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Isolation and characterization of a new *Vesivirus* from rabbits

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Abstract

This report describes the isolation, cDNA cloning, complete genome nucleotide sequence, and partial characterization of a new cultivable calicivirus isolated from juvenile feeder European rabbits (*Oryctolagus cuniculus*) showing symptoms of diarrhea. Absence of neutralization by type-specific neutralizing antibodies for 40 caliciviruses and phylogenetic sequence comparisons of the open reading frame 1-encoded polyprotein with those of other caliciviruses demonstrate that this new calicivirus is a putative novel member of the *Vesivirus* genus which is closely related to the marine calicivirus subgroup. According to its putative classification, this new virus has been named *rabbit vesivirus*.
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Keywords: *Caliciviridae*; *Vesivirus*; RNA genome

Introduction

The *Caliciviridae* include small non-enveloped viruses of approximately 35 nm in diameter with single-stranded, positive-polarity RNA genomes of 7.4–8.3 kb (Kapikian et al., 1996). This family has been divided phylogenetically into the genera *Vesivirus*, *Lagovirus*, *Norovirus*, and *Sapovirus* (Green et al., 2000). The member viruses of each genus share a common genomic organization and a high degree of sequence similarity. Nonetheless, the genomic organization of two of the genera (*Vesivirus* and *Norovirus*) whose genomes code for three major open reading frames (ORFs) is different from that of the *Lagovirus* and *Sapovirus* which only encoded two ORFs. The major difference between these two genomic organizations is encoding of the major capsid structural component (VP60) in *Vesivirus* and *Norovirus* in a separate open reading frame whereas in the other two genera (*Lagovirus* and *Sapovirus*) the corresponding coding sequences are fused at the 3′-terminus of a larger ORF1 (Clarke and Lambden, 2000).

Calicivirus gene expression relies upon the production of the non-structural proteins from a large polyprotein, encoded by ORF1, that is processed into functional products (König et al., 1998; Liu et al., 1996; Martín Alonso et al., 1996) and the translation of the structural components from an abundant subgenomic mRNA found in infected cells and also packaged into virions of some caliciviruses (Parra et al., 1993). A minor structural component encoded by ORF3 in *Vesivirus* and *Norovirus* and by ORF2 in *Lagovirus* and *Sapovirus* is made by a novel termination-dependent reinitiation mechanism (Meyers, 2003).

A few defined biological roles have been assigned to some of the calicivirus non-structural proteins: NTPase and

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possibly RNA helicase similar to picornavirus 2C in *rabbit hemorrhagic disease virus* (RHDV) (Marín et al., 2000) and *Norwalk virus* (NV) (Pfister and Wimmer, 2001); cysteine-protease similar to picornavirus 3C, responsible for the polyprotein processing in RHDV (Boniotti et al., 1994), *Southampton virus* (SV) (Liu et al., 1996), *feline calicivirus* (FCV) (Sosnovtseva et al., 1999), *Camberwell virus* (Seah et al., 2003), and *Chiba virus* (Someya et al., 2002) and RNA-dependent RNA polymerase similar to picornavirus 3D-protein in RHDV (López Vázquez et al., 1998), NV (Ng et al., 2004), and FCV, although in this last virus the precursor protein protease–polymerase is the active form of both activities (Wei et al., 2001). Another polyprotein processing product identified in *primate calicivirus* Pan-1 (Dunham et al., 1998), RHDV (Machín et al., 2001), and NV (Daughenbaugh et al., 2003) is the viral protein linked to the viral genome (VPg) for which a role in initiation of RNA synthesis and recruitment of translation initiation factors has been suggested. Several additional mature products still have an undefined function. The major structural capsid protein has been described to be synthesized as a precursor cleaved by the 3C cysteine protease in FCV (Sosnovtsev et al., 1998), *canine calicivirus* (Matsuura et al., 2000), and *primate calicivirus* Pan-1 (Rinehart-Kim et al., 1999), whereas in most caliciviruses no such precursor or VP60-processing exist. A basic protein of 11–20 kDa encoded by ORF3 is reported to be a minor capsid component in FCV (Herbert et al., 1996; Sosnovtsev and Green, 2000) and in NV (Glass et al., 2000).

Vesivirus and *Lagovirus* include animal pathogens that cause a variety of diseases, such as gastroenteritis, vesicular lesions, respiratory infections, reproductive failure, or hemorrhagic disease. *Vesivirus* also infect humans causing vesicular disease and possibly hepatitis and abortion (Smith et al., 2002a, 2002b). *Norovirus* and *Sapovirus* mainly infect humans, although some animal pathogens have been also included in these genera. *Norovirus* is the major cause of epidemic, non-bacterial gastroenteritis worldwide in humans of all age groups (Green et al., 2000).

Vesivirus are the only caliciviruses that replicate in cell culture, with the exception of *porcine enteric calicivirus* (PEC), that has been successfully adapted to primary porcine kidney cells and to continuous pig kidney cell lines, growing at low titter (Parwani et al., 1991), and *murine norovirus 1* (MNV-1) which provided the first tissue culture model for a *Norovirus* (Wobus et al., 2004). Many *Vesivirus* are of proven marine origin but have emerged periodically as terrestrial animal pathogens (Smith, 2000; Smith and Boyt, 1990), the best documented of these emergence being that of *vesicular exanthema of swine virus*, which was also the first recognized calicivirus.

In this report, we describe the isolation from rabbits, purification, partial characterization, and full genome cloning and sequencing of a new calicivirus with sequence similarity and genome organization indicating it is a *Vesivirus*.

Results

Isolation and characterization of a new calicivirus from rabbits

Five dead or diseased animals belonging to a small Oregon rabbit producer, all undersized and having shown mild to severe diarrhea, were brought to a veterinary clinic. All five animals displayed intestinal pathology, while the livers of three of them had rare white linear foci. Two dead rabbits were parasitized by coccidia and there was heavy growth of *Escherichia coli* evident in pooled intestinal contents of dead animals. *E. coli* was isolated from liver tissue derived from the same animals. Given the presence of multiple possible disease agents, disease etiology was difficult to ascertain. Nevertheless, after the observation by direct electron microscopy of calicivirus-like particles in the pooled intestinal content of dead rabbits we attempted their isolation for further characterization. For this purpose, the viruses in the clarified fecal samples were concentrated and inoculated onto porcine kidney cell lines (see Material and methods) that then were examined for cytopathic effects at various times after infection. In this way, a virus isolate causing cytopathic effect in the porcine cell line was purified and further adapted to growth using Vero cells (VMK).

The resulting purified virus produced cytopathic effects on VMK cells, characterized by the appearance of an increasing number of refringent rounded cells detaching from the culture flasks and forming clear plaques under appropriate conditions. The virus growth curve, using a multiplicity of infection (moi) of 1, showed a log phase starting 5 h post-infection (hpi) reaching high titers (TCID/50 $>10^9$) at 10–12 hpi.

Structural, biochemical, and virological methods were used to characterize this virus isolate, starting from purified virions obtained from infected VMK cell cultures. Transmission electron microscope examination of purified virus preparations showed typical calicivirus particles (Fig. 1A) consisting of naked virions of about 30 nm in diameter decorated with surface depressions characteristic of caliciviruses. All the capsids were isometric and particle size was uniform.

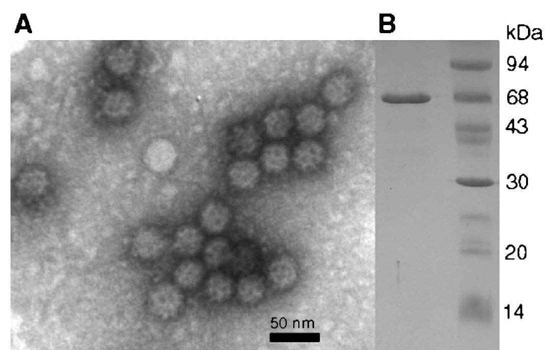

Fig. 1. Electron micrograph (A) and SDS–PAGE analysis (B) of purified RaV virions.

To characterize the protein composition of the purified virions, a sample was subjected to SDS–PAGE in 12% gels. After Coomassie blue staining, only a major protein component was observed, with an electrophoretic mobility slightly greater than that of the bovine serum albumin protein marker (68 kDa), suggesting a relative molecular mass of 67 kDa (Fig. 1B). The identity of this protein was first investigated by MALDI-TOF mass spectrometry (see Material and methods). Twenty of the 22 peptide masses investigated (data not shown), ranging from 800.38 to 2265.20 Da, matched those of equivalent peptides from the major capsid component of WCV, SMSV, VESV, or *bovine enteric calicivirus* (BoCV-Jena), all except the latter members of the *Vesivirus*. Two peptides, of Mr. 800.382 and 1131.556, respectively, could not be identified in the databases. This fact indicated that the new virus was indeed a calicivirus and prompted us to investigate its serum neutralization relationship to known independent SMSV and VESV serotypes (Smith and Latham, 1978). No neutralization occurred of 100 TCID50 of RaV by 20 antibody units of typing serum for any of the 40 calicivirus types analyzed. These results demonstrated that, despite the structural similarities found in the major structural component, the new virus was antigenically distinct from known marine caliciviruses and unrelated to other rabbit caliciviruses such as RHDV. These findings suggested the need to purify, clone, and sequence the full-length virus genome in order to define at a molecular level the identity of this apparently new virus isolated from rabbits.

Preliminary studies on the nature and polarity of the RaV genome were achieved by infectivity analyses of the extracted nucleic acid from purified virions using monolayers of VMK cells (see Material and methods). These experiments demonstrated the infectious nature of the viral genome (data not shown), for cytopathic effects could be observed 48 h post-transfection (hpt) using as little as 50 pg of viral RNA. To investigate the role of the genomic protein (VPg) that has been described attached to the genome 5′-end in other caliciviruses, the viral RaV genome was treated for 1 h at 37 °C with 250 μg ml^{-1} of proteinase K. The resulting treated RNA was much less infective, as indicated by the fact that 0.2 μg was needed (4000 times higher than the untreated RNA) to obtain cytopathic effects at 48 hpt. These results could not be attributed to the presence of residual viral particles in the purified proteinase K-untreated RNA samples, as no cytopathic effects were observed when the transfections were made in the absence of lipofectin.

Molecular cloning and sequencing of the full-length RaV genome

The nucleic acid of purified RaV virions was extracted and analyzed by denaturing formaldehyde-agarose gels. As previously shown for other caliciviruses (Meyers et al., 1991; Neill, 2002), two major RNA species of about 8 and 2.6 kb were found (not shown), corresponding to the RaV genomic (gRNA) and subgenomic RNA (sgRNA), respectively, as deduced from Northern blot studies using specific 5′ and 3′ RaV cDNA-probes (data not shown). The RNA extracted from the RaV virions then was used as the template for one-step RT-PCR amplification of the full-length RaV genome using oligonucleotides RaV14 and RaV1 (Table 1). A prominent 8.3-kb PCR-product was obtained that was gel-purified prior to being cloned into the

Table1
Oligonucleotides used in this work

Oligo name	Nucleotide sequence	Length	Genomic site	Polarity
RaV1	TTTTTTTTTTTTTTTTCCTAATGCAATCTACC	31	3′-UTR	Reverse
RaV2	AACTAGTCCGTTTTGTAGAAGC	22	ORF3	Reverse
RaV3	GGCAATTCTACTACATCAAAGC	22	3D	Forward
RaV4	TCCGTAGAGATCGGTCAGATC	21	capsid	Reverse
RaV5	ATGGCAAACCACGCTCTCGGC	21	3D	Forward
RaV6	GATGATGAGTACGATGAGTGG	21	VPg	Forward
RaV7	TTCGTCATCGAGGGAAC	17	capsid	Forward
RaV8	TGTCTGTCGAAGTACCC	17	capsid	Reverse
RaV9	GTCGATGATCACGGTGT	17	3C	Forward
RaV10	AGCCTTGACAACAGCTG	17	3D	Reverse
RaV11	CAGCATCGGTGTCATCGGCGCC	22	VPg	Reverse
RaV12	AACTGCACACGAGAACAGTGGG	22	2C	Forward
RaV13	CAACCCAACAATCACAGCTGAC	22	2C	Forward
RaV14	GTAAATGAGAATTTGAGCTATGGC	24	5′-UTR	Forward
RaV15	TAACAACACCGAGGGATCACC	21	3A	Reverse
RaV16	GACAATCGCTTTGCAAGGGCG	21	2C	Reverse
RaV17	GATATCATGCTGCACACGGCG	21	2C	Forward
RaV18	ATTCGGGTTCGTTTCCGGTGG	21	2AB	Reverse
RaV19	ATTTACGCGGACGAAGCTGCC	21	ORF3	Forward
RaV20	TTGGCGGCTCATCTGCG	17	3AB	Forward
RaV21	ACGTTGGGATGGGTTGGTGGGG	22	2A	Reverse
K331Af	TCTGCAGCCGTTTACGCGTCTTGTGCTGAAATTGG	35	unrelated	Forward
K331Ar	CCAATTTCAGCACAAGACGCGTAAACGGCTGCAGA	35	unrelated	Reverse

pGEM-T vector. The cDNA inserts from two independent pGEM-T-RaV derivatives then were fully sequenced in both strands using T7 and SP6 as well as specific RaV primers (see Table 1).

Considering the cloning strategy used for amplification of the full-length cDNA, we confirmed the 5′ and 3′ RaV sequences using an alternative approach. The 5′-end sequence of RaV gRNA was first investigated by attempting the ligation of its 5′ and 3′ ends using proteinase K-treated RaV virion RNA and T4 RNA ligase, as described by Sosnovtsev and Green (1995). The attempts made for further RT-PCR amplification across the ligated RNA ends using specific primers were unsuccessful. As an alternative, we investigated the sequence across the ligated 5′ and 3′ ends of a full-length cDNA. The data obtained after sequencing several PCR amplicons confirmed the initial nucleotide sequence obtained from the full-length cDNAs (Fig. 2C). The native sequence of the 5′-end of the RaV genome was deduced by primer-extension analysis (Fig. 2A) using radioactively labeled RaV21 primer. These experiments resulted in an extended cDNA product of 113 nt (Fig. 2B), as predicted from the initial sequencing data of the full-length cDNA.

Taking into account that the purified RaV RNA also contained large amounts of a sgRNA, presumed to be co-terminal with the gRNA at its 3′ end, similar primer extension analyses were performed using the specific oligonucleotide RaV4 in order to deduce the 5′ residue of this smaller RNA species (Fig. 2A). These experiments resulted in an extended cDNA product of 101 nt (Fig. 2B), indicating that the +1 position of the sgRNA corresponded to residue 5648 (G) of the full-length RaV genome.

The authenticity of the 3′ genome sequence was investigated by ligation of a RaV-unrelated desoxyoligonucleotide to the poly(A) tail of purified virion RNA (see Material and methods) and further PCR amplification and nucleotide sequencing of the 3′ region. After sequencing several independent clones, the RaV 3′ sequence was confirmed to be identical to the one found initially for the full-length cDNA. These experiments also indicated that the average poly(A) tail of the RaV genome was 85 nt in length.

Organization and phylogenetic analysis of the RaV genome

From the sequencing of the full-length cDNA clones constructed in this work, we report that the RaV genome is a single-stranded positive-sense RNA of 8295 nt, excluding the 3′ poly(A) tail. The genome composition (28.4% A, 23.4% U, 22.2% G, and 26% C) revealed a balanced presence of the four nucleotide components.

RaV virions also encapsidated a subgenomic RNA of 2647 nt, which is co-terminal to the 3′-end of the genomic RNA (Fig. 3A). The +1 of this sgRNA has been deduced by primer extension at residue 5648 of the RaV viral genome.

The putative 5′-untranslated (UTR) region of the viral genome was 19 nt in length and shared significant identity

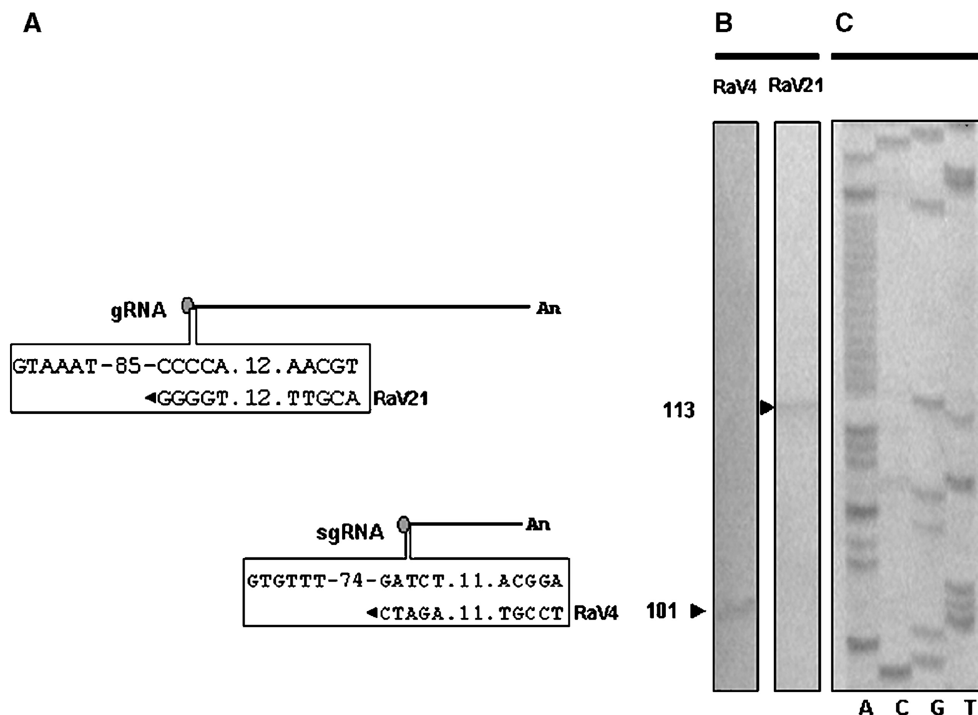

Fig. 2. Analysis of the RaV genome 5′ and 3′ sequences. (A) Schematic diagram of the expected products (113 and 101 nt) of primer extension analysis of genomic or subgenomic RNAs extracted from RaV virions using primers RaV21 (22 mer) and RaV4 (21 mer). The rectangles below the gRNA and sgRNA schemes enclose the abbreviated sequences of the primers annealed to the gRNA and sgRNA 5′ regions. (B) Experimental results of the primer extension analyses depicted in Panel A. (C) Sequencing reaction performed using oligonucleotide RaV21 over a circularized RaV cDNA including the ligated 3′ and 5′-ends.

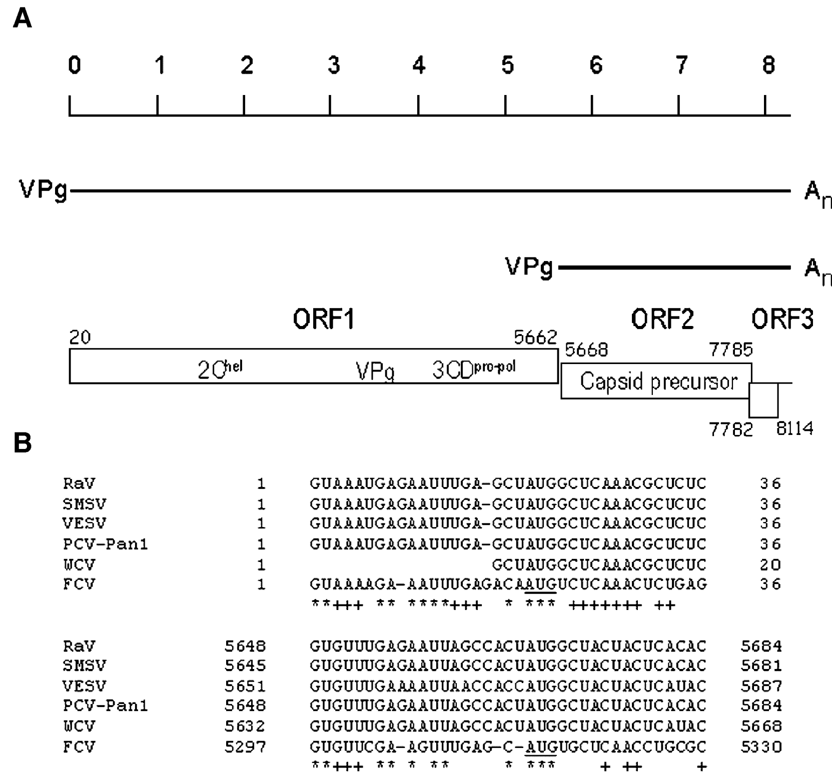

Fig. 3. Organization of RaV genome (A) and sequence conservation analysis of the 5′ genomic and subgenomic RNA sequences (B) among the *Vesivirus*. Numbers flanking the ORFs indicate nucleotides of the RaV genome. The asterisks indicate conserved nucleotides in both genomic and subgenomic 5′ UTRs. (+) Indicate additional identity within the genomic or subgenomic sequences. Initiator AUG is underlined.

with the 5′-UTR of the sgRNA (Fig. 3B). Analysis of the coding sequences in the RaV gRNA predicted the presence of three ORFs (Fig. 3A), indicating a genomic organization similar to that of *Vesivirus* and *Norovirus*. The most 5′ ORF1 was 5642 nt in length (nt 20–5662), encoding a putative polyprotein of 1880 amino acid residues. The deduced polyprotein sequence has well-characterized domains homologous to calicivirus and picornavirus non-structural proteins 2C (helicase-NTPase, residues 590–597 for the GxxGxGKT motif), 3C (cysteine protease, residues 1303–1308 for the motif containing the catalytic cysteine), and 3D (RNA-dependent RNA polymerase, residues 1598–1603 for motif A or 1701–1704 for the YGDD motif C).

ORF2 (nt 5668-7785) starts five nucleotides downstream from ORF1, encoding a 705 amino acid residues protein (Mr. 78,701), a precursor of the mature capsid protein as deduced from estimated molecular weight of 67 kDa of the major structural protein found in purified virions (Fig. 1B). In addition, the MALDI-TOF mass spectrometry analysis of tryptic peptides derived from this viral protein assigned the 22 peptide masses (including the two peptides which could not be found in other capsid proteins from related vesiviruses) to peptide sequences found within the last 490 amino acid residues of the precursor (data not shown). No peptides were identified from the N-terminus to amino acid residue 218 of the ORF2 product, indicating that a mature capsid protein was produced by proteolytic processing of a precursor as have been described for *canine calicivirus* (Matsuura et al., 2000), *feline calicivirus* (Sosnovtsev et al., 1998), and *primate calicivirus* Pan-1 (Rinehart-Kim et al., 1999).

ORF3 (nt 7782–8114) was 332 nt long and its 5′ sequence overlapped 4 nt with the 3′ end of ORF2. The predicted product of ORF3 has 110 amino acid residues, possibly a minor structural component of the virion as described for the equivalent product in RHDV (Wirblich et al., 1996) or NV (Glass et al., 2000).

The 3′ UTR of the RaV genome was 181 nt long, excluding the poly(A) tail, that averaged 85 nt. This region showed much higher uridine content (44.2%) than the rest of the viral genome.

Phylogenetic analyses were performed using the complete sequences of ORF1 polyproteins from several caliciviruses (Table 2), except for those belonging to the *Lagovirus* and *Sapovirus* for which the capsid sequence was excised from its carboxy terminus. The *bovine enteric calicivirus* NB strain (AY082891) not yet assigned to any of the existing genera was also used. The resulting analysis showed that *rabbit vesivirus* belongs to the same genus as VESV, SMSV, PCV-Pan1, and WCV (Ganova-Raeva et al., 2004). Three *Vesivirus* clades were observed in this analysis, the first including the FCV isolates, the second including the new rabbit virus described in this paper, PCV-Pan1, and the marine caliciviruses (SMSV, VESV, and

Table 2
Sequences used for phylogenetic analysis

Virus strain	Abbreviation	Accession no.	Genome length (nt)
Vesicular exanthema of swine virus	VESV-A48	U76874	8284
San Miguel Sea Lyon virus-1	SMSV-1	AF181081	8284
Primate calicivirus-Pan1	PCV-Pan1	AF091736	8304
Walrus calicivirus	WCV	AF321298	8289
Rabbit vesivirus	RaV	This work	8295
Feline calicivirus-F9	FCV-F9	M86379	7690
Feline calicivirus-F4	FCV-F4	D31836	7681
Canine calicivirus	CaCV	AB070225	8513
Isolate 2117	CV2117	AY343325	8091
Rabbit hemorrhagic disease virus-AST/89	RHDV-AST/89	Z49271	7437
Rabbit hemorrhagic disease virus-Iowa/2000	RHDV-Iowa	AF258618	7467
European Brown Hare Syndrome virus-GD	EBHSV-GD	Z69620	7442
Human calicivirus-Manchester	HuCV-Manchester	X86560	7431
Porcine enteric calicivirus-Cowden	PEC-Cowden	AF182760	7320
SRSV-I-NV/8FiiA/68S	NV/68S	M87661	7654
SRSV-I-Southampton/91K	NV-Southampton	L07418	7708
SRSV-I-BS5/Germany	NV-BS5	AF093797	7598
Bovine enteric calicivirus-Jena virus 117/80	BoCV-Jena	AJ011099	7338
SRSV-II-Lordsdale/93K	NV-Lordsdale	X86557	7555
SRSV-II-Hu/NLV/Camberwell/101922/94/AUS	NV-Camberwell	AF145896	7555
Bovine enteric calicivirus-NB/80S	BEC-NB	AY082891	7453

WCV), and the third the *canine calicivirus* and a new recently isolated calicivirus (isolate 2117) of unknown origin (Oehmig et al., 2003).

Discussion

We have partially characterized a new cultivable calicivirus, originally isolated from young rabbits suffering gastrointestinal disorders. The phylogenetic analyses, as well as the genomic organization found after sequencing the full virus genome, clearly place this virus isolate into the *Vesivirus* genus.

The comparison of the genome 5′-UTR regions with those from the subgenomic RNAs from members of the *Vesivirus* showed a high degree of sequence identity (12 out of 22 nt) which was much higher if we compared only the genome 5′-UTR sequences (18 out of 23 nt) (Fig. 3B). The conserved sequences at the genomic and subgenomic RNA 5′-ends must participate in a regulatory process, such as being a signal for RNA packaging, replication, or transcription (Lambden et al., 1995), although no experimental data have been provided supporting any of these functions. Sequence conservation of the first 60 nt of the RaV genome was particularly high with other *Vesivirus* (46–98% identity) in comparison to the *Lagovirus* (10–13%), *Norovirus*, and *Sapovirus* (18–20%) reflecting the phylogenetic relationships using the full-length ORF1 polyprotein (Fig. 4). In addition, the 100% identity found between RaV genome 5′ region and that of the marine-related caliciviruses (SMSV, VESV, and PCV) justified the one-step RT-PCR amplification and successful cloning of the RaV full-length genome reported in this paper. The recently published WCV full-length genome sequence (Ganova-Raeva et al., 2004) lacks the 16 most 5′ nucleotides of the viral genome shared by other reported *Vesivirus* genomes, but does include the AUG start site for ORF1 that has been used successfully as an antisense target for blocking calicivirus replication in vitro and in vivo (Iversen, 2001; Smith et al., 2002b; Stein et al., 2001).

The infectivity analyses performed on VMK cells using the extracted nucleic acid from purified RaV virions and the four-logarithm reduction of transfectivity observed after a proteinase K treatment suggested the presence of a VPg protein linked to the 5′-end of virion RNAs. Immunochemical data using specific antibodies against recombinant VPg also supported the presence of this protein linked to both genome and subgenomic RNAs (not shown). The lower residual infectivity observed using proteinase K-treated RNA should be related to its inefficient translation and replication rather than to the efficacy of the RNA transfection.

The cysteine proteinases of the *Caliciviridae* have been shown to be similar to the 3C-like cysteine proteinases of the picornavirus superfamily. These enzymes cleave the polyprotein encoded by ORF1 and, in the particular case of the *Vesivirus*, the major capsid protein precursor encoded by ORF2. An increasing number of papers have described polyprotein processing in different members of the *Caliciviridae*, such as RHDV (Martín Alonso et al., 1996; Wirblich et al., 1996; Meyers et al., 2000); FCV (Sosnovtseva et al., 1999, 2002); *Southampton virus* (Liu et al., 1996); and *Chiba virus* (Someya et al., 2002). Based upon these data and according to the substrate specificity found (E or Q residues at the P1 position) for the calicivirus 3C-like cysteine proteinases (Wirblich et al., 1995), a hypothetical processing map for RaV polyprotein can be drawn (Fig. 5). Four primary processing sites can be hypothesized following the putative P1 residues E435, Q791, E1070, and E1183. These proteolytic events would generate polypeptide products 2AB (precursor), 2C (a putative NTPase), 3A, 3B (VPg), and 3CD (a bifunctional protease-polymerase enzyme). Similar processing sites have been identified or predicted in other caliciviruses (Fig. 5). Although these sites are strongly supported predicted processing sites in the RaV ORF1 polyprotein, other cleavage sites are possible, as have been described for RHDV (Meyers et al., 2000) and FCV (Sosnovtsev et al., 2002). In addition, considering that the RaV genome is significantly longer than those of other

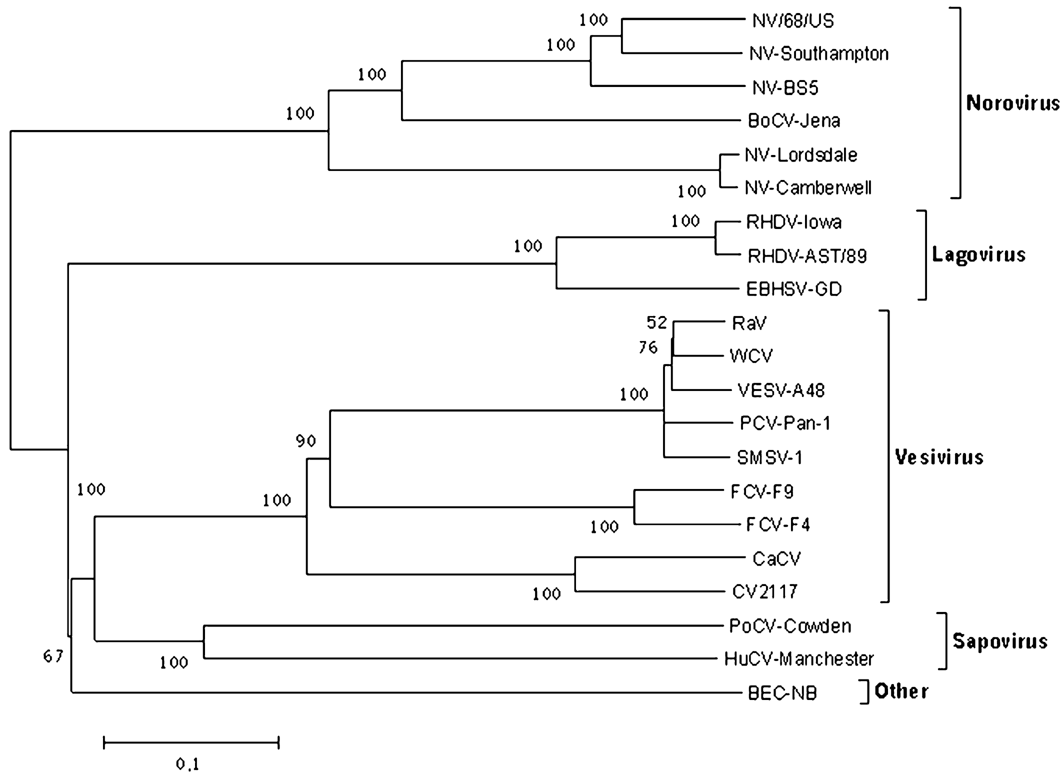

Fig. 4. Unrooted consensus tree of ORF1-encoded proteins of representative calicivirus using the Neighbor-Joining method.

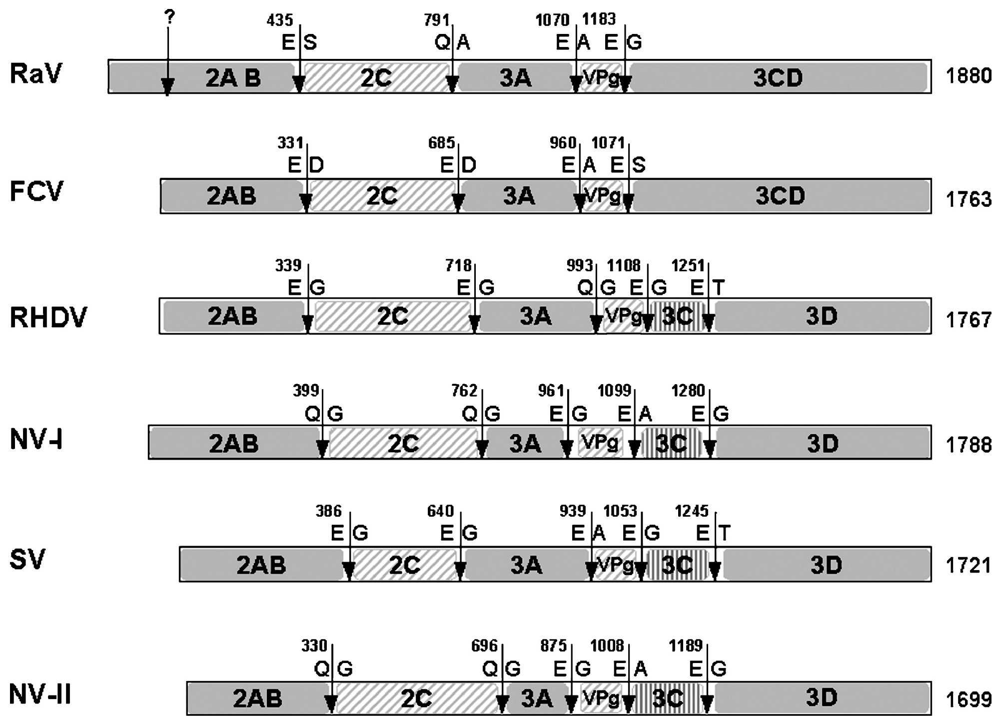

Fig. 5. Predicted RaV polyprotein processing compared to characterized cleavage products found in *Vesivirus* (FCV), *Lagovirus* (RHDV), and *Norovirus* NV-I (Southampton virus/91K) or NV-II (MD145-12/1987S). The hypothetical processing map of a *Sapovirus* (SV) (Manchester virus/93K) is also included. Numbers on the bars referred to the polyprotein residue number corresponding to the P1 residues preceding the cleavage sites indicated by arrows. (?) Indicates a putative processing site as described for Pan-1 (Rinehart-Kim et al., 1999).

caliciviruses, except for congeners as Pan-1 and SMSV, an extra sequence (approximately 461 nt) could be described at the ORF1 5′ region (Fig. 5) encoding a putative protein product (approximately 142–148 amino acid residues) as hypothesized previously for Pan-1 (Rinehart-Kim et al., 1999). This putative protein product was homologous (88–85% identity) to the equivalent regions in related vesiviruses Pan-1, WCV, VESV, and SMSV-1 but not in feline *Vesivirus* strains and no other significant similarities could be found to other sequences in the public data bases.

A fundamental difference has been reported among the *Caliciviridae* concerning the identity of the mature RNA-dependent RNA polymerase (RdRp) and the existence/absence of further processing of the 3CD product. On one hand, the bifunctional proteinase–polymerase 3CD has been described in FCV (Wei et al., 2001) and Pan-1 (Matson et al., unpublished) as the active form of the RNA polymerase, as well as the responsible enzyme for the proteolytic processing of the polyprotein (Sosnovtseva et al., 1999, 2002) and further processing of the 3CD polypeptide was not observed. In clear contrast, 3CD was further cleaved in other caliciviruses, such as RHDV (Wirblich et al., 1996) or NV (Belliot et al., 2005; Hardy et al., 2002), giving rise to smaller mature 3D products which in some cases have been shown to be enzymatically active (Belliot et al., 2005; López Vázquez et al., 1998) and crystallized, showing three-dimensional structures (Ng et al., 2002, 2004) similar to other known RdRps. In agreement with the data found for FCV and Pan-1, our preliminary 3CD expression experiments (not shown) also indicated that no processing could be detected for the RaV product. Consequently, the lack of 3CD cleavage into 3C and 3D could be a feature characteristic of the *Vesivirus*.

This description of RaV provides an additional example of an animal species (the rabbit) that could act as the host for caliciviruses classified into two of the presently defined calicivirus genera, one of them with a genomic organization with two ORFs (*Lagovirus* or *Sapovirus*) and the other showing three ORFs (*Vesivirus* or *Norovirus*). These calicivirus pairs infecting a common host include *Norovirus* and *Sapovirus* in humans, Jena, and NB isolates of *bovine enteric calicivirus*, *porcine enteric calicivirus*, and a partially characterized porcine genogroup II *Norovirus*.

Initial oral infectivity experiments in rabbits using virus adapted to Vero cell culture yielded asymptomatic infection marked by seroconversion and a low number of infected intestinal cells suggesting cell culture and plaque-purification of RaV led to attenuation.

From the point of view of the current virus nomenclature, the description of this *Vesivirus* from rabbits points out that the choice of genus names for the *Caliciviridae* was not intended to recognize the known or potential hosts of origin for strains within a genus. This new virus from rabbits highlights that some rabbit caliciviruses are not in the genus *Lagovirus* and that a calicivirus from lagomorphs is a *Vesivirus*.

Materials and methods

Biological samples

The starting material used in this study was obtained from five New Zealand/California crossbreed European rabbits (*Oryctolagus cuniculus*) presenting in 1995 to the Veterinary Diagnostic Laboratory at Oregon State University College of Veterinary Medicine. Pooled fecal samples from necropsied rabbits were clarified by centrifugation for 10 min at 400 × *g*. The supernatant was removed and re-clarified for 10 min at 7700 × *g*. The resulting supernatant was centrifuged at 21,000 × *g* for 1 h using a refrigerated microcentrifuge and the sediment obtained was suspended in 1:1000 L-cysteine hydrochloride and incubated at 8 °C.

Virus isolation and propagation

The virus was isolated from one rabbit (number 4) and also from a pool of five fecal samples including feces from this animal. The isolate from rabbit number 4 is the prototype strain.

Aliquots from the above L-cysteine hydrochloride solution were removed, inoculated onto PK-15 cells, and cytopathology occurred on the first pass. Then, after two passes in PK-15 cells, the virus was adapted to Vero cells (VMK) in three passages. The plaque purification was carried out then by three plaque passages and stock virus grown from that in monolayers of VMK cells in DMEM medium containing 10% fetal calf serum (FCS) (Smith et al., 1977). The resulting virus isolate was designated *rabbit vesivirus* (RaV).

RaV virion purification

Almost confluent monolayers of VMK cells were infected with the RaV stock virus (7th cell passage) at a multiplicity of infection (moi) of 1. After 20 h at 37 °C, lysis was almost complete. The culture flasks were freeze-thawed twice and the cell debris removed by centrifugation at 2500 × *g* for 10 min at 4 °C. RaV virions were concentrated from the clarified culture supernatants by centrifugation at 120,000 × *g* for 150 min at 4 °C using a Beckman SW28 rotor. The pellet was suspended in phosphate-buffered saline (PBS) and extracted with 1,1,2-trichloro-trifluoroethane prior to a final sedimentation step through 30% sucrose cushions at 160,000 × *g* for 180 min at 4 °C using a SW40Ti rotor. The resulting pellet was suspended in PBS and kept at −20 °C for further use.

Electron microscopy

Sucrose gradient-purified RaV virions were spotted onto Formvar-coated grids, stained with uranyl acetate, blotted dry, and examined with a Jeol 2000 EX-II electron microscope.

Serum neutralization

Neutralization was conducted using 100 $TCID_{50}$ of RaV in VMK cells in 96-well plates with replicates of 4 wells per twofold dilution of test serum. The rabbit virus isolate was typed using of 20 antibody units of heterologous typing antiserum from each of 40 calicivirus types (Smith et al., 1977).

RNA transfection into VMK cells

VMK cells maintained in DMEM medium containing 10% FCS were passaged to 6-well plates and grown to 60–80% confluence over 24 h. At this stage, cell cultures were washed twice with PBS and incubated with the indicated amounts of RaV RNA in the presence or absence of lipofectin (Invitrogen). After 10 min of incubation at 37 °C, the transfection mixture was removed, the culture washed once with PBS, and then DMEM culture medium was added. The transfected cultures were then examined for the presence of cytopathic effects up 72 h post-transfection.

RNA purification, cloning, and sequencing of the full-length RaV cDNA

Viral RNA was isolated from purified RaV virions using the QIAamp Viral RNA Mini Spin Protocol from Qiagen. This RNA was used as template for cDNA synthesis using oligonucleotide RaV1 (Table 1) as the primer and SuperScript reverse transcriptase (Invitrogen) at 42 °C for 1 h. The resulting cDNA was then amplified by PCR using the Expand Long template PCR system from Roche (Penzberg, Germany) and oligonucleotides RaV1 and RaV14 (Table 1) which were deduced from conserved 3′ and 5′ terminal nucleotides (nt) of aligned sequences from *San Miguel sea lion virus* (SMSV) (AF181081), *walrus calicivirus* (WCV) (AF321298), *vesicular exanthema of swine virus* (VESV) (AF181082), and *primate calicivirus* Pan-1 (PCV-Pan1) (AF091736). Briefly, the PCR amplification was performed in one of the buffers supplied by the commercial system, containing 1.75 mM $MgCl_2$, 350 μM of each dNTP, 1 μl of the reverse transcription mixture, and 300 nM of the oligonucleotide primers RaV1 and RaV14. Temperature cycling for the amplification reaction was as follows: 1 cycle of 5 min at 94 °C, 1 min at 55 °C, and 16 min at 68 °C; 35 cycles of 30 s at 94 °C, 1 min at 55 °C, and 16 min at 68 °C; and 1 cycle of 30 s at 94 °C, 1 min at 55 °C, and 30 min at 68 °C. The 8.3-kb product obtained was gel-purified and cloned into the pGEM-T vector (Promega, Madison, WI). The sequence of the recombinant plasmids was determined in both strands using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA) together with T7 and SP6 primers and the specific oligonucleotides listed in Table 1, which were deduced from the sequence data obtained from previous clones. The complete cDNA sequence, including RaV1 and RaV14 primers, was determined from two independently cloned PCR amplicons.

Investigation of the RaV genome 5′ and 3′-terminal sequences

The 5′-end sequence of RaV genome was deduced by ligation of both ends of the RaV cDNA before amplification with T4 RNA ligase (Fermentas, Vilnius, Lithuania) and PCR amplification across the ligated ends. Briefly, first-strand cDNA was synthesized with SuperScript RT using RNA extracted from RaV virions as template and phosphorylated oligonucleotide RaV1 as primer. After RNaseH treatment of the RT reaction, the cDNA ends were ligated with T4 RNA ligase for 3 h at room temperature and used for PCR amplification using oligonucleotide pairs RaV18/RaV19 or RaV21/RaV19. The PCR products of the expected size (714 bp for RaV18/RaV19 or 375 bp for RaV21/RaV19 amplifications) were gel-purified, cloned in pGEM-T vector, and sequenced from both ends with T7 and SP6 primers.

The 3′-end sequence and the poly(A) length of RaV genome were investigated using a RaV-unrelated phosphorylated oligonucleotide (K331Af) which was ligated to the 3′-end of the RaV genomic RNA using T4 RNA ligase. From this material, a cDNA was made using SuperScript RT and primer K331Ar (complementary to K331Af oligonucleotide). The resulting cDNA was then PCR-amplified using oligonucleotides RaV19 and K331Ar. The fragment resulting from this amplification was gel-purified, cloned into the pGEM-T vector, and sequenced from both ends with T7 and SP6 primers.

Primer extension analysis

To deduce the 5′ residue of both genomic and subgenomic RaV RNAs, oligonucleotides RaV4 and RaV21 (see Table 1) were 5′-end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase and used in extension reactions using SuperScript II reverse transcriptase and RNA extracted from RaV virions, which was presumed to consist of a mixture of genomic and subgenomic RNAs. The radioactively labeled extended primers were analyzed in a 6% polyacrylamide–8 M Urea gel electrophoresis in parallel to a sequence reaction of RaV cDNA containing ligated 5′ and 3′-ends and using oligonucleotide RaV21 as sequencing primer.

Northern blot analysis

RNA extracted from RaV virions was also analyzed by formaldehyde-agarose gel electrophoresis and transferred onto nylon filters by capillary blotting. The membranes were then hybridized with cDNA probes of negative polarity derived from both ends of the RNA genome made by in vitro transcription with T7 RNA polymerase in the presence of [α-^{32}P] UTP.

SDS–PAGE and mass spectrometry

Sucrose-gradient purified RaV virions were analyzed by electrophoresis in 12% polyacrylamide gels in the presence of SDS (SDS–PAGE) (Laemmli, 1970). After the separation, the gels were stained with Coomassie blue. The major protein band observed after staining was excised and subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) after in-gel trypsin treatment.

Phylogenetic analysis

Multiple sequence alignments were performed with the ORF1-encoded polyprotein from selected calicivirus sequences (Table 2) using CLUSTAL W program (Higgins et al., 1994) and the MEGA3 software package (Kumar et al., 2004) with the default parameters for global alignments. For *Lagovirus* and *Sapovirus* the region of the capsid protein, which is also included in the ORF1, has been deleted. Phylogenetic trees were calculated from multiple alignments using the Neighbor-Joining method. The final tree represents a consensus for 100 bootstrapped data sets analyzed.

Nucleotide sequence accession number

The sequence of the complete RaV genome has been deposited in the EMBL database under the accession no. AJ866991.

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