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Nucleotide sequence and expression of the spike (S) gene of canine coronavirus and comparison with the S proteins of feline and porcine coronaviruses

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We have cloned, sequenced and expressed the spike (S) gene of canine coronavirus (CCV; strain K378). Its deduced amino acid sequence has revealed features in common with other coronavirus S proteins: a stretch of hydrophobic amino acids at the amino terminus (the putative signal sequence), another hydrophobic region at the carboxy terminus (the membrane anchor), heptad repeats preceding the anchor, and a cysteine-rich region located just downstream from it. Like other representatives of the same antigenic cluster (CCV-Insavc-1 strain, feline infectious peritonitis and enteric coronaviruses, porcine transmissible gastroenteritis and respiratory coronaviruses, and the human coronavirus HCV 229E), the CCV S polypeptide lacks a proteolytic cleavage site present in many other coronavirus S proteins. Pairwise comparisons of the S amino acid sequences within the antigenic cluster demonstrated that the two CCV strains (K378 and Insavc-1) are 93·3% identical, about as similar to each other as they are to the two feline coronaviruses. The porcine sequences are clearly more divergent mainly due to the large differences in the amino-terminal (residues 1 to 300) domains of the proteins; when only the carboxy-terminal parts (residues 301 and on) are considered the homologies between the canine, feline and porcine S polypeptides are generally quite high, with identities ranging from 90·8% to 96·8%. The human coronavirus is less related to the other members of the antigenic group. A phylogenetic tree constructed on the basis of the S sequences showed that the two CCVs are evolutionarily more related to the feline than to the porcine viruses. Expression of the CCV S gene using the vaccinia virus T7 RNA polymerase system yielded a protein of the expected M_r (approximately 200K) which could be immunoprecipitated with an anti-feline infectious peritonitis virus polyclonal serum and which was indistinguishable from the S protein synthesized in CCV-infected cells.

Coronaviruses are large, enveloped, positive-stranded RNA viruses that cause respiratory, enteric and generalized disease in humans and domestic animals. Canine coronavirus (CCV) was first isolated from the faecal specimens of American military dogs with diarrhoeal disease (Binn *et al.*, 1974). It infects dogs of any breed or age, causing depression, anorexia, vomiting and diarrhoea in young animals. The dogs generally recover spontaneously 7 to 10 days after infection, but the diarrhoea may persist for more than 2 weeks. Death may occur 1 to 3 days after the onset of disease, especially in young pups (Carmichael & Binn, 1981). The virus replicates in the enterocytes of the small intestine and has been found in the intestinal lymph nodes. Vaccines to protect against CCV disease are beginning to appear on the market. Parenteral inoculation of dogs with CCV (either attenuated or not) did not result in disease, but the animals were not protected against oral challenge (Carmichael & Binn, 1981).

Feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV), transmissible gastroenteritis virus of swine (TGEV), porcine respiratory coronavirus (PRCV) and CCV possess common antigenic determinants localized on the three major virion proteins (Horzinek *et al.*, 1982; Sanchez *et al.*, 1990). A human coronavirus (HCV; strain 229E) also showed cross-reactivity at the level of the nucleocapsid protein (Horzinek *et al.*, 1982), which led Siddell *et al.* (1983) to

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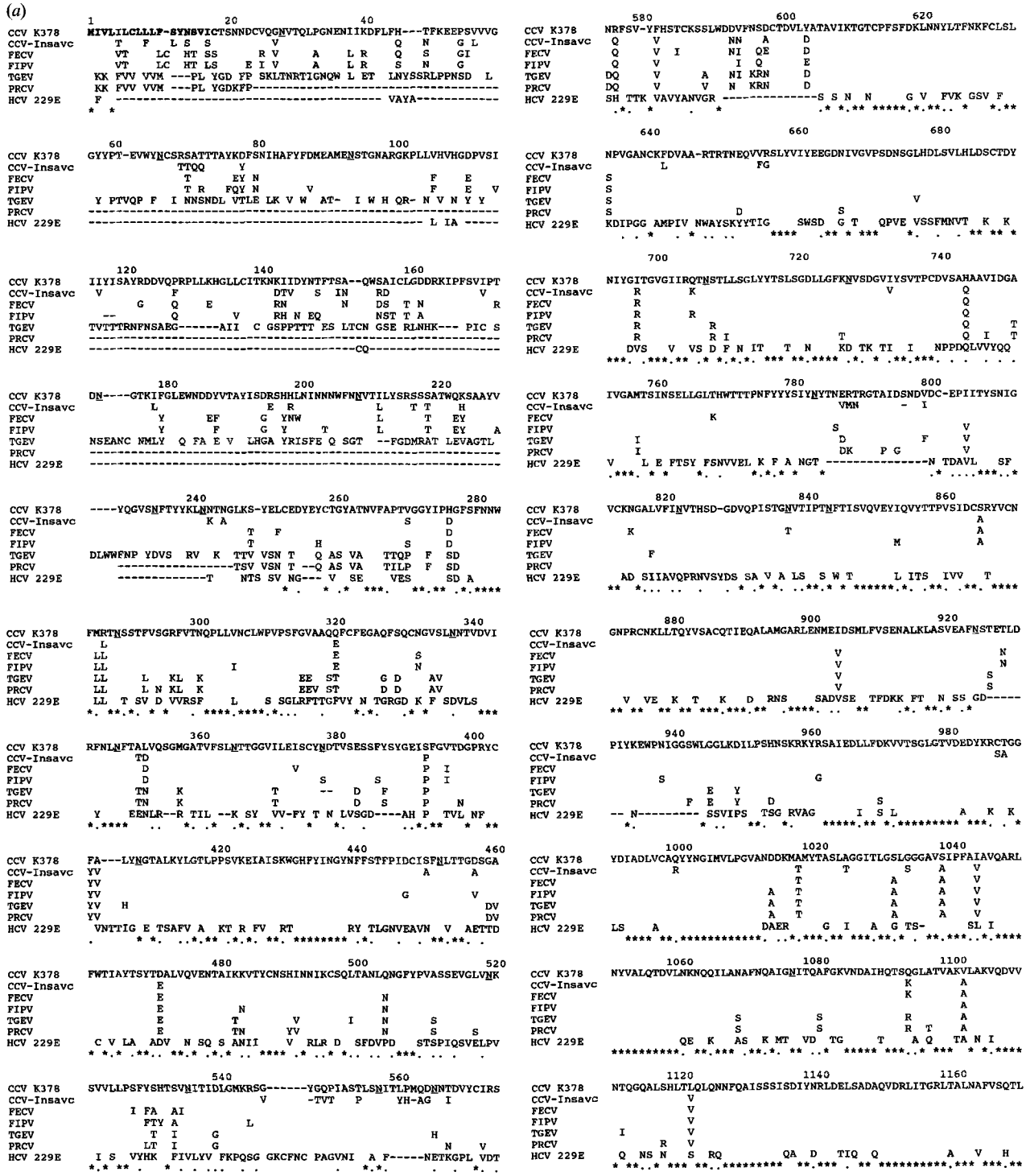

Fig. 1. For legend see opposite.

propose that these coronaviruses belong to one antigenic cluster. However, the cross-reaction was not reproduced in a more extensive study (Sanchez *et al.*, 1990). The CCV virion RNA potentially encodes nine protein species: the 160K spike (S) glycoprotein, the 30K membrane protein (M), the 43K nucleocapsid protein (N), the 9K small membrane protein (SM) and five non-structural polypeptides [designated 1b, 3a, 4, 7a and 7b (Horsburgh *et al.*, 1992) or 6a and 6b (Vennema *et al.*, 1992)].

CCV is the least characterized virus from this antigenic group. Because the S protein of coronaviruses is generally

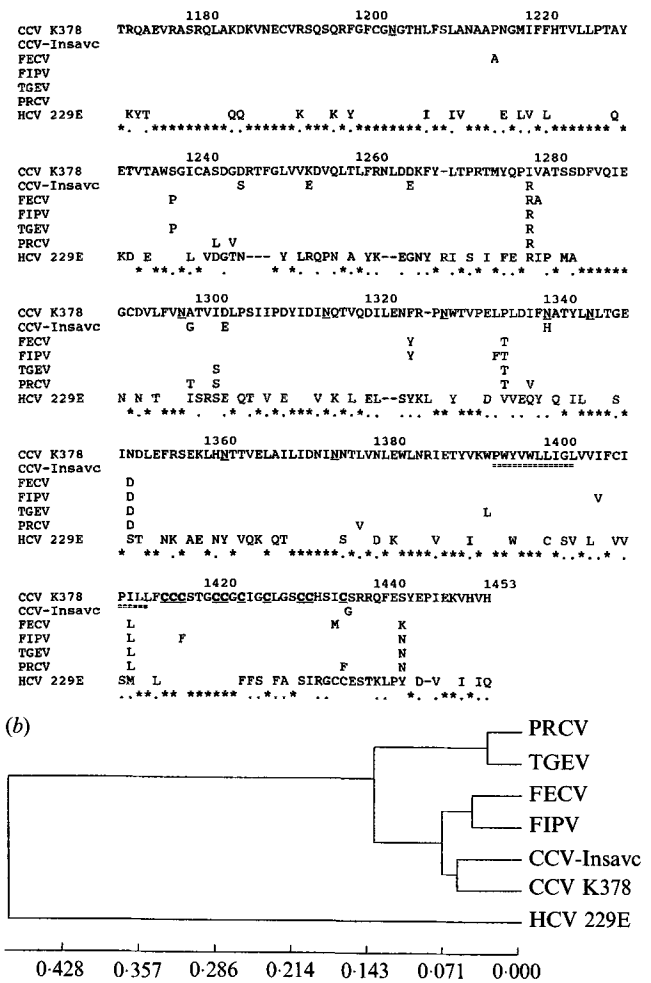

Fig. 1. Amino acid sequence comparisons and evolutionary tree based on the S proteins of dog, cat, swine and human coronaviruses. (*a*) Comparison of the predicted amino acid sequence of the S protein of CCV strain K378 with those from CCV strain Insavc-1 (Horsburgh *et al.*, 1992), from the feline coronaviruses FECV strain 79-1683 (accession Q25539 Geneseq Database) and FIPV strain 79-1146 (de Groot *et al.*, 1987*a*), from the porcine coronaviruses TGEV (Jacobs *et al.*, 1987) and PRCV (Rasschaert *et al.*, 1990), and from the human coronavirus HCV 229E (Raabe *et al.*, 1990). Residues not shown are identical to those of CCV K378. Dashes have been introduced to obtain optimal alignment. The putative amino-terminal signal sequence is printed in bold and the putative membrane anchor (= = = =) is underlined. Potential *N*-glycosylation sites N) and the cysteine-rich (C) region are indicated. Consensus in the sequence is symbolized by an asterisk (complete identity) or a dot (conserved amino acid changes); a blank space indicates the absence of conservation. (*b*) Reconstruction of a phylogenetic tree of the coronaviruses based on the S amino acid sequences. For the calculation of distances between different S protein sequences a 'Unity Distance' table was used by 'Homologies/Distances'. The gap penalty was set to the highest mismatch value in the matrix (1·0).

considered to be a candidate antigen for the development of recombinant DNA-based vaccines, we present here the cloning and sequencing of the gene encoding the S protein of CCV strain K378. We compared this sequence with that of the CCV strain Insavc-1 (Horsburgh *et al.*, 1992) and with other known S sequences of the antigenic cluster to determine their evolutionary relationships. The S gene was then expressed using the vaccinia virus T7 RNA polymerase system.

The CCV strain K378 (Barlough *et al.*, 1984; obtained from Dr H. Flore, Solvay-Duphar, Weesp, The Netherlands) was grown in *Felis catus* whole fetus (fcwf-D) cells (obtained from Dr N. C. Pedersen). RNA was isolated from virus-infected cells and poly(A)$^+$ RNA was used as the template for cDNA synthesis (Gubler & Hoffman, 1983) using oligo(dT) and random hexamer primers (Pharmacia). Poly(dC)-tailed cDNA was annealed to a *Pst*I-digested, dG-tailed pUC9 plasmid (Pharmacia) and the recombinants were used to transform *Escherichia coli* strain PC2495. Ampicillin-resistant colonies were transferred onto nitrocellulose filters, lysed *in situ* (Sambrook *et al.*, 1989) and hybridized with the ^{32}P-labelled S gene of FIPV (de Groot *et al.*, 1987*a*). Plasmid DNA was obtained from 35 positive clones (Birnboim & Doly, 1979), and the inserts from these clones were analysed by restriction enzyme mapping and Southern blotting, using parts of the FIPV S gene as probes (results not shown). Three overlapping cDNA clones were obtained that covered the entire CCV S coding region. These were sequenced using the M13 dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977). The nucleotide sequences were assembled and analysed with the aid of the computer programs of Devereux *et al.* (1984).

The nucleotide sequence obtained contains a single open reading frame (ORF) of 4359 nucleotides and has a coding capacity for a 1453 amino acid polypeptide of predicted M_r 160K. Twenty-seven nucleotides before the start of the S ORF a CTAAAC sequence is found, which serves as the minimal conserved signal for transcription, as has also been observed for FECV, FIPV, TGEV, PRCV and CCV Insavc-1 (Horsburgh *et al.*, 1992; Spaan *et al.*, 1988). The predicted S protein (Fig. 1*a*) contains 31 potential *N*-glycosylation sites (Asn–X–Thr or Asn–X–Ser). Assuming a mean contribution to the total M_r of 2·1K per carbohydrate chain (Hunter *et al.*, 1983), the mature S glycoprotein would be approximately 220K. This value is slightly larger than the apparent M_r of CCV S (see Fig. 2) which may indicate that not all potential glycosylation sites are used. The CCV S protein has a number of other features typical of coronavirus S proteins. First, at the amino terminus of the polypeptide a stretch of 20 mainly hydrophobic amino acids represents the putative signal sequence which is probably cleaved between Cys-18 and Thr-19 as this is the predicted signal peptidase recognition site (von Heijne, 1986). Second, at the carboxy terminus (residues 1395 to 1415) a second hydrophobic region is observed which probably serves as the membrane anchor (de Groot *et*

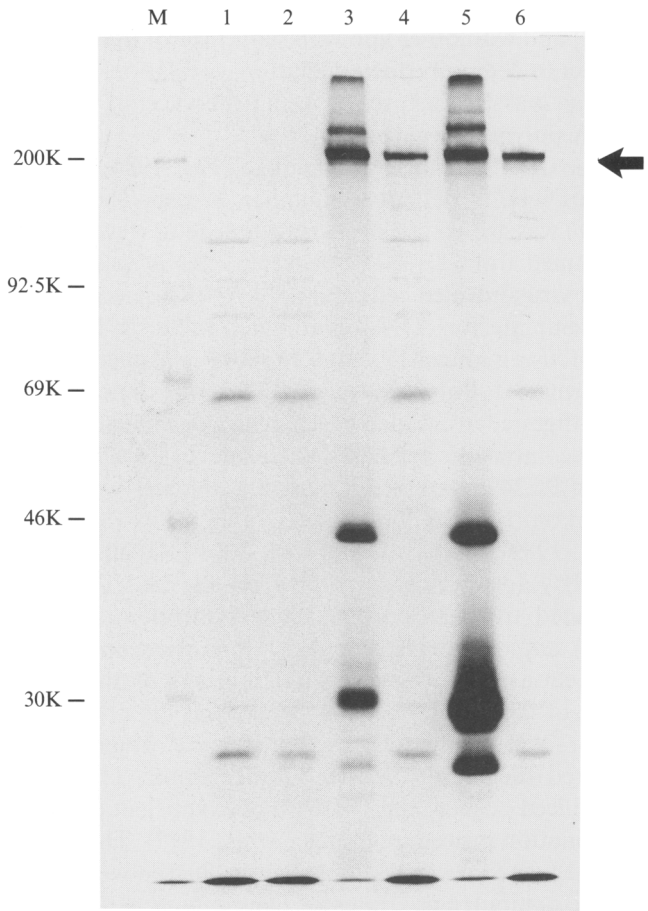

Fig. 2. Expression of the CCV S protein. HeLa cells were infected with the recombinant vaccinia virus vTF7-3 which produces the T7 polymerase (Fuerst *et al.*, 1986) and were transfected 1 h later with pTUGCCVS (lane 4), pTUGFIPVS (lane 6), pTUG3 (lane 2) or were mock-transfected (lane 1). At 16 h post-infection (p.i.) the cells were labelled for 1 h with 100 μCi/ml L-[^{35}S]methionine in methionine-free medium. For comparison, CCV- and FIPV-infected fcwf-D cells were labelled similarly for 1 h starting at 6 h p.i. and subsequently incubated in chase medium for 1 h (lanes 3 and 5, respectively). Cell lysates were prepared and immunoprecipitations carried out using ascitic fluid (A36) derived from an FIPV-infected cat according to Vennema *et al.* (1990*a*). Numbers at the left indicate the positions of marker proteins run in the same gel; the arrow at the right indicates the position of the S proteins.

al., 1987*a*) and is followed by a cysteine-rich region. Furthermore, the C-terminal part contains two regions with heptad repeat periodicity (residues 1068 to 1150 and 1336 to 1381) which were proposed to be essential elements for the formation of the elongated stem structure of this peplomer-forming protein (de Groot *et al.*, 1987*b*). Finally, the CCV S protein does not contain any basic amino acid sequences related to the motifs RRXRR or RRAHRR (where X is F, S, H or A) which are the sites at which mouse hepatitis virus (MHV) and infectious bronchitis virus (IBV) S proteins are proteolytically cleaved to yield the S1 and S2 polypeptides (Spaan *et al.*, 1988). Like the FIPV and TGEV S proteins, the CCV S protein is probably not cleaved, which is consistent with experimental data (see Fig. 2). However CCV (like FIPV) is capable of inducing cell fusion in feline and canine cells, which shows that protein cleavage is not required for cell fusion activity. This conclusion is in agreement with recent results reported for MHV S which is normally cleaved. When cleavage was prevented by mutation of the cleavage site, this did not abolish the fusion potential of the expressed protein (Stauber *et al.*, 1993; Taguchi, 1993). Consistent with this observation, cleavage site mutants of MHV isolated from persistently infected cells had also retained their cell fusing capacity, albeit to a much lower extent (Gombold *et al.*, 1993).

Table 1. *Sequence comparisons of S proteins within the CCV antigenic cluster (identical and similar amino acid residues)**

	K378	Insavc-1	FECV	FIPV	TGEV	PRCV	HCV
K378	**100·0**	93·3	93·2	92·4	82·8	89·6	51·7
Insavc-1	95·2	**100·0**	92·1	91·4	81·9	88·5	51·6
FECV	96·0	95·0	**100.0**	95·3	84·1	90·0	52·0
FIPV	95·5	94·8	97·2	**100·0**	83·4	90·0	52·7
TGEV	89·3	88·5	90·2	90·0	**100·0**	95·5	52·0
PRCV	93·5	92·6	94·0	93·9	97·3	**100·0**	52·8
HCV	70·8	70·4	70·9	71·5	71·5	72·1	**100·0**

* Percentages of identical (right above 100% diagonal) and similar (left below 100% diagonal) amino acid residues calculated from pairwise sequence alignments of the different S proteins (UWGCG gap program using gap penalty of 1·00 and gap length weight of 0·1).

The evolutionary relationships and conserved or variable structural features were analysed using a computer-based comparison of the CCV K378 spike protein sequence with those of the S proteins of CCV Insavc-1 (Horsburgh *et al.*, 1992), FECV 79-1683 (accession Q25539 Geneseq Database), FIPV 79-1146 (de Groot *et al.*, 1987*a*), PRCV (Rasschaert *et al.*, 1990), TGEV (Jacobs *et al.*, 1987) and HCV 229E (Raabe *et al.*, 1990); the results are shown in Fig. 1(*a*) and Table 1. The S sequences of the two CCV strains have 94 amino acid differences (93·3% identity) in addition to a one amino acid deletion in the Insavc-1 strain (Ser at position 797). They appear to differ about as much from each other as they do from the feline coronaviruses. The similarities of the two CCV S sequences with those of FIPV and FECV (91·4% to 93·2% identity) are much higher than with the two swine coronaviruses PRCV and TGEV (81·9% to 89·6% identity). On the basis of its S sequence, HCV is clearly less related to the other members of this antigenic cluster (51·6% to 52·8% identity); clearly this virus has evolved differently (see also Fig. 1*b*).

It has been noted before (de Groot *et al.*, 1987*a*; Luytjes *et al.*, 1987; Raabe *et al.*, 1990) that most

Table 2. *Sequence comparisons of N- and C-terminal portions of S proteins**

	K378	Insavc-1	FECV	FIPV	TGEV
K378	**100·0**	87·0	83·6	81·2	37·8
Insavc-1	95·0	**100·0**	82·2	80·8	38·8
FECV	95·7	94·6	**100·0**	89·6	41·2
FIPV	95·3	94·1	96·8	**100·0**	38·3
TGEV	93·6	92·2	94·3	94·1	**100·0**
PRCV	92·0	90·8	92·5	92·8	96·5
HCV	51·5	51·2	51·5	52·1	51·5

* Percentages of identical amino acid residues of different S proteins calculated from pairwise sequence alignments of residues 1 to 300 (right above 100% diagonal) and starting from residue 301 (left below 100% diagonal), using the UWGCG gap program with gap penalty 1·00 and gap length weight 0·1.

variations in the coronavirus S proteins are found in the amino-terminal domain. Our sequence comparisons confirm this point. Considering the data illustrated in Fig. 1 (*a*) the S proteins can be divided into a moderately conserved amino-terminal domain and a highly conserved carboxy-terminal domain. This distinction is shown in Table 2 where the identities between the S proteins have been calculated separately for the amino-terminal 300 residues and for the remainder of the polypeptides. In the amino-terminal domain the highest identity scores are observed for the two canine viruses (87·0%) and for the two feline viruses (89·6%). Pairwise comparisons of the canine and feline sequences with each other yielded identities varying from 80·8 to 83·6%. The TGEV S protein is considerably different from the canine and feline S sequences in its amino-terminal domain (from 37·8 to 41·2% identity). In contrast, in its carboxy-terminal part TGEV S protein can be considered to be very closely related to these same canine and feline viruses, with identities amounting to 92·2 to 94·3%. These figures are only slightly lower than those obtained when the feline and canine sequences are mutually compared in this region (94·1 to 96·8%). Interestingly, the K378 strain of CCV is almost as closely related to FIPV and FECV as it is to the Insavc-1 CCV strain in the carboxy-terminal region of the S protein. When percentage similarities are compared over the whole protein (Table 1), the K378 strain appears to be even more similar to the feline viruses than to the other CCV strain. This pattern of sequence similarities may reflect the geographical origin of these coronaviruses: the two feline coronaviruses and CCV K378 originate from the United States whereas CCV Insavc-1 is a British isolate.

To construct a phylogenetic tree from the comparative data (Fig. 1 *a*) we have used a program that compares sequences on the basis of distance matrix files using the neighbour-joining method described by Saitou & Nei (1987). It appears from the resulting tree (Fig. 1 *b*) that the canine coronaviruses are evolutionarily more closely related to the feline than to the porcine coronaviruses. The human coronavirus HCV 229E is more distant within the antigenic cluster. IBV-M41 and MHV-A59 are most distantly related (data not shown), a finding that is in keeping with the fact that these coronaviruses belong to a separate antigenic cluster (Siddell *et al.*, 1983).

In order to express the CCV S ORF, the gene was assembled from three overlapping cDNA clones. After removal of the 5′ non-coding region using a PCR strategy (not shown), the coding region was cloned into the *Pst*I and *Eco*RI sites of the polylinker of pBlueScript KS$^-$. From this plasmid a *Bam*HI/*Eco*RI fragment was cloned into the vaccinia virus T7 expression vector pTUG3 (Vennema *et al.*, 1991). In parallel, a *Bam*HI fragment encoding the FIPV S protein (de Groot *et al.*, 1987*a*; Vennema *et al.*, 1990*a*) was also cloned into pTUG3. The resulting constructs pTUGCCVS and pTUGFIPVS were used to express the respective S proteins. HeLa cells infected with the recombinant vaccinia virus vTF7-3, which produces T7 RNA polymerase (Fuerst *et al.*, 1986) were transfected with the plasmids and subsequently labelled with [^{35}S]methionine (Amersham). The expressed products were analysed electrophoretically (Laemmli, 1970) after immunoprecipitation using a polyclonal FIPV-specific antiserum.

As shown in Fig. 2, the CCV S construct specifically induced the synthesis of a protein of M_r approximately 200K (lane 4), which is close to the expected M_r of the glycosylated spike polypeptide (see above). No such protein was detected after transfection with pTUG3 alone (lane 2) or after mock transfection (lane 1). The product comigrated with the S proteins synthesized in cells infected with CCV (lane 3) or FIPV (lane 5). The CCV expression product also comigrated with the FIPV S gene product (lane 6), in agreement with their similar predicted M_rs. Collectively, these results prove that the ORF cloned, sequenced and reconstructed indeed specifies the CCV S protein.

CCV causes gastroenteric disease in dogs, often resulting in death of the more susceptible younger animals (Carmichael & Binn, 1981). Several conventional vaccines have been tested but none induced long-lasting protection. With the notorious exception of FIPV, antibodies to the S protein of which can enhance the infection process (Vennema *et al.*, 1990*b*), the S protein of coronaviruses generally appears to be the prime candidate to be the basis for a vaccine (Spaan *et al.*, 1990). The bona fide expression of the CCV S gene reported in this paper may therefore provide the basis for the development of a recombinant vaccine which, when suitably presented, e.g. through an adenovirus carrier, may induce protection.

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