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Organization of the canine calicivirus genome from the RNA polymerase gene to the poly(A) tail

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In recent years a wealth of data has become available about the caliciviruses that infect humans, as well as those which infect a range of animal species, notably cats, rabbits, pigs and marine animals. However, in the two decades since the earliest reports of calicivirus infection in dogs, very little has become known about the epidemiology, pathogenicity and molecular biology of the caliciviruses that may infect canines. In 1990, a canine calicivirus (CaCV) was isolated from a 2-month-old diarrhoeic domestic dog in Japan. This virus, which can be grown in cultured cells of canine origin, has the classic 'Star of David' morphology of caliciviruses, and the one major structural protein was shown to be immunogenic in dogs. In this study, a 3·8 kb region of the genome of this CaCV isolate from the RNA polymerase gene to the 3′ poly(A) tail was cloned and sequenced, and phylogenetic analysis was undertaken in order to establish the relationship of CaCV to other animal and human caliciviruses. This CaCV isolate had a nucleotide sequence, genomic organization and phylogenetic position closest to, but clearly distinct from, both feline calicivirus and San Miguel sea lion virus isolates. These findings suggest that CaCV represents a new clade of animal caliciviruses, presumably as a member of the recently proposed new genus *Vesivirus*.

Introduction

Caliciviruses are important veterinary and human pathogens. In the human household environment, small round-structured viruses (SRSVs) are a major cause of epidemic gastroenteritis, and 'classic' human caliciviruses (HuCVs) cause paediatric diarrhoeal disease in humans, while among pets feline calicivirus (FCV) and rabbit haemorrhagic disease virus (RHDV) are major causes of disease in these animals. In recent years these caliciviruses have become the subject of extensive research and numerous studies have addressed the molecular biology, epidemiology and evolutionary aspects of these viruses (for recent reviews see Clarke & Lambden, 1997; Smith *et al*., 1998).

In contrast, very little is known about calicivirus infection in the domestic dog. Some reports have described caliciviruses antigenically similar to FCV causing glossitis, enteric infections, vesicular genital disease or diarrhoea in dogs (Evermann *et al*., 1981, 1985; Crandell, 1988; San Gabriel *et al*., 1996). In 1985, Schaffer *et al*. (1985) isolated canine calicivirus proper (CaCV) from the faeces of a diarrhoeic dog. These reports have left many questions unanswered, such as the prevalence, epidemiology and molecular characteristics of the caliciviruses infecting canines.

The subject virus of this study is the only known Japanese CaCV isolate. As described previously, this virus was isolated in 1990 from a 2-month-old domestic dog showing intermittent watery diarrhoea, vomiting, general depression and anorexia, leading to death (Mochizuki *et al*., 1993). Electron microscopic and physical characteristics classified this isolate as a calicivirus. The observations that this virus (designated No.48 strain) could be cultured only in cells of canine origin, was infective to pups, and did not show antibody cross-reactivity with FCV suggested that it should be classified as a canine calicivirus. Recently, the capsid protein of this isolate was identified and shown to react in Western blotting with antibodies of CaCV-infected dogs (San Gabriel *et al*., 1997).

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The objective of the present study was to sequence the RNA-dependent RNA polymerase (pol) region, the capsid gene and the short 3′ open reading frame (ORF) of this CaCV isolate and establish the phylogenetic position of CaCV among animal and human caliciviruses.

Methods

Cloning and sequencing. No.48 strain was propagated in Madin–Darby canine kidney cells as described (San Gabriel *et al*., 1997). Three regions of the genome were amplified and cloned separately. First, RT–PCR was carried out to verify the presence of two motifs characteristic of caliciviruses named GLPSG and YGDD, after their respective amino acid sequences. To do this, poly(A)–RNA was extracted from 1 µl of clarified infected cell supernatant using an RNA micro-isolation Spin Cartridge system (Life Technologies), followed by RT–PCR of the total product using an RNA PCR kit (AMV, version 2.1, Takara). Reverse transcription was carried out using random primers, while cDNA was produced using a primer set based on the motifs mentioned above (primer GLPSG, 5′ GGVCTVCCATCTGGWTTSCC 3′; primer YGDD, 5′ AATCTCATCATCACCATA 3′; Green *et al*., 1994). The resulting fragment was cloned into pT7Blue(R) (Novagen) and sequenced on both strands using fluorescence-labelled primers recognizing the plasmid arm m13 sequences, using the T7 polymerase AutoRead sequencing kit (Amersham Pharmacia).

Second, the presence of a third motif, KDEL (Jiang *et al*., 1993), was verified using a degenerate primer constructed upstream of the expected KDEL motif and based on the published sequences of FCV and SMSV in this region (primer KDEL-F1, 5′ CTMCCWCAYGARTACRCMMT-TGG 3′). This corresponds to nt 4214–4236 of the FCV strain F9 (GenBank no. M86379). The antisense primer was constructed based on the sequence obtained from the GLPSG–YGDD region (primer CaCV2, 5′ GCCGATCCCACAAGAAGACAGTG 3′). RT–PCR, cloning and sequencing were performed as above.

Third, the complete 3′ end of No.48 was amplified essentially as described by Ando *et al*. (1997). In brief, total RNA was extracted from 250 µl of cell supernatant using Isogen-LS (Nippon-Gene) according to manufacturer's instructions and dissolved in 20 µl H_2O. Poly(A)-RNA from a 5 µl aliquot was then bound to oligo(dT) latex particles, the particles were washed and resuspended in 400 µl of RT–PCR reaction mix [50 mM Tris–HCl pH 8·7; 40 mM KCl; 2·5 mM $MgCl_2$; 0·5 mM of each dNTP; 2 mM DTT; 0·5% (v/v) Triton X-100; 2% (v/v) glycerol; 0·4 U/µl human placental RNase inhibitor (Takara); 0·25 mM each of primers GLPSG and VN (primer VN, 5′ GCTGGAGTCTAGA$(T)_{25}$VN 3′); 3 U/µl Superscript II (Life Technologies); and 10 U of a mixture of *Taq* and *Pwo* polymerases (Expand Long Template PCR system, Boehringer)]. Seventy-five µl of the mixture was transferred to a fresh reaction tube and RT–PCR was performed with the thermocycling format described by Ando *et al*. (1997). The amplified DNA was purified and subjected to a second round of semi-nested PCR in order to obtain sufficient material for subsequent cloning and sequencing. The semi-nested PCR sense primer used was constructed based on the sequence obtained from the GLPSG–YGDD region (primer CaCV1, 5′ AGGGT-GTGGATGTGAATTGG 3′), with VN as antisense primer. PCR reaction conditions were as above.

RT–PCR products from the third amplified region were also cloned into pT7Blue(R) and sequenced, first using m13 primers to verify the extreme sequences and further using fluorescence-labelled primers designed from the obtained sequences. The sequence of the overlapping region, approximately nt 2300–2500 on the final sequence, was therefore based on data from both strands.

For all nucleotides of each of the three fragments analysed, sequence results of at least five clones were used to construct a total sequence length of 3794 nt.

Phylogenetic analysis. Sequences were translated into amino acid sequences and aligned with known calicivirus sequences using Genetyx-Win (Software Development Corp) and improved by visual inspection. The alignments were input into ClustalW (version 1.74, Thompson *et al*., 1994) and PHYLIP modules (version 3.57c, Felsenstein, 1993) to produce phylogenetic trees. Tree construction was done without outgroup specification but with bootstrap analysis (ClustalW, 1000 trials with seed 111, including gap positions, without multiple substitution correction).

Results and Discussion

RT–PCR of the three targeted regions resulted in single bands of expected sizes when compared to published sequences for the 3′ half of animal and human caliciviruses. Sequencing of the three regions demonstrated that fragments 1, 2 and 3 were 124 nt, 449 nt and 3279 nt, respectively, between primers.

Alignment of the ORFs with relevant published sequences showed that the No.48 strain has extensive identity to other caliciviruses.

The polymerase gene

The amplified pol gene fragment was 1082 nt long, encoding 359 aa. This compared to 357 aa for the corresponding region of FCVs, 361 aa for San Miguel sea lion viruses (SMSVs) and between 343 and 348 aa for RHDV, HuCV (strain Manchester, Ma) and SRSVs. The pol sequence included the presence of the conserved amino acid motifs KDEL, GLPSG and YGDD, showing further molecular evidence of this virus indeed being a calicivirus.

As shown in Table 1, the No.48 strain pol gene sequence showed highest similarity to SMSV (63–66%), followed by FCV (56–58%). In comparison, similarity among strains of the same calicivirus type was much higher (FCV, 89–92%; SMSV, 95%; SRSVs, 93%).

The capsid gene

The capsid gene ORF of the No.48 strain consisted of 2076 nt encoding an expected protein of 691 aa. In FCVs, the capsid protein is between 668 and 671 aa, while the length of the SMSV capsid protein is 702–704 aa. In contrast, the predicted capsid protein of RHDV, HuCV and SRSVs is significantly shorter, at 530–579 aa.

Viruses in the FCV/SMSV groups are known to produce capsid precursor proteins that are processed by a viral protease to yield the mature capsid protein, which is the major immunogen of the caliciviruses (Fretz & Schaffer, 1978; Carter, 1989; Shin *et al*., 1993). In a preliminary experiment, we investigated the presence of a precursor capsid protein for CaCV by Western blotting. When cells infected with No.48 strain were treated at 45 °C for 1 h and analysed by Western

Table 1. Amino acid similarity of the polymerase region of the polyprotein

Pairwise percent identity between calicivirus strains was calculated from deduced amino acid sequences of the polymerase gene region with the Genetyx-Win software package (Software Development Corp) using the method of Lipman & Pearson (1985).

Strain*	No.48	F4	F9	CFI/68	Urbana	SMSV1	SMSV4	RHDV	Ma	NV	So
No.48		56	56	56	58	66	63	37	43	31	31
F4			89	89	91	60	61	39	45	32	32
F9				90	92	61	62	41	47	31	31
CFI/68					91	60	62	40	47	31	32
Urbana						63	64	39	46	32	32
SMSV1							95	38	43	30	30
SMSV4								37	43	29	29
RHDV									39	27	29
Ma										31	32
NV											93
So											

* Abbreviations, GenBank accession numbers and original sources of the comparison strain sequence data are as follows: F4 (FCV F4, D31836, Oshikamo *et al.*, 1994); F9 (FCV F9, Z11536, Carter *et al.*, 1992); CFI/68 (FCV CFI/68, U13992, Neill, 1990); Urbana (FCV Urbana, L40021, Sosnovtsev & Green, 1995); SMSV1 (U15301, Neill *et al.*, 1995); SMSV4 (U15302, Neill *et al.*, 1995); RHDV (M67473, Meyers *et al.*, 1991); Ma ('classic' human calicivirus Manchester, X86560, Liu *et al.*, 1995); NV (SRSV Norwalk, M87661, Jiang *et al.*, 1993); So (SRSV Southampton, L07418, Lambden *et al.*, 1993).

Table 2. Amino acid similarity of the deduced capsid protein

Top right, calculations based on the whole capsid gene region; bottom left, calculations based on the conserved B region.

Strain*	No.48	F4	F9	CFI/68	Urbana	SMSV1	SMSV4	SMSV17	RHDV	Ma	NV
No.48		37	37	38	36	40	41	40	25	27	31
F4	54		89	89	90	45	46	45	26	27	19
F9	54	96		89	89	46	46	44	30	28	30
CFI/68	55	95	96		90	45	45	44	37	27	29
Urbana	54	96	95	95		45	46	45	25	29	17
SMSV1	57	64	63	64	64		70	71	27	25	19
SMSV4	56	59	59	60	59	81		82	33	25	19
SMSV17	57	61	60	61	61	82	91		35	26	28
RHDV	35	36	33	36	34	36	34	35		31	19
Ma	39	41	41	38	41	35	35	37	36		27
NV	31	30	30	29	29	30	27	28	29	27	

* Abbreviations, GenBank accession numbers and original sources of the comparison strain sequence data are as in Table 1 with the following exceptions: F4 (D90357, Tohya *et al.*, 1991); F9 (M86379); CFI/68 (U13992, Neill *et al.*, 1991); SMSV1 (M87481, Neill, 1992); SMSV4 (M87482, Neill, 1992); SMSV17 (U52005, Matson *et al.*, 1996).

blotting, a band with an M_r of 75000 appeared in addition to the usual band with an M_r of 57000. The immune serum used was a polyclonal mouse serum produced using SDS–PAGE gel-purified No.48 strain mature capsid protein (San Gabriel *et al.*, 1997), which was specific for No.48 capsid protein in subsequent Western analysis (results not shown). This result suggested that, like FCV and SMSV, the CaCV capsid protein is also first synthesized as a precursor protein, which is then proteolytically processed into a smaller mature capsid protein.

The capsid precursor of FCV is cleaved by a virus-encoded proteinase which removes the N-terminal 125 aa (Sosnovtsev *et al.*, 1998) between glutamic acid and alanine residues. This

Table 3. Nucleotide and deduced amino acid similarities of ORF3

Top right, calculations based on nucleotide sequences; bottom left, calculations based on the deduced amino acid sequences.

Strain*	No.48	F4	F9	CFI/68	Urbana	SMSV1	SMSV4	SMSV17	RHDV	Ma	NV	So
No.48		46	48	50	49	43	48	50	51	43	46	46
F4	27		85	85	87	59	51	61	48	49	47	48
F9	27	93		84	84	47	50	49	45	48	50	45
CFI/68	28	96	96		87	46	49	49	45	48	44	52
Urbana	28	94	96	98		46	50	51	46	47	47	47
SMSV1	32	27	26	26	27		69	69	50	44	49	60
SMSV4	34	26	24	24	24	67		85	57	46	53	51
SMSV17	29	26	25	25	25	69	89		48	44	46	46
RHDV	26	22	24	22	23	14	18	16		42	46	44
Ma	48	28	25	18	18	25	19	25	24		48	48
NV	15	22	20	22	20	18	39	24	27	17		65
So	12	33	30	31	30	57	31	31	57	16	72	

* Abbreviations, GenBank accession numbers and original sources of the comparison strain sequence data are as in Tables 1 and 2.

proteinase has a high specificity for the P1 position (Glu), but a more relaxed amino acid requirement for the P1′ position (Ala). Alignment of the No.48 strain sequence with FCV and SMSV in the same region showed that the corresponding sequence in No.48 has the same P1 residue as FCV. The sequence has six residues identical to SMSV (FRAESD, corresponding to P4–P2′ of FCV) and four residues identical to FCV (FR, E, D). The presence of a number of conserved amino acids in the SMSV, CaCV and FCV virus groups around the FCV precursor cleavage site suggests that this may be the cleavage site of many or all of the viruses in these groups.

The capsid gene of animal viruses is generally taken to consist of five distinct regions A–E (Neill, 1992). Region B is the most highly conserved among caliciviruses, and contains two conserved motifs, named PPG after their amino acid sequence (Numata *et al.*, 1997).

In the No.48 strain, one PPG motif was present as such, while the second PPG was present as PPT. This threonine residue is not unique, since it is also found in Norwalk virus (NV), the prototypical SRSV strain.

As is clear from Table 2, similarity of the capsid region of No.48 to any of the other caliciviruses was relatively low compared to similarities among strains of the same type, whether based on amino acid sequences of the whole capsid region or on the conserved B region only. Analysis of nucleotide sequences of the various whole capsid genes gave similar results, since similarity of No.48 to other caliciviruses was found to be 44–52%, while that within the FCV and SMSV groups was significantly higher (69–79%).

The small 3′ ORF

Analogous to other caliciviruses, the No.48 genome contained a small ORF immediately downstream of the capsid gene (ORF3) encoding a putative basic protein of 133 aa. This size was intermediate between FCVs, SMSVs and RHDV (106–117 aa) on the one hand, and HuCVs on the other (Ma, 162 aa; NV, 198 aa; So, 197 aa).

The ORF3 of No.48 shared no more than about 50% nucleotide and amino acid similarity with any of the analysed comparison strains, as is shown in Table 3.

Phylogeny

Fig. 1 shows inferred phylogenetic relationships between calicivirus strains based on the amino acid alignments of the pol gene, the capsid gene and the small 3′ ORF. The phylogenetic tree of the pol region (Fig. 1*a*) and the 3′ ORF (Fig. 1*d*) showed a distinct branch for No.48 originating between the FCV and SMSV clades, while the trees based on the complete capsid gene (Fig. 1*b*) and on the conserved B region only (Fig. 1*c*) showed a similarly distinct branch for No.48, but originating between the SMSV/FCV area and the RHDV/NV/Ma area. These results seem to suggest that while FCV, SMSV and CaCV are at present clearly separate virus clades, they share ancestral roots that may at one time have been closely related.

Genomic features

Analysis of two distinguishing features in the 3′ half of caliciviruses is shown in Table 4. The nucleotide sequence of the junction between the ORFs of the non-structural polyprotein (ORF1) and the capsid (ORF2) is conserved among viruses of the same calicivirus type (for review see Clarke & Lambden, 1997).

In RHDV and HuCV strains, the polyprotein gene and the capsid gene are fused into one large ORF lacking a polymerase

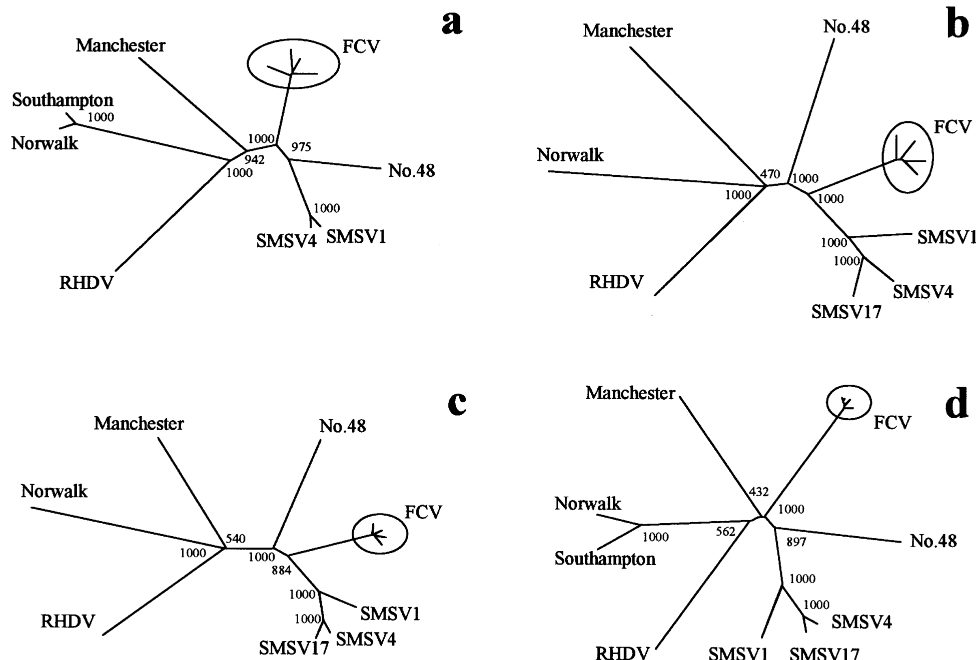

Fig. 1. Phylogenetic analysis of the polymerase, capsid and ORF3 regions of CaCV and selected animal and human calicivirus strains. Tree topology was based on the neighbour joining method. Bootstrap values noted indicate the number of times each branching was found in 1000 bootstrap analyses. Branch lengths indicate phylogenetic distances calculated from distance matrices of deduced amino acid sequences. Abbreviations are as in Tables 1 and 2 and the text. FCV indicates clustering of the four feline calicivirus strains analysed. (*a*) RNA-dependent RNA polymerase regions; (*b*) complete capsid regions; (*c*) conserved region B of the capsid genes; (*d*) the small 3′ ORF.

Table 4. Genomic features

Strain*	Polymerase/capsid gene junction†				Length of the 3′ non-coding region‡
No.48	tga	gct	*atg*		235
F4	tga	gc	*atg*		44
F9	tga	gc	*atg*		44
CFI/68	tga	gc	*atg*		43
Urbana	tga	gc	*atg*		46
SMSV1	tag	ccact	*atg*		182
SMSV4	tag	ccact	*atg*		178
SMSV17	tag§	ccact	*atg*		178
VESV	tag§	ccacc	*atg*		179
RHDV		aatgtt	*atg*		59
Ma		tttgag	*atg*		82
NV			*atg*	atgatggcgtg taa	66
So			*atg*	atgatggcgtg taa	77

* Strain names and sources of sequences are as in the legends to Tables 1 and 2, except for VESV (accession no. U76874, unpublished).
† Nucleotide sequence of the polymerase gene stop codon (underlined) and the capsid gene start codon (in italics).
‡ Length of the region between the short 3′ ORF and the poly(A) tail of the genome (nucleotides).
§ Stop codon identity inferred from available sequence data.

stop codon. In the case of SRSV the termination codon of ORF1 overlaps the start codon of ORF2 by 17 nt [ATG$(N)_{11}$TAA]. FCV, SMSV and vesicular exanthema of swine virus (VESV) share the structure of having a frame-shift between ORF1 and ORF2, with 2 nt (FCV) or 5 nt (SMSV/VESV) in between. We found that the No.48 strain has 3 nt between the termination codon of ORF1 and the start codon of ORF2, a unique sequence not seen in any published calicivirus sequence. The presence of 3 nt between the two ORFs makes them in-frame in the event of read-through errors, possibly allowing for low levels of fusion protein species between the polymerase and capsid proteins.

The length of the non-coding region between ORF3 and the poly(A) tract of calicivirus genomes is somewhat conserved among calicivirus types. We found that No.48 strain had a longer 3′ terminal non-coding region than any published sequence (235 nt), the next longest regions being those of SMSV and VESV (178–182 nt). Interestingly, FCV, which showed relatively high identity to No.48 strain in the pol and capsid gene sequences has a short region (43–46 nt), similar to human and rabbit sequences.

Our findings that the No.48 strain genome has no more than about 50% sequence identity to other caliciviruses, while having hereto unknown sequence features between ORF1 and ORF2, and between ORF3 and the poly(A) tail suggest that this virus did not arise through simple one-time rearrangement of genomic sequences from other caliciviruses like FCV or

SMSV, but instead constitutes a distinct calicivirus genotype. The phylogenetic uniqueness of CaCV presupposes the existence of a significant virus pool in nature. This is in accordance with previous work describing areas both in the USA and in Japan with high rates of seropositivity in dogs to CaCV (Mochizuki *et al.*, 1993; Schaffer *et al.*, 1985). The reported low pathogenicity of CaCV for dogs may facilitate an unrestricted and hereto largely undetected presence of this pathogen in nature.

In this context it should be noted that the International Committee on Taxonomy of Viruses has recently proposed the establishment of the new genus *Vesivirus* within the family *Caliciviridae*, including VESV, and probably FCV and SMSV (Pringle, 1998). The sequence data presented here for a CaCV point to a high identity of this calicivirus to the caliciviruses from feline and sea lion origin (and VESV), both in phylogenetic similarity and genome organization. When the complete sequence of a CaCV becomes available it may be possible to establish conclusively that CaCV indeed belongs to the *Vesivirus* genus.

In conclusion, CaCV No.48 strain was found to share the distinguishing amino acid motifs found in the polymerase and capsid genes of caliciviruses, but this strain did not show high sequence identity to known calicivirus sequences. Sequence similarities, genomic organization and phylogenetic analysis of ORFs 1, 2 and 3 showed that this virus is most closely related to, but distinct from, feline and San Miguel sea lion caliciviruses.

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