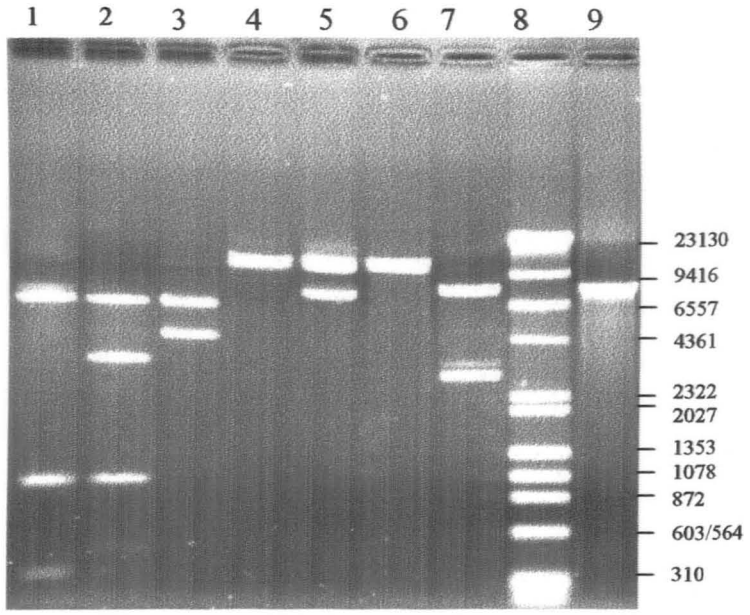
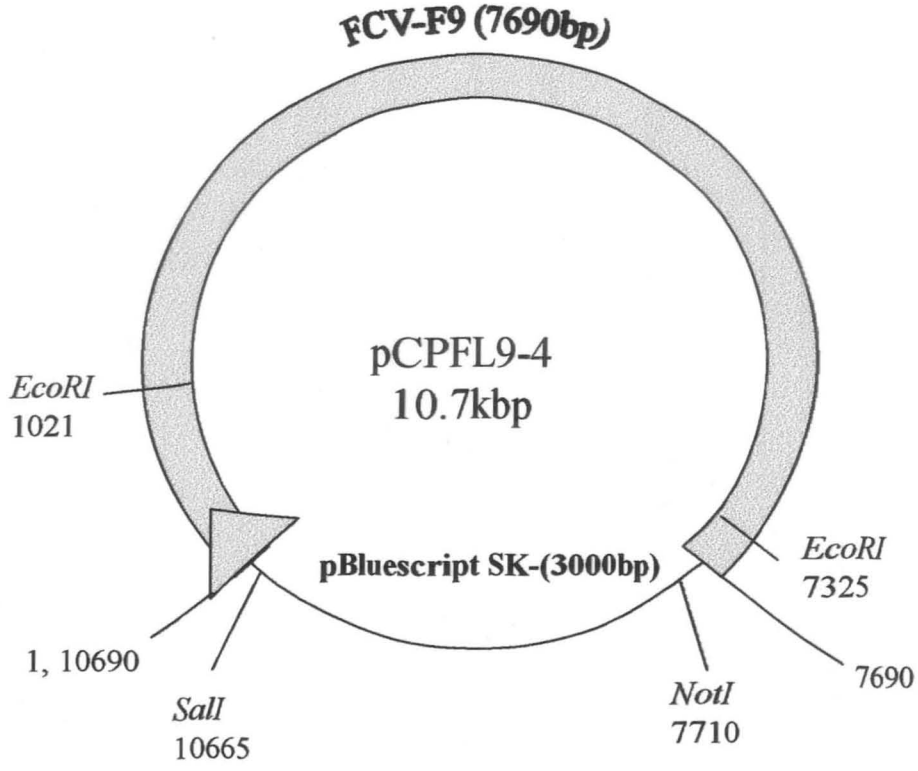


Fig. 10b



Figs. 10. Restriction mapping of pCPFL9-4. All restriction enzyme sites correspond to their predicted positions. **Figb 10a.** Lane 1 is uncloned full-length PCR product cut with *EcoRI*. Lane 2 is pF9FL2 cut with *EcoRI*. Lanes 3, 4, 5, 6, and 7 are pCPFLF9-4 cut with *EcoRI* (lane 3), *NotI* (lane 4), uncut (lane5), *SalI* (lane 6), *NotI / SalI* (lane 7), and uncut purified PCR product (lane 9). Molecular markers are in lane 8 and consist of lambda *Hind III* digest and ϕX174 *Hae III* digest. **Fig. 10b**. Putative map of pCPFLF9-4 based on known published sequences of F9 and SK-

reaction kit; Perkin Elmer). The results confirmed that in each case the T7 BEG/ GEN 3 FCV F9 amplicon had been correctly cloned into the *NotI* / *SalI* sites of plasmid SK-

In Vitro Trancription and RNA transfection into cells.

In order to determine if the cloned FCV F9 genome downstream of the T7 promoter encoded an infectious genome, RNA transcripts were generated from six plasmids. using the Ribomax Large Scale RNA Production System (Promega) according to the manufacturer`s instructions. Briefly, 10μg of each plasmid was linearised with *NotI* followed by a phenol chloroform extraction and ethanol precipitation using standard protocols. Then a transcription mix was prepared consisting of 20μl T7 Transcription 5X Buffer, 30μl rNTPs (25mM ATP, CTP, UTP and 2mM GTP), approximately 10μg linear plasmid DNA, 7.5μl of ribo m^7 G Cap analog 40mM, 10μl T7 RNA polymerase enzyme mix made up to a total volume of 100μl with nuclease free water and incubated at 37°C for 4 hours. The transcription mixture was incubated with DNase for a further 15minutes at 37°C, to degrade any remaining DNA while maintaining the integrity of the RNA. The RNA was purified by phenol chloroform extraction followed by ethanol precipitation using standard protocols, resuspended in 100μl of nuclease-free water, and stored at -20°C until used in transfection experiments.

Subsequently, 25μl of each RNA sample was incubated with 163μl Opti-Mem medium (Gibco BRL) and 12μl Lipofectin (Gibco BRL) for 30 minutes at room temperature, and a further 800μl of Opti-Mem medium was added. Monolayers of CRFK cells (approximately 1 X 10^5 cells) were washed twice with fresh Opti-Mem

medium. The transfection mixture was then added and incubated for 5hrs at 37°C. Following incubation, the medium was removed and 4ml of Eagle`s minimum essential medium (Gibco BRL) containing 1% foetal calf serum (FCS) (Sigma) was added. Monolayers were observed for characteristic FCV cytopathic effect (cpe) for 24-36 hrs post –transfection. However no typical signs of FCV infection were observed, even when cell monolayers were freeze thawed and titrated. Transfections were repeated twice for each of the six clones but the results still suggested that these potential full-length clones were uninfectious. One possible reason for failing to recover infectious virus from these plasmids is that errors introduced into the individual amplified molecules during PCR rendered the genomes inactive. It was therefore decided to attempt to recover infectious virus from *in vitro* transcripts prepared directly from the purified amplicon mixture prior to cloning. On this occasion, cpe was observed and successfully passaged, suggesting the presence of functional genomes in the amplified mixture. Infectivity was not recovered from plasmid SK- which was used as a negative transfection control. It was not possible to repeat this experiment on the same amplified batch due to insufficient purified product. Therefore, a new batch of amplified FCV F9 was prepared from pF9FL2. Attempts to recover infectious virus by *in vitro* transcription from this amplicon were unsuccessful. This suggested that the virus recovered in the first experiment may have been due to contamination of cell cultures by wild-type virus. Alternatively, it is possible that if the first experiment was a genuine result, that the second experiment failed to work because of one of several reasons including PCR error, DNA quantity and purity. Unfortunately, time constraints on the duration of this project prevented us from resolving this issue further.

DISCUSSION.

Infectious cDNA clones represent important tools for studying the molecular genetics of viral replication by allowing the study of the phenotype of site-specifically mutated genotypes. In this study we have attempted to generate infectious clones for FCV using a potentially rapid and efficient method that places the full-length genome under transcriptional control of a T7 RNA polymerase promoter (Huang et al., 2003, Lindberg et al., 1997, Martino et al., 1999, Tellier et al., 1996). The technique requires three steps. Firstly, long-range RT PCR is used to amplify the entire genome and places a T7 promoter immediately upstream of the 5' end of the genome. Secondly, this product is cloned to facilitate subsequent DNA manipulation. Finally, synthetic genomes are synthesised by *in vitro* transcription and transfected into permissive cells to recover infectious virus.

In this study, we have been successful in producing full-length PCR products both from RNA isolated from infectious virus and from an FCV F9 DNA genome copy. The latter amplicons were also cloned into a plasmid vector. Although we were unable to recover infectious virus from any of these clones, we have developed all the necessary steps required to generate infectious clones by this protocol.

This approach to generating infectious clones has depended on improvements in PCR technology specifically in the areas of fidelity and processivity that have greatly facilitated the amplification of longer PCR products. Indeed PCR amplification of up to 35kb of DNA with high fidelity and yield from lambda bacteriophage double stranded DNA templates has been achieved (Barnes, 1994). For RNA viruses, where a reverse transcription step is required prior to PCR, generation of long DNA products

has generally been restricted to products of less than 10 kb. Most probably the integrity and purity of RNA templates and the reverse transcription reaction are the critical parameters prior to the amplification of the cDNA (Thiel et al., 1997). Such technology has been used to produce infectious clones of several important RNA viruses including Japanese encephalitis virus (Gritsun & Gould, 1995, Gritsun & Gould, 1998, Zhang et al., 2001), coxsackie B6 (Martino et al., 1999), infectious bursal disease virus (Huang et al., 2003). We have been able to amplify products of the anticipated size by RT-PCR from RNA isolated from several strains of FCV. Successful amplification was dependent on the polymerase used, and frequently associated with strong amplification of other smaller non-specific products. As is frequently the way with scientific publications, difficulties with protocols are rarely discussed. However, low product yield and amplification of non-specific products has been frequently observed for other RNA virus systems (personal communication Raymond Tellier).

Although product of the anticipated size was obtained in this study for FCV, the amount of product was variable and low. This was a major limitation for both the definitive characterisation of these amplicons as genome equivalents and also eventually to their successful cloning. Difficulties that would make the FCV genome a difficult target for PCR amplification include not just its length but also the potential for both the 5' (this thesis manuscript 3) and 3' end of the FCV genome to form potential stem-loop structures (Seal et al., 1994). Incorporation of such secondary structure-prone sequences in primers would lead to primers forming hairpins and dimers limiting amplification. In this study, we have attempted to minimise this by extending the 5' primer beyond a region predicted to form a stem loop. Although the

use of long PCR to generate infectious clones has worked well for several viruses, this has remained problematic for other viruses with a similar genome organisation. For example several attempts have been made to amplify Hepatitis E virus genome by long range PCR without any success (personnel communication S. Emerson). Some of the problems encountered may include complicated secondary structure causing a segment of the genome to be 'skipped' during the PCR reaction. In future studies, it may be necessary to attempt further characterisation of putative full-length amplicons to confirm their integrity. However, this would not be immediately required if infectious virus was recovered.

In order to facilitate cloning of long PCR products we adapted a similar protocol to that of previous studies and incorporated restriction enzyme sites in the 5' and 3' genomic PCR primers. These were specifically chosen to minimise the likelihood of the same restriction sites occurring within the amplified genome. For amplicons derived by RT-PCR from viral RNA, low product yield led us to use a commercial kit for cloning long PCR products to attempt to clone these products directly thereby minimising downstream DNA manipulations and associated product loss. Unfortunately, this was unsuccessful, possibly due to limited product yield. However, if such an approach could be developed further it would simplify the cloning procedure and also allow the design of shorter genomic PCR primers. Direct cloning of PCR products using such a prepared vector would abrogate the need for a restriction enzyme site in the 5' genomic primer. This restriction enzyme site had proved difficult in this study where the initial choice of a six-cutter *SmaI* had to be changed to *SalI* because FCV F9 had a *SmaI* site within it. The *NotI* site could be maintained in the 3' genomic primer to facilitate plasmid linearization prior to *in vitro* transcription.

Being an eight-cutter it is much less likely to be found by chance within an FCV genome. This protocol of direct cloning of PCR products and linearisation of recombinant plasmids by *NotI* should allow infectious clones to be generated from most FCV genomes without the requirement for prior sequence information.

Difficulties over product yield for of amplicons derived by RT-PCR and the resultant failure to clone these products led us to use a previously cloned but non-infectious genome of FCV F9 as template. This clone was produced previously in this laboratory using two overlapping sub-genomic fragments that were amplified, ligated together and sub-cloned into a mammalian expression vector downstream of the Rous Sarcoma Virus (RSV) long terminal repeat promoter (Glenn, 1997). Several reasons have been put forward to explain the failure to recover infectious virus from this vector (Glenn, 1997). The RSV promoter would theoretically result in the addition of three non-viral nucleotides to the 5' end of the FCV genome, which has been shown in some other viruses to greatly reduce infectivity (Dore et al., 1990, Heaton et al., 1989). In addition, transcripts from this vector would be devoid of a 3' poly-A tail. This has subsequently been shown to be necessary for infectivity. Using the FCV Urbana infectious clone, Sosnovtsev and Green (1995) showed that synthetic transcripts containing less that 10 adenine residues showed greatly reduced infectivity (Sosnovtsev & Green, 1995). It is also possible that the genome cloned in pF9FL2 is a non-functional genome as previously reported (Eggen et al., 1989, Holt & Beachy, 1991, Lai et al., 1991). This may be due to the amplification of an already defective genome present in the original virus isolate (Holland, 1990) or the introduction by PCR of inactivating substitutions that may not have been picked up in the original sequence analysis (Eckert & Kunkel, 1992). The FCV genome in pF9FL2 was

sequenced and shown to contain a small number of point mutations (Glenn, 1997) relative to the published sequence for FCV F9 (Carter et al., 1992). However as Glenn has suggested there is currently no way of determining whether the published F9 sequence is that of an infectious virus since it too was produced by cloning.

When pF9FL2 was used as a template in this study, amplicons of the anticipated size were obtained and whilst this product was still fairly limited, it was generally of a better yield with fewer non-specific products than derived by RT-PCR from viral RNA. Following *NotI / SalI* digestion, these plasmid-derived amplicons were successfully cloned into *NotI / SalI* digested pSK- as judged by partial sequence analysis and restriction fragment analysis. However, none of the clones analysed by *in vitro* transcription and transfection experiments produced infectious virus. Since the primers used in this study should result in the generation of a faithful 5' genome (or with one additional non-viral nucleotide) and in a poly-adenylated 3' end, it seems most likely that the FCV F9 genome cloned in pF9FL2 may have been in some way defective, ultimately making it an unsuitable template for the recovery of infectious virus. It is also possible that the additional guanosine nucleotide incorporated in T7 BEG between the T7 promoter and the start of the FCV genome, may render the FCV genomes that contain it non-infectious. However, in coxsackie B6 virus, this additional nucleotide was added and worked successfully, and during subsequent replication the extra G is lost by the virus (Personnel communication Raymond Tellier). In future studies, we plan to attempt to clone amplicons from pF9FL2 using GEN3 and a modified T7BEG without this extra G residue.

In conclusion, we have successfully achieved two of the steps necessary for producing an infectious clone. Specifically, we have developed long range PCR protocols that allow the amplification of putative full-length equivalents under the control of T7 RNA polymerase promoter, and cloned these large amplicons into plasmids. Future studies will be aimed at optimising those protocols specifically with reference to facilitate downstream amplicon characterisation and manipulation. Subsequently, screening a sufficient number of cloned amplicons by attempting to recover virus following *in vitro* transcription should allow us to obtain an infectious clone for any FCV isolate.

MANUSCRIPT 5

Attempts to identify a putative cellular receptor for feline calicivirus from Crandell Feline Kidney Cells and feline embryo cell line A by a virus overlay protein-binding assay.

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ABSTRACT.

Cellular receptors play an important role in viral pathogenesis. In order to multiply, a virus must first infect a cell following attachment to specific receptors on the surface of cells. To date, the host cell receptor for feline calicivirus (FCV) has not been identified. FCV replicates exclusively in feline cells, which suggests that its receptor may be a cell surface molecule specific to this host species. In this study, we have used a virus overlay protein-binding assay (VOPBA), which utilises the specific interaction between a virus and its receptor, to attempt to identify a putative cellular receptor for FCV. In earlier experiments a protein band of approximately 60kDa was detected suggesting this approach may be an appropriate one to use. Unfortunately this result proved inconsistent and not reproducible despite several attempts of optimisation. Reasons for the failure of the VOPBA to detect a candidate FCV cellular receptor are discussed together with alternative approaches that may be appropriate in future studies.

INTRODUCTION.

Feline calicivirus (FCV) belongs to the family *Caliciviridae* and is an important pathogen of domestic cats (Burroughs & Brown, 1974). It mainly causes upper respiratory tract disease and acute mouth ulceration (Gaskell & Dawson, 1994), and can also induce a febrile limping syndrome (Dawson et al., 1994, Pedersen et al., 1983). FCV is a small, nonenveloped virus that contains a positive sense, single-stranded, RNA genome of 7.7 kb in length containing three open reading frames (ORFs) (Carter et al., 1992, Neill, 1990, Neill et al., 1991, Sosnovtsev & Green, 1995). Although considerable knowledge of genome organisation has been achieved, the mechanism by which FCV attaches to and gains entry into host cells is poorly understood.

Virus attachment is usually dependent on a specific interaction between a receptor on the cell surface (the cellular receptor; CR) and a receptor ligand on the viral surface (virus receptor ligand; VRL). The interaction of these molecules initiates a chain of dynamic events that ultimately enables entry of the virus into the cell (Schneider-Schaulies, 2000). In the past, intensive studies have highlighted the complexity of these virus–receptor interactions making progress in this area rather slow (Sommerfelt, 1999). However, an increasing number of VRs have now been identified on the surface of respective host cells (Belnap et al., 2000, Mendelsohn et al., 1989). The structure of molecules used by viruses as CRs is highly variable. Attachment to the surface of cells by the adeno-associated viruses requires only the presence of carbohydrates (Summerford & Samulski, 1998). Other viruses, such as echoviruses use specific glycoproteins as receptors, where

both the protein and carbohydrate moieties are necessary for receptor function (Mbida et al., 1992). In contrast, dengue virus uses receptors consisting only of a protein without a carbohydrate moiety (Salas-Benito & del Angel, 1997, Yazi Mendoza et al., 2002).

One of the best characterised CR – VRL interactions is for members of the *Picornaviridae,* which like the caliciviruses, are also small single-stranded RNA viruses (Racaniello, 1990). The cellular receptor has been identified for several members, including coxsackieviruses (Kuhn, 1997, Shafren, 1998, Shafren et al., 1995), rhinovirus (Bella & Rossmann, 1999), and foot-and mouth-disease virus (FMDV) (Duque & Baxt, 2003, Jackson et al., 1997, Jackson et al., 2000). The non-enveloped capsids of picornaviruses interact with a variety of cellular proteins including those of the integrin binding family (Bergelson et al., 1994, Kuhn, 1997, Shafren, 1998, Triantafilou et al., 1999). Following binding, picornaviruses undergo conformational changes leading to virus entry and uncoating of the genome (Arita et al., 1999, Dove & Racaniello, 1997, Kaplan et al., 1990, Pelletier et al., 2003, Racaniello, 1990).

As well as the CR, the VRL has also been mapped for several picornaviruses. In rhinoviruses, the VRL is located in a conserved region at the bottom of a cleft or "canyon" on the surface of the capsid protein VP1, surrounded by variable domains (Kolatkar et al., 1999, Rossmann et al., 2002). This contrasts with the FMDV VRL, where attachment to the CR is mediated by a portion of VP1 forming a disordered loop on the surface of the virus (Fox et al., 1989). This loop contains an Arg-Gly-Asp (RGD) motif characteristic of integrin binding proteins. It is unlikely that FCV uses a cellular

receptor belonging to the integrin family as a conserved RGD motif is not present within the FCV capsid (unpublished observations).

In contrast to the Picornaviruses, neither the CR, nor its VRL, has been definitively identified for any member of the *Caliciviridae*. Indeed, the only caliciviruses for which a putative CR has been identified are the prototype norovirus (Norwalk virus; NV) (Marionneau et al., 2002, Tamura et al., 2000) (Hutson et al., 2002) and rabbit haemorrhagic disease virus (RHDV) (Ruvoen-Clouet et al., 2000). The ability of RHDV to agglutinate certain human erythrocytes ultimately led to the identification of carbohydrate antigens belonging to the histo-blood group family as putative receptors for these viruses (Ruvoen-Clouet et al., 2000). Initial studies with recombinant NV-like particles (VLPs) have suggested that attachment of NV to cells is mediated by a 105 kDa cellular protein, ubiquitously expressed in mammalian cells but which was not identified further (Tamura et al., 2000). However, more recent studies have shown that, as with RHDV, NV binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals (Marionneau et al., 2002). Secretor individuals express ABH antigens in saliva and on most epithelial cells whereas non-secretor individuals have an inactivating mutation in FUT 1 or FUT 2 genes. These genes produce an enzyme which catalyses the conversion of precursors into H antigenic structures.

A number of different experimental approaches have been used for the identification of CRs. Many of the earliest attempts involved morphological studies, using electron microscopy and virus infected tissue culture (Dales, 1973). In addition, kinetic studies

using radiolabeled virus at various concentrations to adsorb to target cells were used to determine the number and class of receptors per cell (Kaplan et al., 1990, Racaniello, 1990). However, although such studies have provided useful information about the specificities and basic structural features of CRs, the data generated does not lead to the isolation or purification of a functional viral receptor (Bass & Greenberg, 1992).

Direct purification and biochemical characterisation have also been used frequently to identify CRs. Usually, purified virus binds to cell membranes either on cell monolayers, in solution following detergent extraction from cells, or bound to a solid phase matrix. Perhaps the most success use of these biochemical methods was demonstrated with coxsackie B virus receptors (Hsu & Crowell, 1989, Hsu et al., 1988, Krah & Crowell, 1982, Krah & Crowell, 1985, Mapoles et al., 1985). The advantage of this approach is the ability to isolate and purify specific cell membrane molecules involved in viral attachment. However, the disadvantages of these strategies include poor affinity of virus-receptor complexes compared to the multivalent binding, which functions *in vivo*, and the need to correlate productive infection with binding to a given entity.

A more recent and simple modification of the affinity-based biochemical approach to receptor identification is the virus overlay protein-binding assay (VOPBA) (Bass et al., 1991). In this assay, cellular membranes are prepared from permissive cells, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted to a nitrocellulose membrane, and probed with virus. Membrane components, which retain high affinity for virus, are detected as bands on the membrane. A major advantage

stated for this approach is the high concentration of receptor that can be achieved on a nitrocellulose membrane for viral attachment, which increases the sensitivity of this assay compared to those based on liquid phase binding (Bass & Greenberg, 1992). Several viruses have been studied by this technique (Crane et al., 1991, Fox & Bloom, 1999, Jin et al., 1994, Ludwig et al., 1996, Salas-Benito & del Angel, 1997) including NV (Tamura et al., 2000). However, to date, no permissive cell lines for the growth of NV have been established, and the 105kDa putative CR referred to above was identified in a variety of non-permissive cell types using virus-like particles (VLPs). These VLPs were generated with recombinant baculoviruses harbouring the capsid protein of NV, and are proven to be morphological and antigenically similar to the native virions (Jiang et al., 1992, Prasad et al., 1999, Prasad et al., 1994).

The purpose of this study was to attempt to identify a putative CR for FCV on the cell surface of permissive feline cell lines using a VOPBA. This is a first attempt to identify the CR for FCV, and was based on the assumption that the FCV CR may be a protein. Since permissive cell lines for the growth of many other caliciviruses have not yet been established, the identification of the FCV CR may create a useful model in which to study the interaction between members of the *Caliciviridae* and their host cells.

METHODS

Several approaches were attempted to identify the CR for FCV, due to inconsistent results on several occasions.

Cells and virus.

Two FCV permissive continuous cell lines, feline embryo cell line A (FEAs) (European collection of cell cultures) (ECACC) (Jarrett et al., 1973) and Crandell Rees Feline Kidney Cells (CRFK) (ECACC) (Crandell et al., 1973) were used for this study. Both cell types were grown in $75cm^2$ flasks with Eagles minimum essential medium (EMEM, Sigma), supplemented with 10% foetal calf serum as required for growth until monolayers were confluent. The virus strain FCV F9 (Bittle et al., 1960) was used throughout.

Virus overlay protein binding assay using crude cell extracts.

In preliminary experiments, crude cell and viral extracts were used in the VOPBA to attempt to prove the utility of this type of assay for the identification of specific virus – cell interactions. The FCV F9 strain used to probe for the VR was grown in FEAs. Infection was allowed to proceed overnight until cytopathic effect (CPE) was complete. Monolayers were frozen at -80°C and thawed to produce a cellular lysate, which was used in later experiments.

Crude extracts of cell membrane proteins were prepared using confluent FEA cells in $75cm^2$ flasks. Briefly, these cells were washed twice with phosphate buffered saline (PBS; 154mM NaCl, 3 mM KCl, 9mM Na_2HPO_4, 1.65mMKH_2PO_4). Then 1ml of PBS was added and the cells were scraped into sterile Eppendorf tubes and centrifuged at 4,000g for 1 min. The supernatants were discarded and the cell pellet resuspended in 1ml

of PBS, aliquoted into fresh Eppendorf tubes, and stored at -20°C. A stock solution of 3X reducing sample buffer was prepared (1ml of 1M Tris, pH 6.8, 4ml of 10% SDS, 2ml of glycerol, 1ml of the reducing agent 2-mercaptoethanol, and 0.1% bromophenol blue dye). Then 20µl of this stock solution was added to 10µl of FEA cellular extract and boiled for 5mins at 95-100°C and immediately placed on ice. Sample volumes of 20µl of this FEA cellular preparation was then loaded into each lane of a 10% polyacyrlamide gel and subjected to electrophoresis at a constant voltage of 120V until the dye front reached the bottom of the gel (approx 45-60 mins). Separated proteins were transferred onto a nitrocellulose membrane by electroblotting at 100V for 1 hour in ice-cold transfer buffer (0.025M Tris-base, 0.192M glycine, and 200ml methanol made to a final volume of 1 litre in distilled water). Ponceau S staining (Sigma) of nitrocellulose blotted proteins was used to confirm successful transfer (data not presented).

Non-specific protein binding with the membrane was blocked overnight by incubating in 5% skimmed milk in 100ml PBS. After blocking, the membrane was washed twice in 0.05% Tween 20 (Sigma) in PBS (PBS-T), for 10 minutes per wash and incubated with FCV F9 (~10^7 $TCID_{50}$) diluted 1:100 in 0.5% (wt/vol) skimmed milk in PBS overnight at 4°C. After a further three washes in PBS-T, each for 10 minutes, the membrane was then incubated for 1 hour at 37°C with mouse monoclonal antibody IG9 (Nova Castra) diluted 1:100 in 0.5% (wt/vol) skimmed milk in PBS. The linear epitope for IG9 resides within the capsid protein of FCV F9 (Carter et al., 1989). The membrane was washed three times in PBS-T for 5 minutes per wash, and incubated for a further hour at 37°C in peroxidase- conjugated anti mouse IgG (Sigma) diluted 1:2000 in 0.5% (wt/vol) skimmed

milk in PBS. Then the membrane was washed twice in PBS-T for 10 minutes per wash, followed by two final washes in PBS for 5 minutes per wash. Finally, membrane-bound peroxidase was developed at room temperature with 3,3`-diaminobenzadine tetrahydrochloride (DAB; Sigma) according to the manufacturer`s instructions.

Controls run on SDS-PAGE gels included FCV F9 (approximately $2x10^4$- $2x10^6$ TCID) as a positive control for IG9 binding and staining, and uninfected tissue culture fluid harvest (10ul) as a negative control. Both controls were treated under the same conditions as for the cellular extract. As a further negative control, duplicate membranes were also probed with uninfected tissue culture fluid harvest instead of FCV F9, with all subsequent steps being performed as previously described.

Virus overlay protein-binding assay using cell surface proteins extracted with mild detergent lysis.

In order to minimise potential disruption to the FCV CR conformation and function, the cell extraction protocol was adapted by using mild detergent cellular lysis and by the addition of protease inhibitors to minimise proteolysis during cell extraction (Bass et al., 1991). Briefly, confluent FEA and CRFK cells in $75cm^2$ flasks were washed twice with PBS, and lysed in PBS containing 2mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and 1% of the mild detergent Nonidet P-40 (NP-40) for 30mins on ice. The cell lysates were scraped into sterile Eppendorf tubes and nuclei were removed by centrifugation at 1000g for 5mins at 4°C, and the supernatant was further centrifuged for

30mins at 11,000g at 4°C. The supernatants were transferred and aliquoted to fresh Eppendorf tubes and stored at -70°C. Total protein concentration was estimated using the standard Bradford assay (Biorad) according to manufacturer`s instructions to be 3.2 μg/ml.

As well as reducing conditions, prepared cell membrane proteins were also separated under non-reducing conditions to minimise the potential loss of VR function. The reducing conditions were as described previously. Non-reducing conditions were the same, except the 2-mercaptoethanol was omitted from the sample buffer. Similar positive and negative controls were run for each experiment.

In order to estimate the protein concentrations on the gel, one gel was incubated in 0.1% Coomassie blue in 40% methanol for approximately 1min, and destained with 50% methanol (Coomassie staining is able to detect 0.1-1μg protein per band). Proteins from a duplicate gel were transferred onto a nitrocellulose membrane by electroblotting at 100V in ice-cold transfer buffer as previously described. To demonstrate that the transfer was successful, Ponceau S (Sigma) staining was performed according to manufacturer's protocols. Following Ponceau S staining, the membrane was de-stained with PBS and developed exactly as described in earlier experiments. In later experiments, the stringency of the wash conditions was reduced to maximise the chances of detecting low affinity virus – CR interactions.

Purification of FCV F9 for use as a probe in VOPBA.

In order to try and improve the sensitivity and specificity of the VOPBA, FCV F9 was purified using Iodixanol (Optiprep; Nycomed, Pharma, Oslo, Norway) (Graham et al., 1994). Iodixanol readily forms self-generating gradients because of its molecular mass and high solubility (1.32g/ml) (Ford et al., 1994). The advantage of using a gradient formed from iodixanol as opposed to those based on sucrose to purify viruses is that iodixanol gradients are isoosmotic, minimising the impact on protein conformation and virus infectivity.

Cell culture medium was removed from a confluent monolayer of FEA cells, in a 162cm^2 flask and the cells infected with 1ml of an FCV strain F9 (approximately 10^7 TCID). Virus was adsorbed to the cells for 1 hour, before 50ml of maintenance medium was added containing 1% foetal calf serum. Virus was allowed to replicate for 24 hours until cpe was complete. A virus cell lysate was prepared by freeze thawing these cells and the lysate stored at -80°C until required. Cellular debris was removed from the viral cell lysate by centrifugation at 1000g for 10 mins at room temperature in a bench top centrifuge (Centaur 2). The supernatant was overlayed onto 5ml of 50% Optiprep solution, in 36ml Sorvall centrifuge tubes (polyallomer). After centrifugation in a swing out Sorvall rotor (AH625) at 100,000g for 4 hours at 4°C, the supernatant was removed leaving 5ml of the medium over the 5ml optiprep cushion. The cushion and the remaining medium was mixed to create a 25% virus Iodixanol solution and transferred to 6ml Sorvall centrifuge tubes (polyallomer thin walled) and cenrifuged in a Sorvall vertical rotor TV1665 at 350,000xg for 2 hours 15 mins at 4°C.

Following centrifugation any observed bands were collected by inserting a 21-gauge hypodermic needle just below the band. A sample was also removed from the top of the gradient for comparative titre analysis. Ten-fold serial dilutions of the sample to be assayed were prepared in growth medium containing 10% foetal calf serum and 100µl samples of each dilution were inoculated onto 4 wells of a 96 well plate, and an FEA cell suspension was added. Viral titres were calculated by the method of (Reed & Muench, 1938), and were expressed as the 50% tissue culture infective dose per ml ($TCID_{50}$/ml). Following centrifugation a single distinct band was observed. After performing a virus titration on the gradient fraction containing this band, the titre was 10^9 $TCID_{50}$ compared to a pre-purification titre of 10^7 $TCID_{50}$. Furthermore, the titre of the virus from the top of the density gradient medium had a titre of only 10^5 $TCID_{50}$. This suggested that we had purified and concentrated the virus.

Detection of virus-cell interactions in a dot blot assay.

In an attempt to simplify the assay procedure for detecting virus-cell interactions, a dot blot assayed was also employed. Briefly, cell membrane extracts were prepared as for the protease / mild-lysis protocol, and dotted onto nitrocellulose membranes (Sigma). In addition, 10µl of FCV F9 (corresponding to approximately 2x 10^4- 2x 10^6 TCID), and 10µl of uninfected tissue culture fluid, were also added as positive development and negative controls respectively. After drying at room temperature, the membranes were blocked, incubated with either FCV F9 or uninfected tissue culture harvest (negative control), and developed as described previously. In addition, another development

system was used for other dot blots. This was essentially the same procedure but the secondary antibody was anti mouse labelled with alkaline phosphatase diluted 1:2000 in 0.5% skimmed milk in PBS. Development occurred using 5-Bromo-4-chloro-3-indolyl phosphate dipotassium salt/ Nitrotetrazolium blue chloride (BCIP/NBT) which produces a dark purple colour for a positive reaction.

RESULTS.

Virus overlay protein binding assays.

The results of preliminary virus overlay protein-binding assays using crude cell extracts showed a positive reaction with a cellular protein of approximately 60kDa (Fig. 1a). This result was of considerable interest, suggesting that the virus–CR interaction was maintained under the assay conditions employed. However, this result was inconsistent. In some cases multiple bands were obtained (Fig. 1b), whilst in others, no positive reaction was obtained (data not presented). The FCV F9 positive control for the staining protocol was reproducibly positive showing a band of approximately 62 kDa consistent with the size of the FCV capsid (Fig. 1c) (additional bands of approximately 80kDa, 100kDa and 135kDa were also occasionally detected as described previously (Carter et al., 1989).

Attempts to improve the sensitivity and specificity of the VOPBA by preparing cellular extracts with mild detergents in the presence of protease inhibitors was unsuccessful. These results were consistently negative using both the crude unpurified and Iodixanol-

purified FCV F9 as a probe under both reducing and non-reducing conditions (unpublished data). Only the positive staining control of FCV F9 produced the expected protein band of 62kDa in all experiments performed.

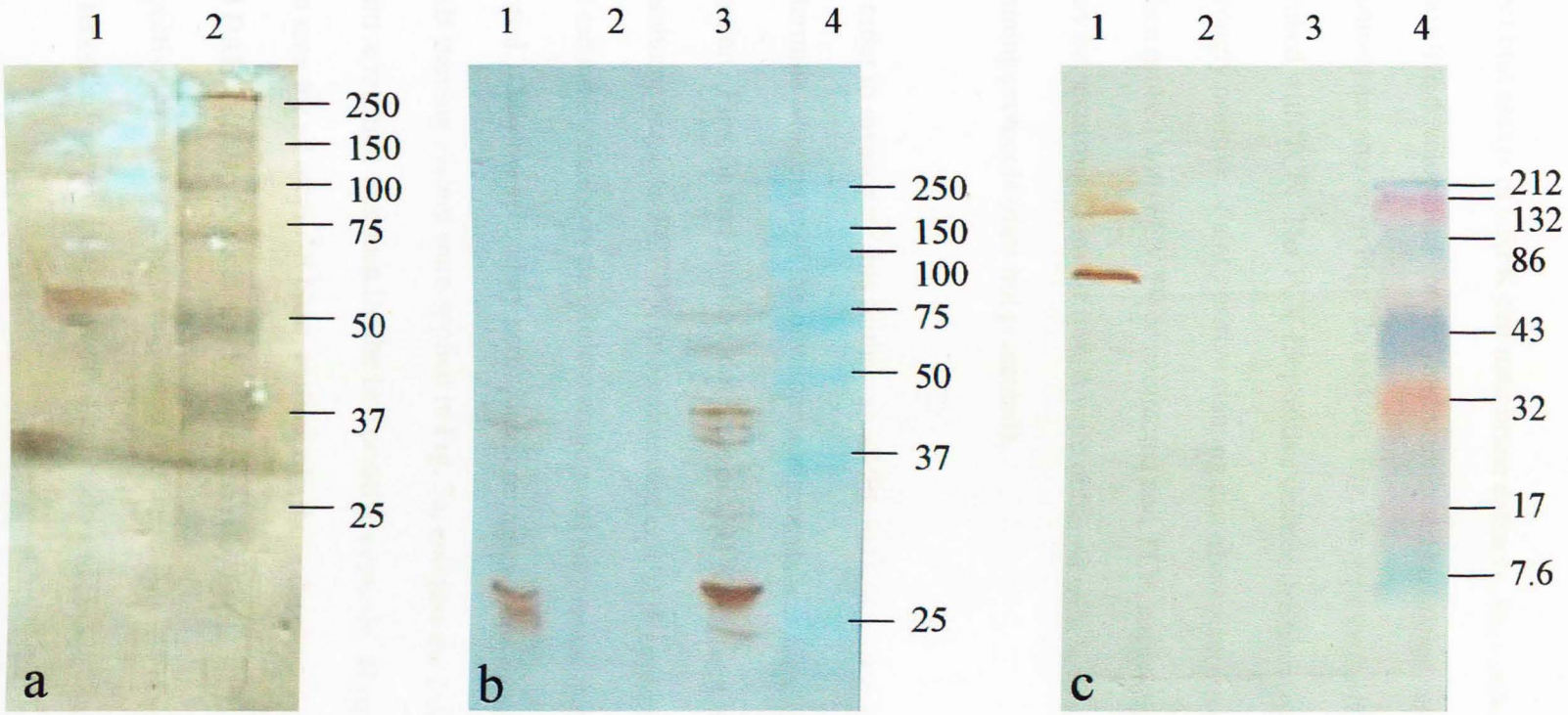

Fig. 1. Representative preliminary results of VOPBA using crude FeA cell and FCV F9 extracts under denaturing conditions. **a**). A protein band of approximately 60kDa was identified in some experiments (lane 1). Prestained molecular weight markers are present in Lanes 2 (Biorad). **b**). Multiple bands were detected in some experiments (lanes 1 and 3). Prestained molecular weight markers are present in Lane 4 (Biorad). Lane 2 was loaded with sample buffer only. **c**). In some cases, no specific bands were detected (lanes 2 and 3). The positive staining control F9 is present in Lane 1 and shows a major band at the known weight of the FCV major capsid protein. Prestained molecular weight markers are present in Lane 4 (Biorad). Molecular weight marker values in kDa are indicated on the right of each figure.

Virus cell interactions using dot blot assays.

Dot blot assays of CRFK cell membrane extracts, FEA cell membrane extracts, FCV F9 (positive development control) and uninfected cell tissue culture fluid (TCF) (negative control) are shown in Figs. 2a and 2b. Fig. 2a is probed with FCV F9 and Fig.2b is probed with TCF. The FCV F9 positive control dot blots in both Figs. 2a and 2b are strongly positive. Faint positive staining can also be seen for both CRFK and FEA cells, when probed with FCV F9 or uninfected cell TCF, suggesting that non-specific binding may be occurring. Similar results were obtained with both the DAB and BCIP/NBT staining protocols (data not presented).

In order to investigate this further, an additional series of dot blots were performed to determine what stage of the development process was responsible for this non-specific reaction. Figs. 3a and 3b show dot blots of CRFK cell membrane extracts, FEA cell membrane extracts, FCV F9, and uninfected cell TCF applied as in Figs.2a and 2b, and the secondary antibody peroxidase conjugated anti-mouse IgG as a positive staining control. Only the secondary peroxidase conjugated anti-mouse IgG antibody and the DAB staining system were applied in Fig. 3a, and just the DAB for Fig. 3b. A strong positive reaction was seen for the two positive controls. However, weak staining was also seen for all other dot blots, particularly those developed with the secondary antibody and DAB, but also to a lesser extent for those developed with DAB alone (Figs 3a and 3b respectively). These results suggested that a non-specific reaction was occurring between the secondary peroxidase-conjugated antibody and components of the feline cell extract.

Attempts to remove this non-specific binding by preadsorbing the secondary antibody with cell membrane extracts proved unsuccessful (data not presented).

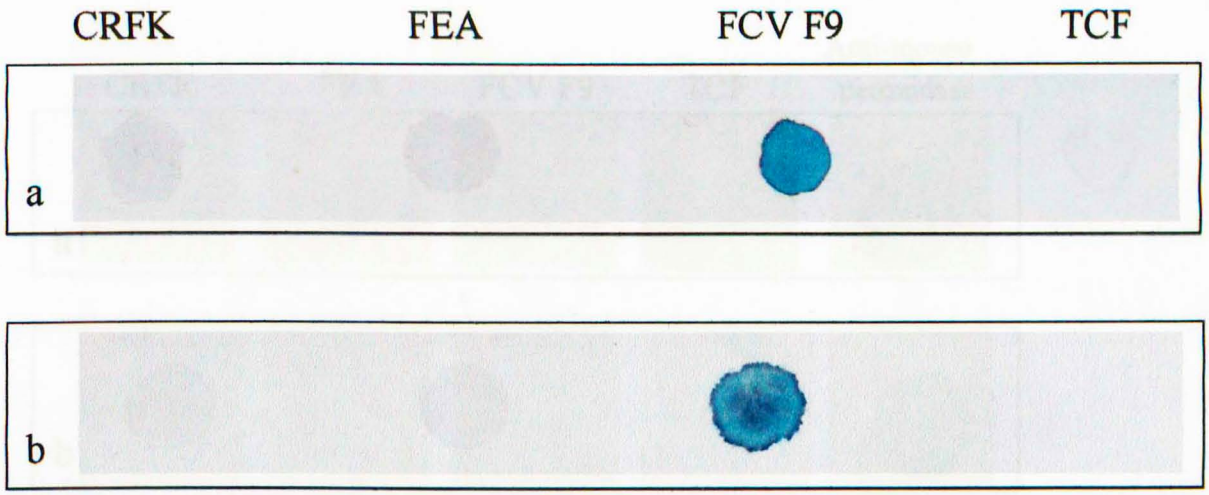

Fig. 2.

Figs. 2a and **2b.** Dot blot assays of CRFK cell membrane extracts, FEA cell membrane extracts, FCV F9 (positive control) and uninfected cell tissue culture fluid (TCF) probed with FCV F9 (Fig. 2a) or uninfected TCF (Fig. 2b). The FCV F9 staining control is strongly positive in both (Figs. 2a.and. 2b). Faint positive staining can be seen for CRFK and FEA cells when probed with FCV F9 or uninfected cell TCF, suggesting non-specific binding may be occurring. Membranes developed using BCIP/NBT.

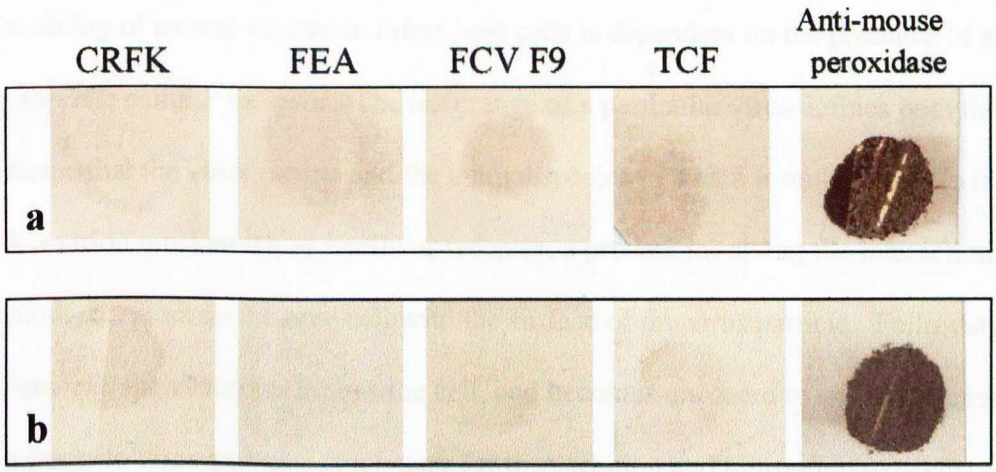

Fig. 3.

Figs. 3a and **3b.** Dot blots of CRFK cell membrane extracts, FEA cell membrane extracts, FCV F9, uninfected cell TCF applied as in Figs.2a and 2b, and the secondary antibody peroxidase conjugated anti-mouse IgG as a positive control. Membranes were developed using secondary antibody peroxidase conjugated anti-mouse IgG and the DAB staining system in Fig. 3a, and DAB alone in Fig. 3b. A strong positive reaction was seen for the two positive staining controls in both Figs. 3a and 3b (anti-mouse peroxidase). However, weak staining was also seen for all other dot blots when probed with either secondary antibody peroxidase conjugated anti-mouse IgG and DAB or DAB alone (Figs 3a and 3b).

DISCUSSION.

The ability of animal viruses to infect host cells is dependent on the presence of an appropriate cellular receptor. The host range of a particular virus defines both the types of tissue that the virus infects and the animal species in which it multiplies. To infect a cell, a virion must attach to a cellular receptor, a process involving the interaction of one or more receptors on the host cell with the surface of the virus particle. Following attachment, the virion penetrates the cell, and becomes uncoated to make its genome accessible to virus and host machinery for transcription and translation to occur. Information on identifying such putative cellular receptors is increasing but the molecular mechanisms concerning these events are not fully understood (Schneider-Schaulies, 2000).

The aim of this study was to attempt to identify the putative CR for FCV as little is known about the mechanism by which the virus enters permissive cells and initiates infection. A virus overlay protein-binding assay was used as several receptor proteins have been identified using this technique including those of encephalomyocarditis virus (Jin et al., 1994), Sendai virus (Gershoni et al., 1986), mouse hepatitis virus (Boyle et al., 1997), and Hantaan virus (Kim et al., 2002). In earlier experiments a band of approximately 60 kDa was detected suggesting that the VOPBA may be a suitable protocol to detect the FCV CR. Unfortunately these results were inconsistent and after several attempts to refine the VOPBA protocol, were not repeatable. It is uncertain at

this stage whether this 60 kDa protein represented a genuine interaction between FCV and cellular protein or whether it was an artifact of the earlier experimental protocol.

There are several reasons that may explain why we were unable to detect a specific interaction between FCV and a putative cellular receptor. For example, it is possible that denaturation of the cellular extracts may have occurred during electrophoresis and blotting. This can still occur even with freshly prepared cellular extracts, although the addition of non-denaturing detergents in the lysis buffer before SDS-PAGE can preserve the protein`s biological activity. This was demonstrated in a study by Fox et al (1999) who identified a cell surface protein from CRFK cells that specifically binds Aleutian mink disease parvovirus (ADV). During the optimization of their protocol, these authors found that the inclusion of detergents decreased the specificity of binding. One of the reasons that detergents may promote non-specific binding is that the detergents are interacting with hydrophobic transmembrane regions of the membrane proteins on the blot. The virions may be nonspecifically interacting with the membrane protein associated detergents. In addition, intercellular proteases are fairly resistant to denaturation and can also degrade sample proteins.

As well as the possible denaturation and inactivation of the cellular receptor, it is also posible that the affinity of FCV for its receptor and the sensitivity of the VOPBA were too low to allow detection of FCV CR. Future studies to identify such a putative cellular receptor could focus on increasing the sensitivity of the assays by radiolabelling FCV.

Such a protocol has recently been used to identify a 30 kDa cell surface protein as a putative cellular receptor for Hantaan virus (Kim et al., 2002).

However, it is also possible that the FCV CR is not a protein, but another type of molecule such as a carbohydrate antigen. This has recently been suggested for RHDV and NV (Marionneau et al., 2002, Ruvoen-Clouet et al., 2000). However, this work was published after the current study was initiated. The assumption was made in our studies that the FCV receptor was likely to be a protein, based on earlier NV work (Tamura et al., 2000), and the specificity of FCV for feline cells, and the initially promising results of the early VOPBAs. Since related viruses often use similar receptors for virus entry, it may be appropriate in future studies to see if FCV also interacts with histo-blood group antigens as recently demonstrated for RHDV and NV. Many virus receptors have been identified this way based on the previous identification of receptors in related viruses. Examples of these include the role of aminopeptidase N as the major cell surface receptor for group 1 coronaviruses including feline coronavirus (Tresnan et al., 1996) and the role of CXCR4 as a common receptor for human and feline immunodeficency viruses (Willett et al., 1997). However, a role for histo-blood group antigens in FCV seems unlikely at this stage since the agglutination phenotype of RHDV and NV that initially suggested such antigens may be involved in attachment for these viruses has not been demonstrated for FCV and to the best of the authors knowledge (see also Studdert, 1978).

It seems likely that future studies aimed at characterising an FCV cellular receptor would require a completely new approach to the VOPBA used in this study. These may include

the development of simple cell attachment assays based on permissive cell lines followed by the identification of specific inhibitors of this attachment assay. Other approaches, including the characterisation of monoclonal antibodies raised against permissive cells, and the transfer of the permissive phenotype to non-permissive cells by cDNA transfection, may also be appropriate.

General discussion and future work.

This thesis was aimed at increasing the understanding of important mechanisms of FCV replication and host cell interactions utilising a wide range of sensitive molecular techniques.

Previous studies by this research group identified two linear epitopes in conserved regions of the major capsid protein (Radford et al., 1999) suggesting they may prove useful in vaccine development, especially as one, ags 4, appeared to be detected by the majority of FCV infected cats. We investigated the antigenic cross-reactivity of ags 4 using antisera obtained from rabbits with a synthetic peptide corresponding to ags 4 conjugated to a carrier protein. Then we demonstrated that this epitope induced an antibody response in rabbits which reacted well with all FCV isolates tested in dot immunoblotting, Western blotting, and immunofluoresence. This confirmed the ability of this epitope to induce an antibody response, which is broadly cross-reactive amongst FCV strains, suggesting such antiserum could prove highly useful as a diagnostic and research tool for the specific detection of a wide range of FCV isolates. The ability of antiserum to ags4 to neutralise FCV was more equivocal, with the two antiserum showing low and undetectable neutralising activity respectively. However, if this neutralising activity is genuine and as broadly reactive as its reactivity in blots and by immunofluorescence, then the potential for ags4 to act as a novel vaccine could be huge.

Future studies to enhance and confirm the specificity of the neutralising response seen will require the synthesis of higher titre antisera. This may be achieved by the use of

other carrier proteins described in manuscript 1 such as tetanus toxoid, *Pseudomonas aeruginosa* toxin A, beta-galactosidase, *Brucella abortus* (killed bacteria), hepatitis B core and surface antigens, all of which have been previously used to improve the immunogenicity of peptides (Baker et al., 2000, Beekman et al., 2001, Lapham et al., 1996). Recently, the use of T helper recognition sites has also been used to increase the immunogenicity of injected peptides (Wang et al., 2002).

These higher titre antiserum could then be used to determine their neutralising activity against a wide panel of FCV isolates *in vitro*. Although the ultimate target species for neutralising is the cat, it would seem most sensible to trial peptide delivery systems again, as in this study, in the rabbit, making use of commercial laboratories for the preparation of antiserum. Once a suitable peptide delivery system has been optimised and providing the neutralising activity is confirmed, it would then be necessary to use ags4 peptides to vaccinate cats. In the natural host, neutralising activity could then be assayed both in cell culture and *in vivo* using challenge experiments. In this regard it is worth mentioning that currently available commercial vaccines for FCV result in relatively low-level antibody titres as determined by *in vitro* virus neutralisation.

Regarding the *Caliciviridae* as a whole, little is known about how these important viruses cause disease at the cellular level. Only members of the vesivirus genus can be grown routinely in cell culture including FCV, SMSV and some isolates of CaCV. Because infections caused by FCV are ubiquitous, and the virus has a narrow host range this is an ideal model system for studying calicivirus replication. Studies by this research group have previously characterised the morphological changes in infected cells which include the cells becoming rounded and refractile. In addition, plasma

membrane blebbing can be observed on the surface of some infected cells (Knowles, 1988). Whilst the exact mechanisms by which FCV induces this cell death are unknown, some of the observed changes are suggestive of a cell undergoing apoptosis. As viruses can actively modulate cell cycle progression and apoptosis we therefore investigated whether the major mechanism of cell death in infection with FCV *in vitro* is associated with the induction of apoptosis. In this study, there was little evidence to support specific induction of apoptosis in FCV-infected cells as assessed by terminal deoxynucleoide transferase-mediated labelling of 3'-OH ends (TUNEL assay). In addition, there was no apparent upregulation of the anti-apoptotic factor Bcl-2. However, there was some suggestion that cell cycle progression was being manipulated by FCV infection as judged by levels of proliferating cell nuclear antigen (PCNA).

These results are at variance with two very recent studies suggesting that apoptosis does occur in FCV infected cells (Al-Molawi et al., 2003, Sosnovtsev et al., 2003). It is not clear why our studies did not demonstrate evidence of apoptosis. Both of the recent studies published used different techniques from our work, including assessment of caspase activity as an indicator of apoptosis. However, different cell lines, and, in one study, a different virus strain was used, in both our work and the published work and this may have influenced the results.

In future studies, it would be useful to repeat the experiments we performed on cell pellets on cover slip grown cells where cellular morphology is maintained and where dual labelling for cellular markers and FCV antigens is more readily achieved. Although we did achieve limited success using cover slips, technical problems

precluded more extensive use in this study. The significance of PCNA upregulation as suggested by our results remains to be determined. Further studies are required to determine the location of FCV replication within infected cells and to further evaluate its effect on cellular function and turnover.

As little information is available on predicted secondary structures in FCV, we analysed regions at the 5`ends of both the genomic and subgenomic RNA molecules of all FCV isolates that have been fully sequenced to date. Putative conserved secondary structures were predicted for all FCV isolates. The significance of these regions was further suggested by the high degree of sequence conservation identified between the 5' ends of both the genomic and sub genomic RNA molecules, suggesting these regions may play a similar role in both molecules. However, it is worth emphasising again the putative status of these structures. Before they can be regarded as definitive, synthetic RNA molecules will be necessary to characterise the significance of these putative structures *in vitro*. These molecules could be produced from a full-length infectious clone (if available). In the absence of an infectious clone, smaller sub-genomic RNA fragments could be generated and used as molecular 'hooks' to identify cellular (and viral) proteins that bind to these regions (Gutierrez-Escolano et al., 2000). In both cases, mutational analysis of predicted structures will allow the precise structure of the RNA molecules in these important genomic regions to be dissected.

Infectious clones have been previously attempted within this research group and although a full-length clone was produced, infectious virus was never recovered. The possible reasons for such failures are discussed in manuscript 4. Improved PCR technology has enabled many studies to generate full-length cDNA in a single round

of long PCR, following the reverse transcription of the viral RNA. Then infectious RNA can be generated directly from the amplicon by *in vitro* transcription since a T7 promoter is engineered in the 5` end primer. It was decided that this approach should be attempted to generate infectious clones of FCV isolates. In principle, the application of long RT-PCR should simplify the construction of infectious clones and diminish some of the problems often encountered especially during the ligation of many sub genomic cDNA fragments prior to cloning. Using this methodology, we were successful in producing long RT-PCR products with some FCV isolates. However, following purification, the yield of putative full length amplicons obtained was in general disappointing and represented a limitation to amplicon and characterisation and cloning. Future work would therefore be aimed at increasing the yield. This could be achieved by attempting to purify and concentrate viral template RNA for example by caesium chloride gradients. It is also possible that further attempts at PCR optimisation may improve product yield, particularly as enzyme technology continues to improve. Readily generated infectious clones of FCV isolates would be particularly beneficial to help understand genomic replication and the molecular mechanisms of virulence.

As mentioned throughout this thesis FCV replicates exclusively in feline cell lines therefore representing a useful model to study the interaction between caliciviruses and cells. As the cellular receptor has not been identified for FCV, and based on the assumption it is likely to be a protein, we attempted to identify a cellular receptor on the surface of feline permissive cell lines. Although cellular receptors have long been a topic of interest, until recently progress in this area has been rather slow. Indeed a variety of strategies have been utilised to detect and characterise specific cellular

receptors. Amongst various methods the most promising approach has been the virus overlay protein-binding assay (VOPBA) used in this study. However, as we were unable to detect the putative cellular receptor for FCV on this occasion other methodologies described in manuscript 5 could be attempted. For example, using a VOPBA assay but probing with radiolabelling virus as described in a recent study (Kim et al., 2002). The possibility that the receptor may not be a protein should also be considered.

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