Analysis of feline calicivirus capsid protein genes: identification of variable antigenic determinant regions of the protein

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Three isolates of feline calicivirus (FCV) designated NADC, KCD and CFI/68 were compared for biochemical, serological and genetic variation within the capsid protein gene. The M_r of the capsid protein from purified virions was approximately 66 000 for the NADC virus isolate, which differed slightly from the relative mobilities of the purified capsid proteins of the KCD and CFI/68 isolates. Polyclonal antisera from either cats infected or rabbits hyperimmunized with the CFI/68 isolate cross-reacted with all three isolates by Western blot analysis. However, these polyclonal antisera to CFI/68 varied considerably in their virus-neutralization titres to the KCD and NADC isolates. Nucleotide

sequence data confirmed the genetic variability among these FCV isolates. Comparison of the predicted amino acid sequence of the capsid protein among isolates revealed two regions of sequence divergence that probably contain the antigenically variable determinants. These hypervariable regions may vary by as much as 55% among isolates of FCV. The amino acid sequence diversity in the hypervariable regions of the KCD and NADC isolates correlated well with the virus-neutralization data and suggests that polyvalent vaccines may be more protective than the commonly used monovalent vaccines.

Feline caliciviruses (FCVs) are associated with respiratory disease in cats which, in most cases, is limited to the upper respiratory tract. The disease ranges from inapparent infection or mild disease to severe respiratory disease and pneumonia. Ulcerations occur in the oral cavity and viral shedding may persist from the nasal and pharyngeal region for 2 weeks post-infection (p.i.) (Kahn & Gillespie, 1971; for review, see Gillespie & Scott, 1973). Investigators analysing FCV isolates from the U.K., U.S.A., Australia and New Zealand have demonstrated that substantial variation exists in serum cross-neutralization among most virus isolates examined (Bittle *et al.*, 1960; Crandell, 1967; Povey, 1974; Kalunda *et al.*, 1975; Burki *et al.*, 1976). Isolates of FCV also differ among one another in plaque morphology (Ormerod & Jarrett, 1978) and in cross-reactivity to monoclonal antibodies (MAbs) (Carter *et al.*, 1989; Toya *et al.*, 1991a). It has recently been postulated that variant

strains resistant to current vaccination procedures may be emerging (Knowles *et al.*, 1990).

Structurally, FCV isolates are non-enveloped with a positive-strand, polyadenylated RNA genome of 7.69 kb in size with a single capsid protein (Carter *et al.*, 1992a). The viral genome codes for a picornavirus-like RNA-dependent RNA polymerase, cysteine protease, a 2C polypeptide and, similar to picornaviruses, the RNA-dependent RNA polymerase is located at the C terminus of the putative non-structural polypeptides (Neill, 1990; Carter *et al.*, 1992a). During replication, a nested set of 3' coterminal transcripts are produced. The most abundant transcript is a 2.4 kb subgenomic RNA which can be detected as early as 2 h p.i. (Neill & Mengeling, 1988; Carter, 1990) and has been identified as the transcript encoding the viral capsid protein (Neill *et al.*, 1991). The capsid protein gene has been sequenced for the CFI/68 isolate (Neill *et al.*, 1991), the F4 isolate (Toya *et al.*, 1991b) and F9 isolate (Carter *et al.*, 1992a, b) of FCV. Although the majority of the protein is highly homologous among isolates, one domain of the polypeptide from residues 396 to 525 of the F9 isolate varies in amino acid sequence by 25% from the same region of the CFI/68 isolate (Carter *et al.*, 1992b). A major portion of this area of the capsid protein has since been designated region E among caliciviruses (Neill,

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Nucleotide sequence data reported in this paper appear in the GenBank database under the accession numbers L09718 (NADC isolate) and L09719 (KCD isolate).

Table 1. One-way cross-neutralization of four FCV isolates using sera obtained from a cat and rabbit pre- and post-inoculation with CFI/68

	CFI/68*		KCD*		NADC*		F9*	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Cat 2669†	< 2	512	< 2	128	< 2	< 8	< 2	128
Rabbit 101†	< 2	4096	< 2	32	< 2	< 8	< 2	2048

* FCV isolates CFI/68, KCD and F9 were obtained from the ATCC. Isolate NADC was obtained from a healthy cat at the Ames, Iowa, animal shelter.

† Reciprocal virus neutralization titres of twofold dilutions of sera from cats and rabbits inoculated with the CFI/68 isolate of FCV, based on 100% plaque reduction of 100 p.f.u., in triplicate.

1992) and may contain the antigenically variable determinants as suggested by both these investigators. Because FCV isolates may vary considerably in serum cross-neutralization among isolates and amino acid sequence variation occurs in the capsid protein, three isolates of FCV, one of which was antigenically distinct by one-way serum cross-neutralization, were compared for biochemical and genetic variation within the capsid protein genes.

The FCVs used during these studies were replicated in Crandell-Reese feline kidney cells (Crandell *et al.*, 1973) and purified in caesium chloride gradients at a density of 1.37 g/ml (Love & Jones, 1974; Komolafe *et al.*, 1981). Antisera prepared as described by Povey (1979) against the FCV CFI/68 isolate (Crandell *et al.*, 1960) in both cats and rabbits were used for Western blot analysis and serum virus neutralization of heterologous FCV isolates KCD (Fastier, 1957), F9 (Bittle *et al.*, 1960) and a recently obtained isolate designated NADC. When an adult seronegative cat was inoculated with the FCV isolate CFI/68, no clinical symptoms were observed. However, sera from this animal had a high neutralizing titre to the CFI/68 isolate, whereas the neutralizing titre to the more recent FCV isolate NADC was essentially non-existent. The cat anti-CFI/68 serum had a neutralizing titre to the KCD and F9 isolates that was intermediate to the CFI/68 and NADC isolates (Table 1). When a rabbit was hyperimmunized with purified FCV CFI/68 capsid protein a stronger antibody response was obtained (Table 1). The rabbit anti-CFI/68 serum had high neutralizing titres to the CFI/68 and F9 isolates as compared to the titres obtained for the KCD and NADC isolates (Table 1). Again, the titre to the KCD isolate was intermediate to the CFI/68 and NADC isolate's serum-virus neutralization titres.

After the capsid proteins from three different FCV isolates were separated by SDS-PAGE (Hames *et al.*, 1981), a slight difference in relative mobilities of the capsid proteins from each individual isolate was ob-

served. The FCV NADC isolate had a capsid protein of slightly greater size than the KCD isolate which was slightly larger than the CFI/68 isolate (Fig. 1*a*). In separate gels, the proteins were transferred (Towbin *et al.*, 1979) to nitrocellulose and reacted with either cat or rabbit antisera to CFI/68 using anti-species conjugate to detect primary antibody (Gershoni & Palade, 1983). The capsid proteins from all three FCV isolates reacted positively with no differences in reactivities among these isolates (Fig. 1*b*). The results of the Western blot analysis demonstrates that there are many linear epitopes shared among the FCV capsid proteins. However, based on the serum-virus neutralization data, the neutralizing epitopes may be highly variable.

Poly(A) RNA isolated at 6 h p.i. (Chirgwin *et al.*, 1979; Glisin *et al.*, 1974; Aviv & Leder, 1972) was used to construct cDNA libraries (Gubler & Hoffman, 1983) to the CFI/68, KCD and NADC FCV isolates using a *NotI*-*SalI* primer-adaptor system (Bethesda Research Laboratories; Kotewicz *et al.*, 1988; D'Alessio & Gerard, 1988). The cDNA libraries were amplified in DH10B (Hanahan, 1985), and mini-preparations for plasmid purification (Zhou *et al.*, 1990) were completed and screened under low stringency hybridization conditions using a radioactively labelled (Feinberg & Vogelstein, 1983, 1984), previously cloned CFI/68 cDNA capsid gene (Neill *et al.*, 1991). Positive clones were identified from each isolate that contained inserts from 2.5 to 3.1 kb in size. The 3' 2400 bases of a 3.1 kb cDNA clone from the NADC isolate and a 3.0 kb cDNA clone of the KCD isolate were sequenced (Tabor & Richardson, 1987; Sanger *et al.*, 1977) using an automated nucleic acid sequencer (Smith *et al.*, 1986; Prober *et al.*, 1987). When compared to the published sequence of the CFI/68 isolate of FCV (Neill *et al.*, 1991), the nucleotide sequences of the NADC and KCD isolates were 81% and 79% similar to the CFI/68 isolate. The nucleotide sequences were confirmed by sequencing through each area of the genome at least three times using oligonucleotide primers, sequencing of subclones and by sequencing of exonuclease III-generated nested deletions (Henikoff, 1987). Two open reading frames (ORFs) were identified in both the NADC and KCD cDNA clones. The first ORF was 2004 bases in length and codes for the capsid gene of FCV (Neill *et al.*, 1991; Toya *et al.*, 1991*b*; Carter *et al.*, 1992*a, b*). This large ORF begins at the ATG located at residue 27 of the NADC and KCD nucleotide sequences and ends with the TGA at position 2031. The second ORF was 318 bases in length for both the NADC and KCD isolates. The start site of this ORF overlaps with the capsid gene stop codon at position 2030. The second ORF stop site occurs at nucleotide 2348 which was followed by a small 3' untranslated region 45 bases long for NADC cDNA or 42 bases in

Fig. 1. Analysis of the capsid protein from three different isolates of FCV by SDS-PAGE and Western blot. Isolates were caesium chloride density gradient-purified, solubilized and electrophoresed in 10 to 20% SDS-polyacrylamide and stained with Coomassie blue (a). Following electrophoresis, the proteins were electroblotted to nitrocellulose paper, incubated with cat antiserum to the CFI/68 isolate, and reacted with horseradish peroxidase-conjugated anti-cat IgG (b). Lanes 1 and 5 contain M_r markers; lane 2, NADC capsid protein; lane 3, KCD capsid protein; lane 4, CFI/68 capsid protein. M_r marker sizes are indicated.

length for the KCD cDNA. All the cDNA clones from the CFI/68, NADC and KCD virus isolates contained approximately 50 adenine residues corresponding to the poly(A) tail.

The derived amino acid sequence for the NADC and KCD capsid proteins are aligned with the corresponding capsid protein sequences of the CFI/68 (Neill *et al.*, 1991), F4 (Toya *et al.*, 1991b) and F9 (Carter *et al.*, 1992a, b) in Fig. 2. The amino acid sequences of the capsid proteins are 88 to 91% similar among the five FCV isolates. As proposed by Neill (1992), calicivirus capsid protein sequences were divided into six regions which may be further delineated based on additional sequence information now available. The first region corresponds to residues 1 to 120 for region A, the NH_2 -terminal portion of the capsid precursor which is theoretically cleaved to produce the functional capsid protein. Region B corresponds to amino acids 121 to 396 and begins with the conserved FRLE-D cleavage sequence. Within region B an important myristylated glycine residue was predicted to occur at position 128 just following the cleavage site of the capsid protein (Grand, 1989; Chow *et al.*, 1987). A putative ATP/GTP-binding site motif (Walker *et al.*, 1982) with the amino acid sequence ADMATGKS was located in the highly conserved B region of the capsid protein from amino acid residues 154 to 161. The C region extends from residues 397 to 401 and is a small island of sequence divergence among FCV capsid proteins. The D region extends from amino acids 402 to 425 and is highly conserved. A region of extensive sequence divergence ranges from residues 426 to 520. This has been designated

Fig. 2. Comparative analysis of FCV capsid protein amino acid sequences. Sequences of the FCV CFI/68 isolate (Neill *et al.*, 1991), F9 isolate (Toya *et al.*, 1991b), F4 isolate (Carter, 1992) were obtained from EMBL/GenBank. The FCV NADC and KCD sequences were obtained by sequencing cDNA using fluorescence-labelled dideoxynucleotides and translating the nucleotide sequence (DNASTAR). Sequences were compared by the ALIGN program (Science and Educational Software) based on the methods of Lipman & Pearson (1985).

Fig. 3. Comparative analysis of predicted amino acid sequences of the FCV 3' protein contiguous with the capsid protein gene. Analysis was conducted as outlined in Fig. 2.

region E (Neill, 1992), which in FCV also contains a highly conserved sequence of 28 amino acids from threonine 461 to threonine 488 surrounded by regions of sequence divergence. The carboxy-terminal region F, extending from residues 521 to 668 of the protein, is highly conserved in amino acid sequence.

The ORF located 3' to the capsid gene codes for a protein of 106 amino acids with a predicted size of approx. 12200. This predicted small basic protein was 90 to 94% similar at the amino acid sequence level among the five FCV isolates (Fig. 3). This small highly conserved basic protein has been hypothesized to be a nucleic acid-binding protein (Neill *et al.*, 1991) similar to those found in plant viruses (Koonin *et al.*, 1991).

The capsid protein amino acid sequences are 89 to 91 % similar among the FCV isolates. As proposed by Neill (1992), the capsid protein could be divided into six regions which were further delineated in this report. A domain corresponding to the E region exhibited sequence divergence at the amino acid level among capsid proteins of FCV isolates. Consequently, the E region most likely possesses the antigenically variable determinants of the capsid protein. Computer alignment (Fig. 2) of this region demonstrates that the E region in the FCV capsid protein has two hypervariable areas, one from residues 426 to 460, and a second from residues 490 to 520, surrounding a highly conserved sequence from amino acids 461 to 489. These were the only areas of the capsid protein which exhibited extensive sequence variation. Hyperimmune sera generated to the CFI/68 isolate partially neutralized the KCD isolate, but failed to neutralize the NADC isolate. The two hypervariable regions of the KCD capsid protein are 66 % similar to the CFI/68 capsid protein, and these regions of the NADC capsid protein are only 55 % similar to the CFI/68 isolate. The hypervariable regions of the CFI/68 were 75 % similar to the F9 isolate, a commonly used vaccine strain (Carter *et al.*, 1992*a, b*). This correlates well with the serological data, and suggests that to induce neutralizing activity to heterologous isolates, an FCV must have at least a 60 % or more sequence similarity within the hypervariable areas of the E region. The increased variability of the NADC region E lends credence to the hypothesis of Knowles *et al.* (1990) that there has been a variation in the spectrum of antigenicity

among FCVs induced by vaccination with single isolates of the viruses. In agreement with Knowles *et al.* (1990), some form of a polyvalent vaccine may be more protective than currently used single isolate vaccines.

The F9 calicivirus capsid protein E region has been expressed (Guiver *et al.*, 1992; Milton *et al.*, 1992) and MAbs produced against this region were neutralizing (Toya *et al.*, 1991*a*; Milton *et al.*, 1992). One of the MAbs produced by Milton *et al.* (1992), 4E7, cross-reactive with the CFI/68 isolate, bound the hypervariable region between residues 422 to 448. Another MAb, 1G9, not cross-reactive with CFI/68, bound to residues 449 to 458 where the greatest amount of sequence divergence occurred between the F9 and CFI/68 capsid proteins (Milton *et al.*, 1992). These are predicted hydrophilic areas (Kyte & Doolittle, 1982) of the protein exhibiting a high antigenic index (Jameson & Wolf, 1988). Consequently, the neutralizing epitopes of FCVs probably exist on the surface of the virus as is the case for human rhinovirus 2 (Appleyard *et al.*, 1990). The hypervariable areas of the E region surround highly conserved amino acids (461 to 489) in this same region (Fig. 2). This sequence, conserved in all FCV isolates so far examined, is an area that according to computer analysis (Emini *et al.*, 1985) contains a hydrophilic region of high surface probability surrounded by hydrophobic regions with low surface probability. These conserved amino acids might possibly be involved with virus attachment with the hydrophilic portion surrounded by hydrophobic areas providing a 'canyon' for virus attachment similar to the situation of rhinovirus binding (Rossmann *et al.*, 1985).

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