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# Isolation of caliciviruses from skunks that are antigenically and genotypically related to San Miguel sea lion virus

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**Abstract**

Caliciviruses were isolated from feces of skunks imported from the north central United States to Canada. Virus isolation was accomplished using adenovirus-transformed human kidney (293) cells, swine testes and Vero cells. Plaque size variants were present, but there was no apparent difference in virus morphology by negative stain or immune electron microscopy. Pigs infected with skunk calicivirus had a slightly elevated body temperature at 3 days postinfection. Although the infected animals seroconverted, no overt clinical signs were observed. Purified infectious genomic skunk calicivirus RNA behaved exactly as San Miguel sea lion virus (SMSV) 1 and 4 genomic RNA in cell culture transfection studies. Of the cell types examined, only primary porcine kidney, 293 and Vero cells supported viral replication. No viral replication was detected in cells of bovine, equine, ovine, caprine or feline origin. The skunk caliciviruses contained a single capsid protein with a relative mobility similar to SMSV virus 1 and 4 capsid proteins. The capsid protein was positive by Western blot analysis with SMSV and vesicular exanthema of swine virus (VESV) antisera. Purified RNA from skunk calicivirus infected cells was subjected to reverse transcription followed by polymerase chain reaction. Nucleotide sequences were identified that had greater than 85% similarity to the 2C and RNA polymerase gene regions of SMSV 1 and 4 and VESV A48. Predicted amino acid sequences of these regions were greater than 95% similar and the partial coding sequence of the polymerase gene contained the YGDD sequence common to positive-strand RNA virus polymerases.

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## 1. Introduction

The *Caliciviridae* are a group of non-enveloped single strand positive sense RNA viruses with a single capsid protein that has an approximate molecular weight of 65,000–70,000 (Black and Brown, 1975–1976; Black et al., 1978; Schaffer, 1979). The genomic RNA is approximately 7.5 kb and a major 2.4 kb subgenomic message produced during infection encodes the capsid protein (Meyer et al., 1991; Neill, 1992). With identification of the human Norwalk agent as a calicivirus (Jiang et al., 1990, 1993) interest in these virus types has increased. Other important members of this family include feline calicivirus (FCV) (Seal, 1994), rabbit hemorrhagic disease virus (RHDV) (Meyer et al., 1991), porcine enteric calicivirus (PEC) (Parwani et al., 1990, 1991), vesicular exanthema of swine virus (VESV) (Burroughs et al., 1978a), and San Miguel sea lion virus (SMSV) (Smith et al., 1973, 1977b, 1979; 1981; Burroughs et al., 1978b). Candidate caliciviruses have been isolated from marine mammals other than sea lions (Akers et al., 1974; Smith et al., 1983b,c; Barlough et al., 1986, 1987; Skilling et al., 1987), cattle (Smith et al., 1983d), chickens (Cubitt and Barrett, 1985), fish (Smith et al., 1980), simians (Smith et al., 1983a; 1985a,b), dogs and mink (Long et al., 1980; Schaffer et al., 1985).

Concern with large animal caliciviruses revolves around the disease vesicular exanthema (VE) of swine because clinically it mimics foot-and-mouth disease (Madin, 1975). Caliciviruses biochemically similar to VESV were also isolated from California sea lions in 1972 on San Miguel island and designated SMSV (Smith et al., 1973). Serologically and biophysically, SMSV isolates are similar to VESV (Burroughs, et al., 1978a,b), and several SMSV isolates have been shown to cause disease in marine mammals and swine (Smith et al., 1983b; Berry et al., 1990). We report herein the isolation of viruses from skunks that are antigenically and genotypically related to SMSV and VESV. However, following inoculation of pigs with these viruses, the only overt clinical sign observed was a slightly elevated temperature in one animal.

## 2. Materials and methods

### *2.1. Virus isolation, replication and cell culture*

Human 293 (Graham et al., 1977), Vero and swine testes cells were cultured using MEM with 10% fetal bovine serum (FBS) and antibiotics (neomycin at 50 $\mu$g/ml and gentamicin at 50 $\mu$g/ml). For virus isolation, frozen skunk feces at a final concentration of 2%, were suspended in phosphate-buffered saline (PBS) containing 2% FBS and antibiotics (Gibco 15240 at 5 ×, neomycin sulfate at 100 $\mu$g/ml, gentamicin at 100 $\mu$g/ml). The suspension was clarified at 1,000 *g* for 20 min and 100-$\mu$l aliquots adsorbed onto $2 \times 10^5$ 293 cells. Specimens were incubated at 37°C and examined daily for viral cytopathic effect (CPE). Cell cultures

positive for CPE were stored at −70°C. All samples were passaged further on 293 or Vero cells by infecting monolayers with 50 μl of CPE-positive cell culture fluid. Three passages of viral plaque purifications were completed using Vero cell monolayers in 6-well plates as described by Precious and Russel (1985). SMSV types 1 and 4 and VESV A48 were replicated and purified using Vero cells as described previously (Neill, 1992).

### 2.2. Negative stain and immune electron microscopy of viral isolates

Skunk sera were obtained from animals using conventional methods (Charlton et al., 1992). Negative stain electron microscopy was performed using 10-μl aliquots of infected cell culture fluid applied directly on a 200 mesh grid and stained with 2% phosphotungstic acid (Kapikian et al., 1972). Immune electron microscopy was also conducted essentially as described (Kapikian et al., 1972). A 25-μl aliquot of clarified (1,000 *g* for 20 min) infected cell culture fluid was incubated for 2 h at 25°C with 25 μl of a 1:5 dilution of the corresponding skunk serum. A 10 μl aliquot of sample was applied to a 200 mesh grid, excess sample was removed with blotting paper and 20 μl of 2% phosphotungstic acid was added. Excess stain was removed with blotting paper and the grid examined by transmission electron microscopy (Kapikian et al., 1972).

### 2.3. Experimental infection of pigs with skunk calicivirus

Two-thirty pound, specific pathogen-free pigs were simultaneously inoculated intravenously with 1.0 ml and orally with 1.5 ml of skunk calicivirus at a titer of $10^5$ $TCID_{50}$/ml. Rectal temperatures of both animals were taken daily for 13 days and the animals were observed for clinical signs. No clinical specimens were collected. The pigs were bled before the administration of virus and at weekly intervals thereafter until day 35. Reciprocal virus neutralization titers of two-fold dilutions of pig sera with skunk calicivirus were based on complete neutralization of 450 $TCID_{50}$ completed in triplicate.

### 2.4. Virus, viral genomic RNA purification and transfection of cultured cells with viral genomic RNA

Skunk caliciviruses, SMSV types 1 and 4 and VESV A48 were purified by centrifugation through isopycnic CsCl gradients at a density of 1.37 g/ml as described previously (Schaffer and Soergel, 1973; Love, 1976). Viral genomic RNA was obtained by denaturing virions in guanidinium isothiocyanate (Chirgwin et al., 1979) and centrifugation of RNA through 5.5 M CsCl as described (Glisin et al., 1974). For transfections, 20 μg of Lipofectin (Life Technologies) was diluted to a final volume of 100 μl in serum-free OPTI MEM, mixed with 0.5 μg viral RNA, incubated for 15 min and brought to 1.0 ml final volume with media. Cells were transfected with 200 μl of the RNA-Lipofectin preparation in 24-well plates. After 3 h at 37°C the inocula were replaced with serum-free OPTI MEM and the cells were observed for 48–72 h for viral CPE. Cell types examined for the ability to support skunk calicivirus and SMSV replication included Vero, Crandell–Reese feline kidney (CRFK), fetal bovine lung (FBL), equine embryonic kidney (EEK),

lamb kidney primary (LKP), goat kidney primary (GKP) and primary porcine kidney (PPK). Cells were cultured and experimentation completed as described previously (Kreutz et al., 1994).

### 2.5. *Polyacrylamide gel electrophoresis of skunk calicivirus capsid proteins and Western blot analysis*

Purified skunk caliciviruses were electrophoresed in denaturing 10% SDS-polyacrylamide gels as previously described (Hames, 1981) and transferred to nitrocellulose (Towbin et al., 1979). Non-specific protein binding activity was blocked with 0.1 M PBS containing 3% fish gelatin and 0.05% Tween 20. Blots were reacted with primary antibody (diluted 1 : 100) for 2 h in blocking buffer followed by 3 washes with 0.1 M PBS, 1% fish gelatin, 0.05% Tween 20. Finally, blots were reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel) followed by 3 washes with 0.1 M PBS, 1% fish gelatin lacking Tween 20. Blots were developed using 4-chloro-naphthol and hydrogen peroxide (Gershoni and Palade, 1983).

Rabbit polyclonal antisera to SMSV and VESV were provided by the U.S.D.A. Foreign Animal Disease Diagnostic Laboratory at Plum Island (gift from Dr. J.A. House). Antisera were produced using cell culture viral inoculum following replication in Vero cells and clarification of cell culture fluids. New Zealand rabbits were inoculated intraperitoneally, intravenously and intramuscularly with $1 \times 10^5$ $TCID_{50}$ of each virus type. At 6 weeks, the animals were boosted i.v. Antisera used during these investigations were to SMSV types 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and VESV types B34, A48, B51, C52, D53, E54, F55, G55, H54, I55. Sentinel rabbit sera were used as a negative control during Western blot procedures.

### 2.6. *Single tube reverse transcription-polymerase chain reaction and nucleotide sequencing of amplified products*

A single tube reverse transcription-polymerase chain reaction (RT-PCR) was conducted essentially as described (Lewis et al., 1992). The nucleotide sequences of SMSV types 1 and 4 were aligned (ALIGN; Scientific and Education Software, Stateline, PA) and areas of conserved sequence in the 2C and pol regions were analyzed by the PRIMER2 (Scientific and Education Software, Stateline, PA) computer program to derive RT-PCR primer oligonucleotides. Genomic RNA was reverse transcribed and cDNA amplified using 100 pmol of each primer. The sense primer was 5′-GTCCCAGTATTCGGATTTGTCTGCC-3′ and the antisense primer was 5′-AGCGGGTAGTTCAGTCAAGTTCACC-3′ for the 2C region. The sense primer was 5′-GCCTTCTGGTATGCCACTAACATCC-3′ the antisense primer was 5′-GACGAGCGGTATGATCTTGTTGGTG-3′ for the pol region. The RT-PCR reactions were incubated for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase at 55°C for 1 h and denatured at 95°C for 5 min. Twenty-five cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and primer extension at 72°C for 10 min was completed using 3 units Amplitaq™ (Perkin–Elmer) polymerase (Saiki et al., 1985).

Following RT-PCR amplification, DNA products were cloned using the TA Cloning System™ (Mead et al., 1991) according to methods provided (Invitrogen,

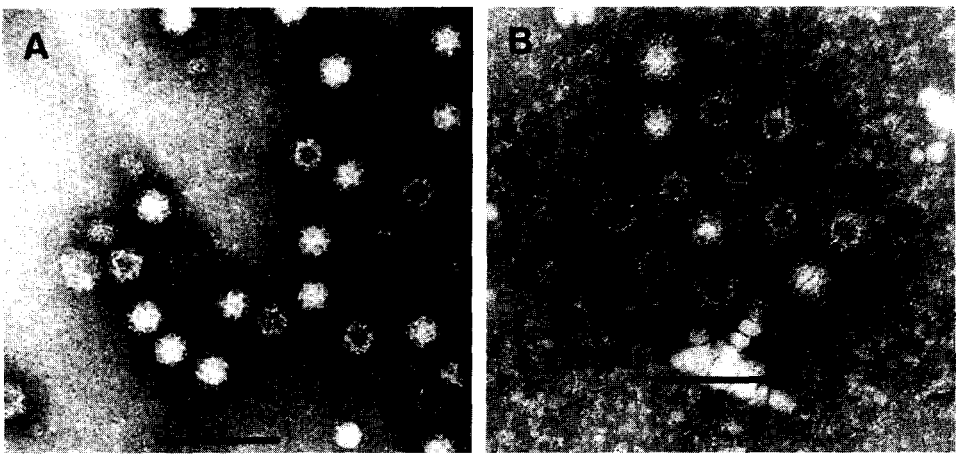


Fig. 1. Negative stain and immune electron microscopy of skunk caliciviruses. Aliquots of infected cell culture supernatants were placed on electron microscopy grids, stained with phosophotungstic acid and examined by transmission electron microscopy (A). Infected cell culture supernatants were also reacted with convalescent skunk sera, aliquots placed on grids and examined by transmission electron microscopy (B).

San Diego, CA). Three recombinants of each isolate were purified for sequence analysis (Seal, 1994). Double stranded sequencing (Tabor and Richardson, 1987) with Taq polymerase (Applied Biosystems Inc.) and fluorescently labeled dideoxynucleotides (Sanger et al., 1977) was performed for analysis with an automated nucleotide sequencer (Smith et al., 1986). Nucleotide sequence editing and final alignments were completed using the GeneWorks 2.3™ programs (Intelligenetics, Mountain View, CA).

The nucleotide sequences for the 2C and pol regions, respectively, have been submitted to GenBank and assigned accession numbers 4-1L, U14667 and U14668; 4-2S, U14669 and U14670; 7-2/3L, U14671 and U14672; SMSV4, U14673 and U14674; SMSV1, U14675 and U14676; VEA48, U14677 and U14678.

## 3. Results

### *3.1. Virus isolation, replication in cell culture and immune electron microscopy*

Filterable agents were isolated from skunk feces that produced viral CPE and replicated in swine testes, human 293 and Vero cells. Viruses were identified from infected cell culture supernatants that had typical calicivirus morphology by negative stain electron microscopy (Fig. 1A). Consequently, viral isolates were further replicated in cell culture and two isolates designated 4 and 7 were plaque purified for further study. Upon plaque purification, large and small plaque variants of isolate 4 were identified and designated 4-1L and 4-2S. Two plaques of isolate 7 were chosen at random and designated 7-2L and 7-3L. Typical calicivirus aggregates were observed for all the viral isolates by immune electron microscopy

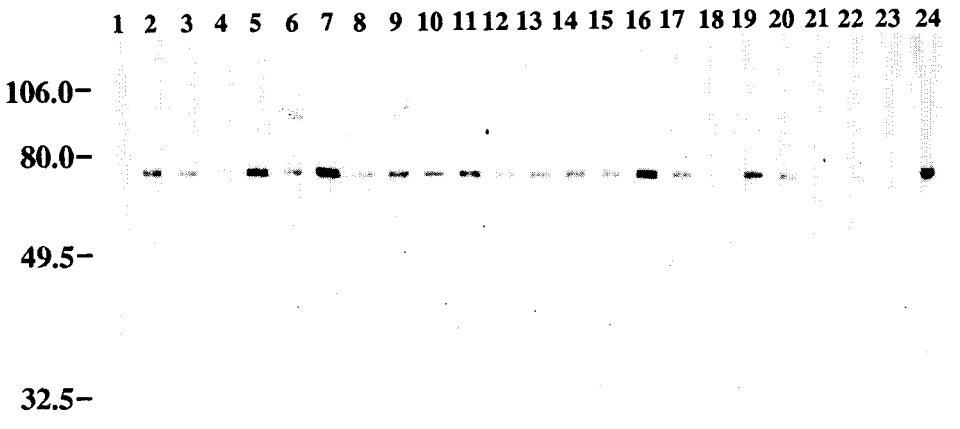


Fig. 2. Western blot reactivities of rabbit antisera to isolates of San Miguel sea lion virus (SMSV) and vesicular exanthema of swine virus (VESV) with purified skunk calicivirus capsid protein. Purified skunk calicivirus (isolate 7-2L) was electrophoresed by SDS-PAGE, transferred to nitrocellulose and reacted with rabbit polyclonal antisera to isolates of SMSV and VESV. Antisera used were to rabbit sentinel control (lane 1), SMSV 1 (lane 2), SMSV 2 (lane 3), SMSV 4 (lane 4), SMSV 5 (lane 5), SMSV 6 (lane 6), SMSV 7 (lane 7), SMSV 8 (lane 8), SMSV 9 (lane 9), SMSV 10 (lane 10), SMSV 11 (lane 11), SMSV 12 (lane 12), SMSV 13 (lane 13), VESV B34 (lane 14), VESV A48 (lane 15), VESV B51 (lane 16), VESV C52 (lane 17), VESV D53 (lane 18), VESV E54 (lane 19), VESV F55 (lane 20), VESV G55 (lane 21), VESV H54 (lane 22), rabbit sentinel control (lane 23) and VESV I54 (lane 24).

utilizing skunk convalescent sera corresponding to each specific isolate. An example is illustrated in Fig. 1B.

### *3.2. Experimental infection of pigs with skunk caliciviruses and transfection of cultured cells with viral genomic RNA*

Two pigs were inoculated with a skunk calicivirus isolate no. 7. One of the two pigs had a slightly elevated body temperature of 40–40.2°C from day 3 to day 6 p.i. The body temperature returned to below 40°C on day 7 and remained at this level for the remainder of the experiment (day 35). During the 35 days, neither animal exhibited clinical signs and feed consumption was normal. Both pigs seroconverted by day 14. One animal had a serum virus neutralization titer of 1:256 while the other animal had a titer of 1:64. No gross lesions were observed in either animal after 35 days.

To determine possible host range of the skunk caliciviruses, viral genomic RNA was used to transfect cultured cells from various domestic animals. Only primary porcine and Vero cells supported viral replication using Lipofectin to transform various cell types. Titers following transfection ranged from $5.7 \times 10^7$ to $1.3 \times 10^8$ PFU/ml using Vero cells for virus titration. Primary cells of bovine, ovine, caprine, equine, or feline origin did not support skunk calicivirus replication following genomic RNA transfection. The same results were obtained using SMSV types 1 and 4 viral genomic RNA during parallel experiments.

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4-2S,2C          .......... .......... .......... .......... ..........
SMSV4,2C         .......... .......... .......... ........V.. ..........
A48,2C           .......... .......... .......... ........V.. ..........
7-2L/3L,2C       .......... .......... .......... .......... ..........
4-1L,2C          .......... .......... .........P. .......... ..........
SMSV1,2C         .......... .......... .......C... .......... ..........
Consensus        VPVFGFVCQN NEIDTVYNLL AAVKARYGAN FNLYKGMTRT AHENSGCGAH

4-2S,2C          .......... .......... .......... .......... ..........
SMSV4,2C         ..........  ...V...S.. .......... .......... ..........
A48,2C           .......... .......... .......... .......... ..........
7-2L/3L,2C       .......... .......... .......... .......... ..........
4-1L,2C          .......... .......... .......... .......... ..........
SMSV1,2C         .......... .......... .......... .......... ..........
Consensus        VHVISREDNF RGKAFTVNRS RLESVPHLEG DSFRRSLGVV MSDKDVTTMF

4-2S,2C          S.........  .........
SMSV4,2C         ..........  .........
A48,2C           ..........  .........
7-2L/3L,2C       ..........  .........
4-1L,2C          ..........  .........
SMSV1,2C         ..........  .........
Consensus        YYIKGKVIND QVNLTELPA
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Fig. 3. Amino acid sequence comparison of the translated nucleotide sequence of amplified products from the genomic 2C region among skunk calicivirus isolates, San Miguel sea lion virus (SMSV) types 1 and 4, and vesicular exanthema of swine virus (VESV) A48. Primary nucleotide sequences were obtained by sequencing cloned reverse transcription-amplification products as described in section 2. Nucleotide sequences were translated and compared using the multiple alignment program of Intelligenetics GeneWorks™ to identify predicted amino acid substitutions and similarities among isolates.

### *3.3. Western blot analysis of skunk calicivirus capsid proteins*

To determine antigenic cross-reactivity of skunk caliciviruses with other porcine caliciviruses, a Western blot analysis using polyclonal antisera to various SMSV and VESV types was completed (Fig. 2). The skunk caliciviruses had a single capsid protein with a similar relative mobility to other animal caliciviruses (approximately 66,000–72,000). Although there was broad cross-reactivity with various SMSV and VESV antisera, specifically weak reactions were obtained with antisera to SMSV type 4 (Fig. 2, lane 4) and VESV types D53 (Fig. 2, lane 18) and G55 (Fig. 2, lane 21). Intense positive reactions were obtained with antisera to SMSV types 5 (Fig. 2, lane 5) and 7 (Fig. 2, lane 7) and with antisera to VESV types B51 (Fig. 2, lane 16) and J56 (Fig. 2, lane 24). At no time were there any positive reactions obtained using a sentinel rabbit sera as a negative control (Fig. 2, lanes 1 and 23). Similar results illustrated in Fig. 2 for skunk calicivirus isolate 7-2L were obtained for isolate 7-3L and for both plaque size variants 4-1L and 4-2S (data not shown).

### 3.4. *Reverse transcription-polymerase chain reaction of viral RNA and nucleotide sequence analysis*

Using oligonucleotide primers to the 2C and pol regions of SMSV types 1 and 4, RT-PCR was utilized to amplify nucleotide sequences from the viral genome. Amplified products were then cloned and nucleotide sequencing was completed to determine similarities among the SMSV types 1 and 4, VESV A48 and the skunk caliciviruses. Sequence analysis of RT-PCR amplified products from the viral genomic 2C region revealed that among the SMSV, VESV and skunk calicivirus isolates an 89% nucleotide sequence identity was shared among all the isolates. The predicted amino acid sequence identity was 95% among all the isolates in the

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7-2/3L,POL      .......... .......... .......... .......... ..........
4-1L,POL        .......... .......... .......... .......... ..........
SMSV1,POL       .......... .......... .......... .......... ..........
A48,POL         .......... .......... .......... ..R....... ..........
SMSV4,POL       .......... .......... .......... .......... ..........
4-2S,POL        .......... .......... .......... .....R.... ..........

Consensus       PSGMPLTSII NSLNHCLMVG SAVVKALEDS GVQVTWNIFD SMDLFTYGDD

7-2/3L,POL      .......... .......S.. .......... ..T....... ..........
4-1L,POL        .......... .......S.. .......... ..T....... ..........
SMSV1,POL       .......... .......... .......... ..A....... ..........
A48,POL         .......... .......... .......... ..S....... ..........
SMSV4,POL       .......... .......... K......... ..S....... ..........
4-2S,POL        .......... .......... K......... ..S....... ..........

Consensus       GVYIVPPLIS SVMPKVFANL RQFGLKPTRT DK.DAEITPI PADEPVEFLK

7-2/3L,POL      .......... .....K.... .......... .......... ...S......
4-1L,POL        .......... .....K.... .......... .......... ...S......
SMSV1,POL       .......... .......... .......... .........R ..........
A48,POL         .......... .......... .......... .........R ..........
SMSV4,POL       .........I .......... .......... .....I.... ..........
4-2S,POL        .........I .......... .......... .....I.... ..........

Consensus       RTIVRTENGV RALLDRSSII RQFYYIKAEN TENWTVPPKK IDTPSRGQQL

7-2/3L,POL      ...G...... .......... ...
4-1L,POL        ...G...... .......... ...
SMSV1,POL       .......... .......... ...
A48,POL         .......... .......... ...
SMSV4,POL       .......... .......... ...
4-2S,POL        .......... .......... ...

Consensus       YNACLYASQH GEEFYTNKII PLV
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Fig. 4. Amino acid sequence comparison of the translated nucleotide sequence of amplified products from the genomic polymerase (pol) region among skunk calicivirus isolates, San Miguel sea lion virus (SMSV) types 1 and 4, and vesicular exanthema of swine virus (VESV) A48. Primary nucleotide sequencing and prediction of amino acid sequence was as described in Fig. 2. The YGDD sequence common to positive-strand RNA virus polymerases is underlined.

2C area of the genome (Fig. 3). Similar results were obtained when analysis of the pol region was completed. The nucleotide sequences of amplified products from the pol region was 87% similar among the SMSV, VESV and skunk calicivirus isolates and 95% similar for the predicted amino acid sequence (Fig. 4).

In both the 2C and pol regions of the genome, sequence differences were observed between the 4-1L and 4-2S plaque size variants of skunk calicivirus. Only two nucleotide sequence substitutions occurred in the 2C region at positions 85 and 302. However, numerous sequence differences occurred between the two 4-1L and 4-2S virus isolates in the pol region of the genome. No differences were detected in the sequence of the 7-2L and 7-3L isolates in either areas of the genome and are listed concurrently as 7-2/3L in the analysis (Figs. 3 and 4).

## 4. Discussion

Viruses were isolated from feces of healthy captive-bred skunks imported from the north central United States to Canada as test animals during unrelated investigations involving use of a potential wildlife rabies vaccine (Charlton et al., 1992). These viruses were identified morphologically as caliciviruses similar to SMSV (Breese and Dardiri, 1977) and VESV (Smith et al., 1978) by negative stain and immune electron microscopy. Norwalk virus is purified from the feces of humans (Jiang et al., 1990) and other animal caliciviruses such as SMSV and canine calicivirus can be recovered from the gastrointestinal system (Smith et al., 1983c; Schaffer et al., 1985). By SDS-PAGE and Western blot analysis the skunk caliciviruses were shown to have a single capsid protein that shares antigenic similarity with serotypes of SMSV and VESV. Using transfection of cells in culture with purified viral genomic RNA, it was demonstrated that viral replication occurred only in porcine and Vero cells. However, cell types from other domestic animals such as bovine, equine, ovine and caprine would not support virus replication. This relates well with the fact that VESV only replicates and causes disease in swine and not in other domestic animals (Madin, 1974). However, it does not strictly conform to a previous study wherein replication of SMSV type 1 was reported in ovine and feline cells (Smith et al., 1977a). In our laboratory SMSV type 1 replication in ovine and feline cell types has not been observed. Replication of SMSV type 1 paralleled what was reported for SMSV type 4, in that only Vero and primary porcine kidney cells supported replication (Smith et al., 1977a).

Nucleotide sequence analysis using RT-PCR to examine the 2C and pol regions of the genomes of these virus types revealed high sequence similarity to SMSV and VESV. All the viruses examined contained the YGDD sequence common to positive-strand RNA virus polymerases as also described for Norwalk virus (Jiang et al., 1990, 1993). Biochemically and biophysically SMSV and VESV isolates are very similar (Oglesby et al., 1971; Soergal et al., 1975, 1976) and the nucleotide sequence analysis further supports the classification among these virus types. Although, there were some weak reactions of skunk calicivirus capsid protein with SMSV and VESV antisera, by Western blot, all the antisera were positive using

this analysis. There appeared to be no correlation of antisera reactivity and chronological isolation of the SMSV or VESV types. It has been demonstrated that among SMSV there are many serotypes that do not cross-neutralize with heterologous antisera (Smith and Latham, 1978), analogous to the situation in feline caliciviruses (Seal, 1994).

Marine caliciviruses have been demonstrated to cause VE in swine (Smith et al., 1973; Smith et al., 1980). Additionally, antibodies to VESV have been detected in feral mammals in southern California coastal areas of the US (Smith and Latham, 1978). Since the skunk caliciviruses were highly related genotypically to SMSV and VESV, pigs were inoculated with this virus. However, infection of pigs with skunk caliciviruses did not produce disease in these animals, although seroconversion occurred. There have also been caliciviruses isolated from various other animals such as mink (Long et al., 1980) and there are reports of a porcine enteric calicivirus (Parwani et al., 1990, 1991). The results of these and our investigations suggest that animal caliciviruses exist in the environment even though no outbreaks of VE have been reported since elimination of the disease was accomplished by improved husbandry practices (Madin, 1975). The phylogenetic relationship of the caliciviruses reported here and their relationship with the mink calicivirus and porcine enteric calicivirus need to be addressed.

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## References

Akers, T.G., Smith, A.W., Latham, A.B. and Watkins, H.M. (1974) Calicivirus antibodies in California gray whales (*Eschrichtius robustus*) and Steller sea lions (*Eumetopias jupatus*). Arch. Gesamte. Virusforsch. 46, 175–177.

Barlough, J.E., Berry, E.S., Skilling, D.E., Smith, A.W. and Fay, F.H. (1986) Antibodies to marine caliciviruses in the Pacific walrus (*Odobenus rosmarus divergens* Illiger). J. Wildl. Dis. 22, 165–168.

Barlough, J.E., Berry, E.S., Goodwin, E.A., Brown, R.F., DeLong, R.L. and Smith, A.W. (1987) Antibodies to marine caliciviruses in the Stellar sea lion (*Eumetopias jubatus* Schreber). J. Wildl. Dis. 23, 34–44.

Berry, E.S., Skilling, D.E., Barlough, J.E., Vedros, N.A., Gage, L.J. and Smith, A.W. (1990) New marine calicivirus serotype infective for swine. Am. J. Vet. Res. 51, 1184–1187.

Black, D. and Brown, F. (1975–1976) A major difference in the strategy of the calici- and picornaviruses and its significance in classification. Intervirology 6, 57–60.

Black, D.N., Burroughs, J.N., Harris, T.J.R. and Brown, F. (1978) The structure and replication of calicivirus RNA. Nature 274, 614–615.

Breese, S.S. and Dardiri, A.H. (1977) Electron microscopic observations on a virus transmissible from pinnipeds to swine. J. Gen. Virol. 36, 221–225.

Burroughs, J.N., Doel, T.R., Smale, C.J. and Brown, F. (1978a) A model for vesicular exanthema virus, the prototype of the calicivirus group. J. Gen. Virol. 40, 161–174.

Burroughs, J.N., Doel, T. and Brown F (1978b) Relationship of San Miguel sea lion virus to other members of the calicivirus group. Intervirology 10, 51–59.

Charlton, K.M., Artois, M., Prevec, L., Cambell, J.B., Casey, G.A., Wandeler, A.I. and Armstrong, J. (1992) Oral rabies vaccination of skunks and foxes with a recombinant human adenovirus vaccine. Arch. Virol. 123, 169–179.

Chirgwin, J.M., Przybla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18, 5294–5299.

Cubitt, W.D. and Barrett, A.D.T. (1985) Propagation and preliminary characterization of a chicken candidate calicivirus. J. Gen. Virol. 66, 1431–1438.

Gershoni, J.M. and Palade, G.E. (1983) Protein blotting: principles and applications. Anal. Biochem. 131, 1–15.

Glisin, V., Crkvenjakov, R. and Byers, C. (1974) Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13, 2633–2637.

Graham, R.L., Smiley, J., Russel, W.C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59–72.

Hames, B.D. (1981) An introduction to polyacrylamide gel electrophoresis. In: B.D. Hames and D. Rickwood (Eds.), Gel Electrophoresis of Proteins: A Practical Approach. IRL Press, Oxford, pp. 1–91.

Jiang, X., Graham, D.Y., Wang, K. and Estes, M.K. (1990) Norwalk virus genome cloning and characterization. Science 250, 1580–1583.

Jiang, X., Wang, M., Wang, H. and Estes, M.K. (1993) Sequence and genomic organization of Norwalk virus. Virology 195, 51–61.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T.S., Kalica, A.R. and Chanock, R.M. (1972) Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J. Virol. 10, 1075–1081.

Kreutz, L.C., Seal, B.S. and Mengeling, W.L. (1994) Early interaction of feline calicivirus with cells in culture. Arch. Virol. 136, 19–34.

Lewis, J.G., Chang, G.J., Lanicotti, R.S. and Trent, D.W. (1992) Direct sequencing of large flavivirus PCR products for analysis of genome variation and molecular epidemiological investigations. J. Virol. Methods 38, 11–24.

Long, G.G., Evermann, J.F. and Gorham, J.R. (1980) Naturally occurring picornavirus infection of domestic mink. Can. J. Comp. Med. 44, 412–417.

Love, D.N. (1976) Feline calicivirus: purification of virus and extraction and characterization of its ribonucleic acid. Cornell Vet. 66, 498–512.

Madin, S.H. (1975) Vesicular exanthema. In: H.W. Dunne and A.D. Leman (Eds.), Diseases of Swine, 4th edn. Iowa State University Press, Iowa, pp. 286–307.

Mead, D.A., Pey, N.K., Herrnstadt, C., Marcil, R.A. and Smith, L.M. (1991) A universal method for the direct cloning of PCR amplified nucleic acid. Biotechnology 9, 657–662.

Meyer, G., Wirblich, C. and Thiel, H.J. (1991) Rabbit hemorrhagic disease virus. Molecular cloning and nucleotide sequencing of a calicivirus genome. Virology 184, 664–676.

Neill, J.D. (1992) Nucleotide sequence of the capsid protein gene of two serotypes of San Miguel sea lion virus: identification of conserved and non-conserved amino acid sequences among calicivirus capsid proteins. Virus Res. 24, 211–222.

Oglesby, A.S., Schaffer, F.L. and Madin, S.H. (1971) Biochemical and biophysical properties of vesicular exanthema of swine virus. Virology 44, 329–341.

Parwani, A.V., Saif, L.J. and Kang, S.Y. (1990) Biochemical characterization of porcine enteric calicivirus: analysis of structural and nonstructural viral proteins. Arch. Virol. 112, 41–53.

Parwani, A.V., Flynn, W.T., Gadfield, K.L. and Saif, L.J. (1991) Serial propagation of porcine enteric calicivirus in a continuous cell line. Arch. Virol. 120, 115–122.

Precious, B. and Russell, W.C. (1985) Growth, purification and titration of adenovirus. In: B.W.J. Mahy (Ed.), Virology: a Practical Approach. IRL Press, Oxford, pp. 193–205.

Saiki, R.K., Scharf, F., Faloona, F. Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnhem, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350–1354.

Sanger, F., Nickles, S. and Carlson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.

Schaffer, F.L. (1979) Caliciviruses, In: H. Frenkel-Conrat and F.L. Wagner (Eds.), Comprehensive Virology, Vol. 14. Plenum, New York, pp. 249–284.

Schaffer, F.L. and Soergel, M.E. (1973) Biochemical and biophysical characterization of calicivirus isolates from pinnipeds. Intervirology 1, 210–219.

Schaffer, F.L., Soergel, M.E., Black, J.W., Skilling, D.E., Smith, A.W. and Cubitt W.D. (1985) Characterization of a new calicivirus isolated from feces of a dog. Arch. Virol. 84, 181–195.

Seal, B.S. (1994) Analysis of capsid protein gene variation among divergent isolates of feline calicivirus. Virus Res. 33, 39–53.

Skilling, D.E., Barlough, J.E., Berry, E.S., Brown, R.F. and Smith, A.W. (1987) First isolation of a calicivirus from the Steller sea lion (*Eumetopias jubatus*). J. Wildl. Dis. 23, 534–538.

Smith, A.W. and Latham, A.B. (1978) Prevalence of vesicular exanthema of swine antibodies among feral mammals associated with the southern California coastal zones. Am. J. Vet. Res. 39, 291–296.

Smith, A.W., Akers, T.G., Madin, S.H. and Vedros, N.A. (1973) San Miguel sea lion virus isolation, preliminary characterization and relationship to vesicular exanthema of swine virus. Nature 244, 108–110.

Smith, A.W., Madin, S.H., Vedros, N.A. and Bankowski, R.A. (1977a) Host range comparisons of five serotypes of caliciviruses. Am. J. Vet. Res. 38, 101–105.

Smith, A.W., Prato, C.M. and Skilling (1977b) Characterization of two new serotypes of San Miguel sea lion virus. Intervirology 8, 30–36.

Smith, A.W., Skilling, D.E. and Ritchie, A.E. (1978) Immunoelectron microscopic comparisons of caliciviruses. Am. J. Vet. Res. 39, 1531–1533.

Smith, A.W., Akers, T.G., Latham, A.B., Skilling, D.E. and Bray, H.L. (1979) A new calicivirus isolated from a marine mammal. Arch. Virol. 61, 255–259.

Smith, A.W., Skilling, D.E., Dardiri, A.H. and Latham, A.B. (1980) Calicivirus pathogenic for swine: a new serotype isolated from opaleye, *Girella nigricans*, an ocean fish. Science 209, 940–941.

Smith, A.W., Skilling, D.E. and Latham, A.B. (1981) Isolation and identification of five new serotypes of calicivirus from marine mammals. Am. J. Vet. Res. 42, 693–694.

Smith, A.W., Skilling, D.E., Ensley, P.K., Benirschke, K. and Lester, T.L. (1983a) Calicivirus isolation and persistence in a pygmy chimpanzee (*Pan paniscus*). Science 221, 79–81.

Smith, A.W., Skilling, D.E. and Ridgeway, S. (1983b) Calicivirus-induced vesicular disease in cetaceans and probable interspecies transmission. J. Am. Vet. Med. Assoc. 183, 1223–1225.

Smith, A.W., Ritter, D.G., Ray, G.C., Skilling, D.E. and Wartzok, D. (1983c) New calicivirus isolates from feces of walrus (*Odobenus rosmarus*). J. Wildl. Dis. 19, 86–89.

Smith, A.W., Mattson, D.E., Skilling, D.E. and Schmitz, J.A. (1983d) Isolation and partial characterization of a calicivirus from calves. Am. J. Vet. Res. 44, 851–855.

Smith, A.W., Skilling, D.E., Anderson, M.P. and Benirschke, K. (1985a) Isolation of a primate calicivirus Pan paniscus type 1 from a douc langur (*Pygathrix nemaeus* l.). J. Wildl. Dis. 21, 426–428.

Smith, A.W., Skilling, D.E. and Benirschke, K. (1985b) Calicivirus isolation from three species of primates: an incidental finding. Am. J. Vet. Res. 46, 2197–2199.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughs, P., Dodd, C., Connel, C.R., Heines, Kent, S.B.H. and Hood, L.E. (1986) Fluorescence detection in automated DNA sequence analysis. Nature 321, 673–681.

Soergal, M.E., Smith, A.W. and Schaffer, F.L. (1975) Biophysical comparisons of calicivirus serotypes isolated from pinnipeds. Intervirology 5, 239–244.

Soergel, M.E., Akers, T.G., Schaffer, F.L. and Noma, A.T. (1976) Amino acid composition of three immunological types of a calicivirus, San Miguel sea lion virus. Virology 72, 527–529.

Tabor, S. and Richardson, C.C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 84, 4767–4771.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.