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NOTES

Genetic Relatedness of the Caliciviruses: San Miguel Sea Lion and Vesicular Exanthema of Swine Viruses Constitute a Single Genotype within the *Caliciviridae*

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The San Miguel sea lion viruses (SMSV) and vesicular exanthema of swine viruses (VESV) are related morphologically and antigenically, but little has been done to determine their genotypic relationship to each other and to other caliciviruses. To examine this relationship, reverse transcriptase PCRs were performed by using oligonucleotide primer sets designed to amplify portions of the 2C RNA helicase-like and RNA-dependent RNA polymerase regions with total cellular RNA purified from virus-infected cell cultures as a template. The 2C RNA helicase primers directed the amplification of this region from eight SMSV serotypes, five VESV serotypes, and four related viruses. The RNA polymerase primer sets amplified products from all these viruses except one. Phylogenetic comparison of the caliciviruses demonstrated that SMSV, VESV, and four related viruses are closely related while being distinct from feline calicivirus, the human caliciviruses (small, round-structured viruses), and rabbit hemorrhagic disease virus and that they should be classified as a single genotype within the *Caliciviridae*.

Members of the *Caliciviridae* are small, nonenveloped viruses composed of a single capsid protein. They contain a single, plus-sense, polyadenylated RNA genome of 7.5 to 8 kb in length (3, 8, 11). The nonstructural polypeptides are encoded as a polyprotein in the 5′ end of the genomic RNA, while the single structural protein is encoded in the 3′ end (3, 8, 11, 15, 17). The identities of the calicivirus nonstructural polypeptides (2C [RNA helicase], 3C [cysteine protease], and 3D [RNA-dependent RNA polymerase] proteins [3, 8, 11, 14]) have been suggested by similarity to functional or highly conserved amino acid motifs in the nonstructural proteins of the picornavirus superfamily.

Two groups within the *Caliciviridae*, i.e., the prototype calicivirus, vesicular exanthema of swine virus (VESV), and San Miguel sea lion virus (SMSV) can cause vesicular disease in susceptible mammals. The disease is characterized by fever and oral, as well as extremity, vesicle formation. Infection seldom results in death; however, it is often associated with reproductive failure (24). SMSV was first isolated from aborting California sea lions in 1972 by Smith et al. (22), who described a virus that had striking similarities to VESV but was not neutralized by any VESV antisera. Since that time, at least 16 additional serotypes have been isolated from a number of marine mammals (24). When introduced into swine, a number of SMSV isolates cause a vesicular disease essentially identical to that caused by VESV (22, 25, 26).

SMSV and VESV, while serologically distinct, are virtually indistinguishable in other respects, including disease symptoms and virus morphology (22, 24). Although the virus serotypes are distinct in that they are neutralized by homologous antisera only (5, 23), several immunological tests have demonstrated that these viruses are antigenically related (29, 30). Viruses morphologically and physicochemically related to VESV and SMSV have been isolated from other mammalian hosts. Primate calicivirus (PCV; *Pan*-1) was isolated from a pygmy chimpanzee displaying an oral vesicle (27). The virus was reisolated from the same animal six months later, indicating a possible persistent infection. Bovine calicivirus (BCV) (Tillamook virus; *BCV*-Bos 1) was isolated from calves from a dairy herd with respiratory tract problems (23). When inoculated into swine, this virus causes vesicular disease similar to that caused by VESV. Cetacean calicivirus (CCV; Tur-1) was isolated from a dolphin with vesicular skin lesions (28) and was shown to also infect California sea lions. Skunk calicivirus (SCV) was recently isolated from captive skunks exported to Canada from the north central United States (20). The skunks were used for testing of an oral rabies vaccine, and the SCV was found in the feces during routine virus isolation. No clinical symptoms were associated with this virus. Passage in pigs caused no clinical symptoms but did result in seroconversion (20).

Information concerning the relatedness of caliciviruses has become available recently for several diverse members of the family. Feline calicivirus (FCV) strains have been shown to be very closely related to other FCV strains, with major differences existing primarily within one or two regions of the capsid

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proteins (3, 17, 19, 21). Within the larger of the two divergent regions is found a run of 24 highly conserved amino acids. High-level conservation in this region may be the reason that there is only a single serotype of FCV (19). One neutralizing epitope has been determined to be present on the N-terminal border of the large divergent region (12). Rabbit hemorrhagic disease virus (RHDV) has been sequenced in its entirety (11) and has been shown to be closely related to another virus, European brown hare syndrome virus (32), but these viruses show only limited sequence similarities to other caliciviruses. The complete genomic sequences of Norwalk and Southampton viruses have been determined, as have selected regions amplified by PCR from a number of related small round-structured viruses (2, 4, 6–10, 31). Comparison of capsid protein and RNA-dependent RNA polymerase coding regions have demonstrated that, among human caliciviruses, two groups can be segregated on the basis of sequence homologies. These two groups, the Norwalk virus (NV) genogroup and the Snow Mountain agent genogroup, were only roughly 63% similar at the amino acid level within the RNA-dependent RNA polymerase polypeptide (31). The NV and Snow Mountain agent groups are antigenically distinct (4, 7, 31).

A recent report (18) described the use of reverse transcription (RT) coupled with PCR to amplify portions of the 2C-helicase-like and RNA-dependent RNA polymerase regions of the SMSV and VESV genomic RNAs. These two regions were chosen on the basis of conservation of sequences and to analyze sequences compared in previous studies of the human caliciviruses (2, 4, 6–10, 31). The primers for these regions directed the amplification of products from the RNA helicase regions of eight SMSV and five VESV serotypes as well as BCV and SCV and from the RNA polymerase sequences of 11 of these viruses. Products were not obtained when mink calicivirus or FCV RNA was used as a negative control. Previous reports (1, 19) have demonstrated the utility of PCR for obtaining DNA fragments from RNA viruses for sequence analysis to determine their genetic relationship. We report here work to resolve the relationship of the SMSV and VESV groups at the genetic level and to correlate this relationship to other members of the *Caliciviridae* for which nucleotide sequences are available.

SMSV serotypes 1, 2, 4, 5, 6, 7, 13, and 14 and Tillamook calicivirus (BCV) were a kind gift of J. Pearson (Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, Iowa). VESV isolates 1934B, A_{48}, C_{52}, E_{54}, and I_{55}; PCV; and CCV were part of the calicivirus collection at the Foreign Animal Disease Diagnostic Laboratory (Animal and Plant Health Inspection Service, U.S. Department of Agriculture), Greenport, N.Y., and were a kind gift of James House. SCV was recently isolated from captive skunks exported to Canada from the north central United States (20). All viruses were propagated in Vero cells, and total cellular RNA was prepared from infected cells as previously described (18).

Single-tube RT-PCRs were performed with total cellular RNA from calicivirus-infected cells (18). The sequences of the primer sets were as follows: Hel1, 5′-GTCCCAGTATTCGG ATTTGTCTGCC; Hel2, 5′-AGCGGGTAGTTCAGTCAAG TTCACC; Pol1, 5′-GCCTTCTGGTATGCCACTAACATCC; Pol2, 5′-GACGAGCGGTATGATCTTGTTGGTG. A third primer (antisense) for the RNA polymerase region, used instead of Pol2, was needed to obtain products from all viruses. The sequence of this primer (Pol3) was 5′-GGTGGAACGG TCCAATTTTCAGTG. PCR products were cloned with the TA cloning system according to the manufacturer's directions (Invitrogen, San Diego, Calif.). Individual clones were sequenced by using the fmol DNA Sequencing kit (Promega, Madison, Wis.), and all reaction products were sequenced directly from the PCR fragment (both strands) by using the PRISM DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) or the Direct PCR Sequencing kit (U.S. Biochemical, Inc., Cleveland, Ohio). Computer analysis of sequence similarities was completed with GeneWorks software (Intelligenetics, Inc., Mountain View, Calif.) by the unweighted pair group method with arithmetic mean (13).

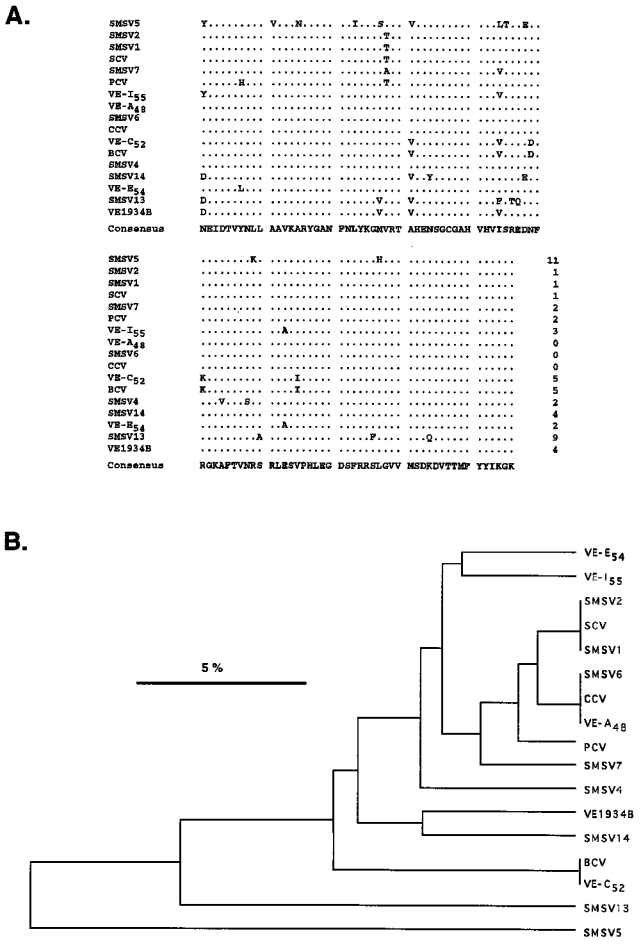

FIG. 1. Alignment and predicted phylogenetic analysis of a portion of the 2C-helicase-like regions of 17 animal caliciviruses. (A) Alignment analysis of the amino acid sequences deduced from the DNA sequences of the PCR products from the indicated viruses. The derived consensus sequence is illustrated at the bottom of the alignment. The numbers at the end of the alignment represent the numbers of amino acid residues that differ from the consensus sequence. (B) Phylogenetic tree, generated by the unweighted pair group method with arithmetic mean (13), of the amino acid sequences derived for the 17 caliciviruses. The horizontal bar represents a distance of 5% divergence in amino acid sequence.

Amplification, sequencing, and amino acid alignment analysis of the 2C-like region. Primer sets which amplify portions of the 2C-like and RNA-dependent RNA polymerase-encoding sequences of SMSV and VESV genomes have been described (18). These primers were shown to be specific for SMSV and VESV and did not direct amplification of a product from FCV or mink calicivirus (18). They have not been tested with RHDV or NV. The 2C primer set used in this study successfully amplified an approximately 350-bp DNA fragment from the 17 viruses tested. All reaction products were equal in size

as determined by agarose gel electrophoresis and showed no obvious differences. These fragments were either cloned for sequence analysis or sequenced directly from the PCR product. All 17 fragments were found to be 357 bp in length. Each contained a single open reading frame which encoded a polypeptide with an amino acid sequence similar to that of the FCV 2C region (14). The derived amino acid sequences were aligned by using GeneWorks software (Fig. 1A). The amino acid residues encoded by the sequences to which the primers annealed during amplification were omitted from analysis to eliminate introduced bias from the PCR primer sequences. All 17 viruses are closely related. Alignment of the 2C RNA helicase amino acid sequences reveals an overall identity of 74%. Individually aligned, the two most divergent viruses are SMSV 5 and SMSV 13, which had an identity of 84% at the amino acid level. Three groups of viruses show 100% identity in this portion of the 2C-like region. These are SMSV 1 with SMSV 2 and SCV, SMSV 6 with VESV A_{48} and CCV, and BCV with VESV C_{52} (Fig. 1B). The remainder of the viruses have amino acid similarities interspersed between 84 and 100%. The numbers following the alignment in Fig. 1A are the numbers of changes from the consensus sequence. The SMSV 6-CCV-VESV A_{48} group shows no deviation from this consensus. The dendrogram in Fig. 1B illustrates definite relationships among certain viruses. However, there is no clear delineation between any virus or group of viruses indicating a division into subgroups.

Amplification, sequencing, and amino acid alignment analysis of the RNA-dependent RNA polymerase region. Use of the RNA-dependent RNA polymerase primer set (primers Pol1 and Pol2) resulted in amplification of fragments from most, but not all, viruses. Amplification was unsuccessful with SMSV 6 and 7, VESV C_{52} and I_{55}, CCV, and PCV. A new primer, Pol3, was designed on the basis of the sequences obtained from the amplifiable viruses. Pol3, when used with Pol1, yielded a DNA product of 419 bp, and it was used successfully to obtain DNA fragments from all of the remaining viruses except CCV. The PCR products obtained with Pol1 and Pol2 were uniform in size (520 bp) following agarose gel electrophoresis with the exception of the product derived from SMSV 14 (18). The SMSV 14 product was smaller than the others by 60 to 80 bp as judged by the difference in relative mobility following agarose gel electrophoresis (data not shown). Following cloning and sequencing, it was found that this fragment contained a deletion of 82 bp near its 3′ end. Despite this, the deletion left the reading frame intact. Sequence analysis of the SMSV 13 product showed that it contained an insertion or duplication at the 3′ end of the amplified region. These extra sequences, approximately 23 bp in length, apparently resulted in the duplication of the primer annealing sequences, resulting in a nucleotide sequence that fortuitously allowed amplification. The exact nature of the insertion or duplication is unknown. It will require cloning of the region or direct sequencing of the genomic RNA to determine exactly what changes occurred. These results indicate that there is some tolerance of change in the amino acid sequences at the C-terminal portion of the RNA polymerase without a resulting loss of apparent ability of the virus to replicate. This ability to tolerate change in this region may explain the failure to generate a product from CCV and the inability to obtain products from six viruses with the Pol1 and Pol2 primer set.

Alignment analysis of the RNA polymerase sequences also showed that these viruses are closely related. Because of the changes found in the 3′ ends of the SMSV 13 and 14 fragments, the corresponding regions of all fragments were left out of the alignment analysis of all viruses. The region examined

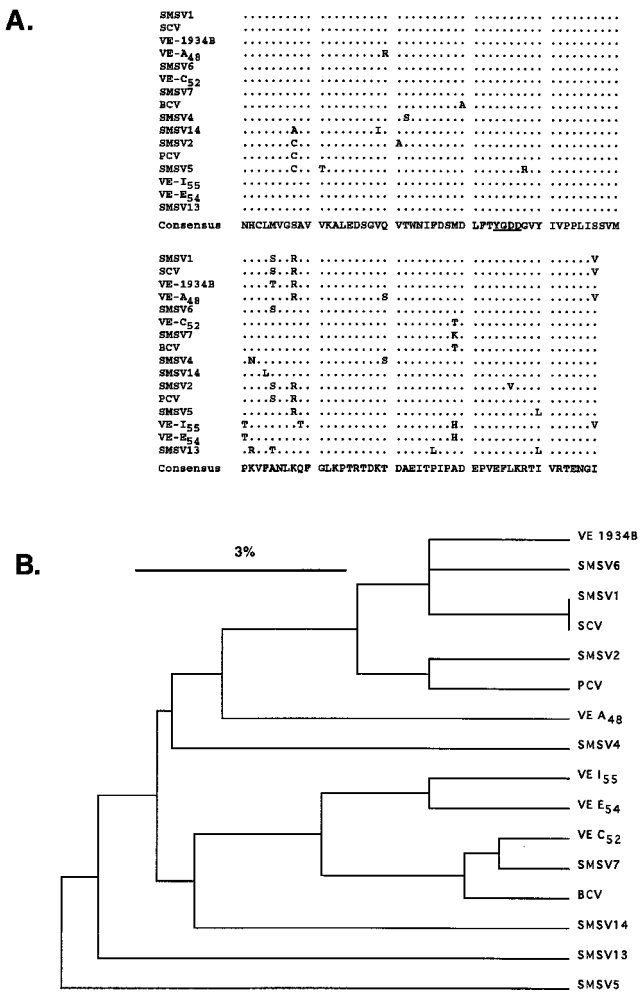

FIG. 2. Alignment and predicted phylogenetic analysis of a portion of the RNA-dependent RNA polymerase regions of 16 animal caliciviruses. (A) Alignment analysis of the amino acid sequences derived from the DNA sequences of the PCR products from the indicated viruses. The derived consensus sequence is illustrated at the bottom of the alignment. The conserved YGDD motif is underlined. (B) Phylogenetic tree for the 16 viruses, constructed from the derived amino acid sequences, using the unweighted pair group method with arithmetic mean (13). The horizontal bar represents a distance of 3% divergence in amino acid sequence.

was reduced to the amino acids encoded by the 5′ 291 bp of all amplified fragments (Fig. 2). As with the 2C helicase region, the amino acids which were encoded by the sequences to which the primers annealed were omitted from this analysis. The alignment of these sequences is illustrated in Fig. 2A. The RNA-dependent RNA polymerase region shows greater conservation than the 2C helicase sequence, with an overall sequence identity of 79%. The two most divergent viruses are SMSV 5 and VESV I_{55}, with a sequence identity of 91%. The region showing the greatest number of changes is the 10 to 20 amino acid residues following the YGDD motif which is present in all viruses. The only viruses in this analysis showing identical sequences were SMSV 1 and SCV. There were no sequences that were identical to the alignment consensus sequence. Figure 2B illustrates the dendrogram generated from alignment of the RNA polymerase amino acid sequences. Overall, the same relationships that were found with the 2C helicase region were still evident. The BCV-VESV C_{52} group-

ing is present, although there is one residue change and it now includes SMSV 7. The SMSV 1-SCV grouping is present, with SMSV 2 having a single residue change. Also included in this branch are SMSV 6 and VESV 1934B. VESV A_{48} is alone in this analysis, its relationship to CCV unknown because of the inability to amplify a product from this virus. It is also not closely related to SMSV 6 in this region. SMSV 5 and 13 are again located on the outside of the phylogenetic tree. There appears to be a slight division into groups in this analysis. However, on the basis of the high degree of conservation, even between the two most divergent viruses, there is not enough difference in amino acid sequences to suggest that these viruses constitute more than a single genotype.

The primer set used for amplification of the 2C helicase region was the most dependable in obtaining product from these viruses. By using this set, amplification products were obtained from all 17 viruses examined in this study. The 2C helicase sequences had a high degree of conservation among these viruses, with no major changes being noted. The region amplified did not contain the nucleotide binding motif (GXGXXGK/T [14]), but yet it showed a high degree of similarity (84%) between the most divergent viruses. A consensus sequence was formed from the alignment of these sequences (Fig. 1). The most divergent virus, SMSV 5, deviated from the consensus sequence at 11 of 97 residues. The majority of the changes from the consensus sequence appear to be spontaneous and do not show any relationship to those of other viruses. One possible exception to this was observed at position 41 of Fig. 1A, where 11 of the viruses have an alanine residue and the remaining 6 have a valine. A second is seen in Fig. 2A at position 57, where 7 viruses encode an arginine and the remaining 10 have lysines. These functionally conservative changes are probably results of selection for a functional protein rather than an indication of lineage. Analysis of these sequences also provides no strong indication of specific lineage in these viruses as indicated by the chronological order of virus isolation. The oldest calicivirus in this analysis, VESV 1934B, which was isolated in 1934, shows similarities to some of the most recent calicivirus isolates, such as SMSV 14 (Fig. 1B) and SMSV 6 (Fig. 2B). The viruses isolated from nonmarine mammals (BCV, PCV, and SCV) had a high degree of homology with the SMSV and VESV isolates, demonstrating that they too belong to the same genotype. SMSV 1 and SCV are identical in sequence in both regions at both the amino acid and nucleic acid levels. Additional analysis of sequences within the capsid region also shows complete identity at the nucleotide level (16). It is possible that SCV is SMSV 1 that was introduced into the skunk population, but preliminary serological evidence indicates a significant serum neutralization difference between the two viruses (16).

Comparison of the two dendrograms presented in Fig. 1A and 2A shows that several close relationships among specific viruses are present in both regions analyzed (e.g., for BCV and VESV C_{52}). These relationships were supported by a recent publication by Ferris and Oxtoby (5). They described the relationships between SMSV, VESV, and related viruses observed by performing an enzyme-linked immunosorbant assay. As expected, most of these viruses were reactive only to type-specific antisera. However, cross-reactivity between BCV and VESV C52 (Fig. 1B and 2B) and between SMSV 6 and CCV (Fig. 1B) was observed. Sequencing of the capsid protein genes of these viruses will be needed to examine the relationships in greater detail.

Phylogenetic comparison of caliciviruses. Evidence that the SMSV-VESV group of viruses belong to a single, unique genotype, based on the data presented here, is presented in Fig. 3.

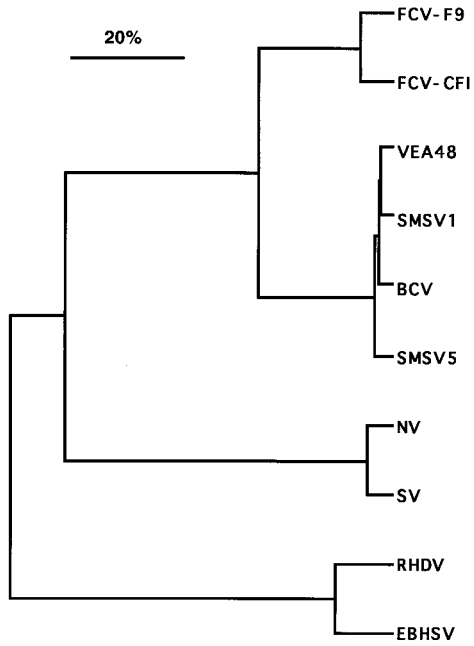

FIG. 3. Phylogenetic analysis of the RNA-dependent RNA polymerase regions of four members of SMSV-VESV and related virus group with the same regions from two members of the FCV genotype, two members of the human calicivirus genotype (NV and other small round-structured viruses), and two members of the RHDV genotype. The horizontal bar represents a distance of 20% amino acid change. The two human viruses shown (NV and Southampton virus [SV]) are the only viruses for which equivalent sequences are available. RHDV and European brown hare syndrome virus (EBHSV) are the only members of this genotype with sequences available.

A phylogenetic comparison of the amino acid sequences of the RNA-dependent RNA polymerase regions of FCV (3, 14), human caliciviruses (NV [8] and Southampton virus [9], for which equivalent sequences were available), RHDV (11) and European brown hare syndrome virus (32), and the SMSV-VESV group (only four representative sequences were used in this analysis) was made. These sequences fall into four distinct groups. Each group is separated by at least 25% amino acid sequence divergence, showing that there is no close relationship except within a group. Interestingly, the FCV and SMSV-VESV groups show the greatest sequence similarity and represent the caliciviruses which can be propagated in vitro. SMSV and VESV show greater similarity within group than the members of the other three groups, and yet each member is serologically distinct in that it is neutralized only by homologous antisera (5, 23). It is thought that this specificity is mediated by one or possibly two small regions of the capsid protein (12, 19). The capsid protein sequences in these viruses which have been analyzed (N termini of the capsid precursor proteins) show sequence homologies similar to those of the two regions discussed above (15, 16). Analysis of the regions which may contain the serotype determinants is under way. The results presented here indicate that SMSV, VESV, and the four related viruses should be classified in a single genotype within the *Caliciviridae*.

Nucleotide sequence accession numbers. All DNA sequences have been deposited with the GenBank database. The nucleotide sequences of the 2C reaction products have the accession numbers U15301 (SMSV 1), U15302 (SMSV 4), and U18470 through U18484 (BCV; CCV; PCV; SCV; SMSV 13, 14, 2, 5, 6, and 7; and VESV 1934B, A_{48}, C_{52}, E_{54}, and I_{55},

respectively). The nucleotide sequences of the RNA-dependent RNA polymerase amplification products have the numbers U15301 (SMSV 1), U15302 (SMSV 4), and U18730 through U18743 (SMSV 2, 5, 6, 7, 13, and 14; VESV 1934B, A_{48}, C_{52}, E_{54}, and I_{55}; BCV; PCV; and SCV, respectively).

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