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Short Report: Whole Genome Analysis of Sierra Nevada Virus, a Novel Mononegavirus in the Family *Nyamiviridae*

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Abstract. A novel mononegavirus was isolated in 1975 from ticks (*Ornithodoros coriaceus*) collected during investigation of an outbreak of epizootic bovine abortion (EBA) in northern California. It was originally designated "bovine abortion-tick virus" (BA-T virus). The EBA is now known to be associated with a deltaproteobacterium infection, and not a virus. The BA-T virus had remained uncharacterized until now. We have determined by electron microscopy, serology, and genome sequencing that the BA-T virus is a fourth member of the newly proposed family *Nyamiviridae*, and we have renamed it Sierra Nevada virus (SNVV). Although antigenically distinct, phylogenetically SNVV is basal to Nyamanini virus (NYMV) and Midway virus (MIDWV), two other tick-borne agents. Although NYMV was found to infect land birds, and MIDWV seabirds, it is presently unknown whether SNVV naturally infects birds or mammals.

Members in the order *Mononegavirales* are non-segmented, negative-strand RNA viruses encoding 5–10 open-reading frames (ORF).[1] Ebola, rabies, and measles are just a few members of this order, and causative agents of serious human infectious diseases. In addition to the four well-established families—*Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*—the order *Mononegavirales* also includes *Nyamiviridae,* a novel and recently proposed family.[2] *Nyamiviridae* presently has only three members: Nyamanini virus (NYMV), Midway virus (MIDWV), and soybean cyst nematode virus 1 (SbCNV); the first two comprise a novel genus, *Nyavirus*.[3]

Here, we report the characterization of a novel virus, originally designated as bovine abortion-tick (BA-T) virus, that was isolated in 1975 from ticks (*Ornithodoros coriaceus*) collected during investigation of an outbreak of epizootic bovine abortion (EBA) in northern California. The EBA is an infectious disease associated with abortion and/or premature birth in cattle grazing foothills and montane pastures in the Sierra Nevada mountain range of California, Nevada, and Oregon.[4] Subsequent experimental studies in heifers have indicated that BA-T virus was not the causative agent of EBA.[5,6] The disease has now been associated with a deltaproteobacterium transmitted by the same tick species,[7,8] but the BA-T virus has remained uncharacterized. Because the original designation, bovine abortion-tick (BA-T) virus, is misleading, we propose instead the name "Sierra Nevada virus" (SNVV) for the new virus.

The SNVV was originally isolated at the University of California/Davis in Vero cell cultures inoculated with a homogenate of *O. coriaceus* ticks collected in northern California (exact site unknown). The isolate was initially sent to the Rocky Mountain Laboratory in Hamilton, MT and was subsequently sent to the University of Texas Medical Branch (UTMB) for further study. Initial studies at UTMB indicated that the virus produced a viral cytopathic effect in both Vero and BHK cells within 48 hours after inoculation. Likewise, intracranial inoculation of newborn mice with SNVV produced illness and death within 2–3 days. Animal use was done under protocol no. 9505045, approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Medical Branch.

In a cross-box complement fixation assay comparing NYMV and MIDWV mouse brain antigens and a SNVV Vero cell antigen with specific hyperimmune mouse ascitic fluids (immune sera) prepared to the three viruses, NYMV and MIDWV were distinct but showed a close antigenic relationship (Table 1). In contrast, SNVV immune serum only reacted with the homologous antigen, indicating that it is antigenically distinct (Table 1). In ultrathin sections of infected Vero cells, transmission electron microscopy showed virion formation at the cell surface (Figure 1). Virions of variable sizes from 200 to 400 nm were observed budding from the host cell plasmalemma. Although the morphology of SNVV is similar to NYMV and MIDWV, the virion size of the former is about twice the size of the latter two viruses.[3]

Whole-genome sequencing and phylogenetic analysis were carried out to further characterize SNVV. Virus genomic RNA was isolated by processing cell supernatants through a 0.45 μM filter to remove eukaryotic cells, enrichment for intact virions with a Vivaspin 20 column (Sartorius, Goettingen, Germany) followed by treatment with 2 U Turbo DNase (Ambion, Austin, TX), 1 U Benzonase (Novagen, Madison, WI), and 2 U Rnase One (Promega, Madison, WI) for 1 hour at 37°C. Viruses were then pelleted through a 10%/15%/20% sucrose gradient by spinning at 39,000 rpm for 1.5 hours. Viral pellets were resuspended in nuclease free water and the presence of virus was confirmed by electron microscopy. Pelleted virus was lysed and genomic material purified using Trizol (Invitrogen, Carlsbad, CA) or the Viral RNA Mini Kit (Qiagen, Valencia, CA). Extracted Viral RNAs were reverse transcribed and barcoded using the SISPA methodology.[9] This virus was sequenced as part of a multiplex of other samples. A multiplex sequencing pool was built using equimolar amounts of each barcoded DNA; this pool was purified with a MinElute PCR kit (Qiagen, Valencia, CA). The virus pool was sequenced on the HiSeq2000 (Illumina, San Diego, CA) with 100 basepair (bp) paired-ends.

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TABLE 1
Results of complement fixation tests comparing Nyamanini, Midway, and Sierra Nevada viruses

Antigen	Complement Fixation Test			
	Immune serum			
	Nyamanini (Tick-39)	Midway	Sierra Nevada	Control
Nyamanini	**512*/128**	32/32	0	0
Midway	128/512	**1024/≥ 512**	0	0
Sierra Nevada	0	0	**64/≥ 2**	0
Control	0	0	0	0

*Reciprocal of the highest antibody titer/reciprocal of highest antigen titer. 0 = < 8.

Demultiplexed reads were quality trimmed using the trim.pl script (http://wiki.bioinformatics.ucdavis.edu/index.php/Trim.pl), and de-novo assembly was performed using Velvet[10] with a kmer of 51, and an expected coverage of 300. Contigs were ordered by ABACAS.pl[11] and two gaps in the assembly were closed by polymerase chain reaction (PCR) and Sanger sequencing. To validate the assembly, reads were mapped back to the assembled genome using SMALT (http://www.sanger.ac.uk/resources/software/smalt/). A coverage plot was generated in R using a pileup file of the mapped reads (Figure 2). The SNVV genome was deposited in GenBank, under accession no. KF530058.

The 11,840 nt SNVV segment sequence may still be missing close to 190 nucleotides at its 3′ end, based on the alignment to the NYMV genome. It nevertheless potentially encodes six protein-coding genes: ORFI (353 aa), ORFII (274 aa), ORFIII (399 aa), ORFIV (145 aa), ORFV (599 aa), and ORFVI (1918 aa). Genomic organization of SNVV is highly syntenic to that of NYMV and MIDWV (Figure 3). A BLASTN analysis indicates that the SNVV genome has a 73% identity with NYMV and MIDWV. Because of the high similarity to NYMV and MIDWV, we have named the six ORFs according to the nomenclature for NYMV and MIDWV: ORFI, nucleocapsid protein (N); ORFII, X protein; ORFIII, phosphoprotein (P); ORFIV, matrix protein (M); ORFV, glycoprotein (G); ORFVI, RNA-dependent RNA polymerase (RdRP).[2]

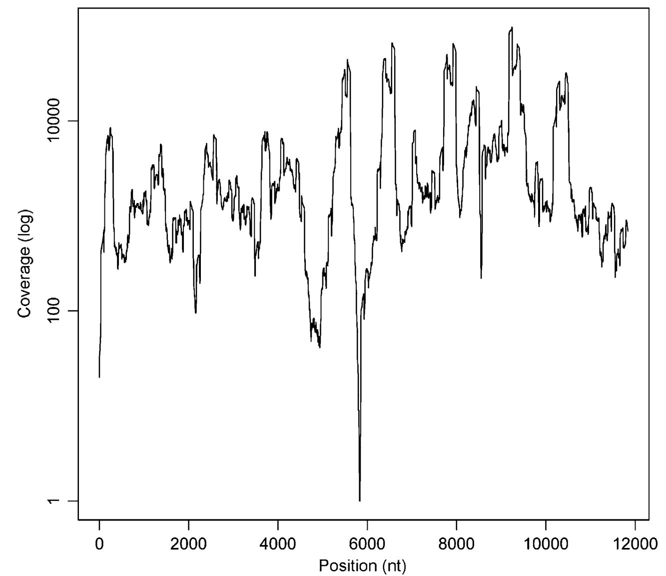

FIGURE 2. Coverage plot in log scale showing the number of mapped reads across the genomic assembly. Low coverage regions were polymerase chain reaction (PCR) amplified and sequenced by Sanger.

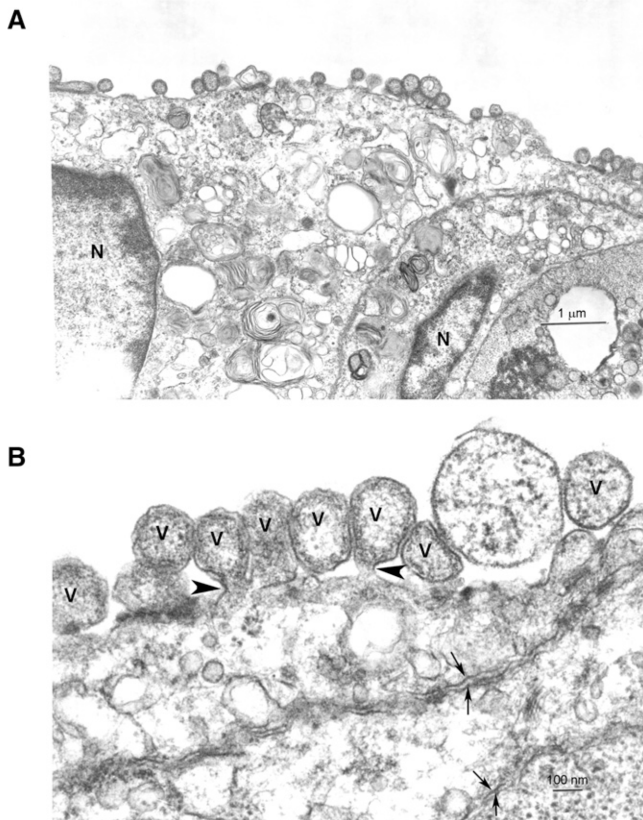

FIGURE 1. Transmission electron microscopy of Vero cells infected with Sierra Nevada (BA-T) virus. (**A**) Virions forming at the cell surface. N = nuclei of adjacent cells. Bar = 1 µm. (**B**) High power of a portion of an infected Vero cell with budding (thick arrowheads) virions (v). Thin arrows indicate cell membranes of adjacent cells. Bar = 100 nm.

To place SNVV in a phylogenetic context, BLASTX queries were performed using the RdRP gene against the GenBank non-redundant (nr) database. The first 1,000 protein matches were downloaded from GenBank, and the type species of each genus in the order *Mononegavirales* was retained (see Supplemental Table 1 for accessions). These protein sequences were aligned with those of SNVV, NYMV, MIDWV, and SbCNV using MUSCLE[12] and the resulting multiple sequence alignment was inspected and manually adjusted in Seaview Version 4.4.0.[13] Character sets for phylogenetic analysis were selected using Gblocks[14] and a maximum likelihood phylogenetic tree was generated with RAxML-HPC, using the GTR substitution model, rates across sites modeled on a gamma distribution and 1,000 bootstrap replicates. Our analysis places SNVV within the *Nyamiviridae* family, and indicates it is basal to the Nyavirus genus containing NYMV and MIDWV (Figure 4A).

Using MEME, a motif-based sequence analysis tool,[16] we identified conserved transcription termination motifs (3′-AG(G/A)AA(U/A)GUUUUU-5′) downstream of every SNVV virus ORF (Table 2). Similar motifs have also been reported for other viruses in the family *Nyamiviridae*, indicating further conservation across this family.[3,17] A study of the paramyxovirus simian virus 5 (SV5), also a mononegavirus, suggests that the poly-U tract in every termination motif may function as a template for polyadenylation, and a genome spacer separating

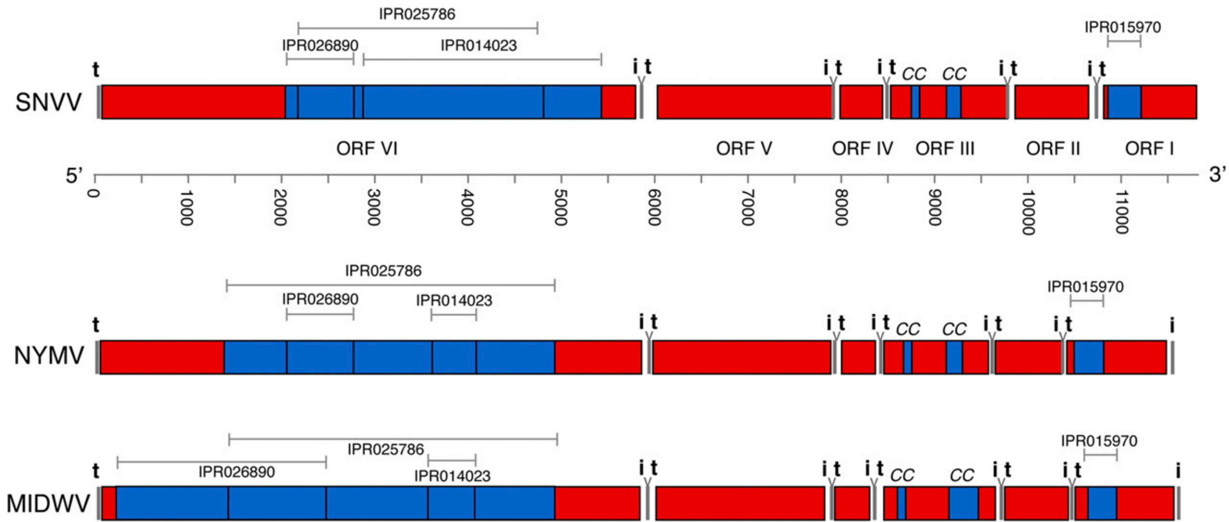

FIGURE 3. Schematic of the annotated Sierra Nevada virus (SNVV) genome, in comparison to Nyamanini virus (NYMV), and Midway virus (MIDWV). Coordinates of interpro domains are annotated on each genome. i = site of transcription initiation motif; t = site of transcription termination motif; *CC* = putative coiled-coil domains (see Figure 4C).

each gene end from the next gene start.[18] It still remains to be determined whether the same function applies for the transcription termination motifs seen in the *Nyamiviridae*.

Conserved transcription initiation motifs 3′-CGUUGGNN GG(G/U)-5′ were also found upstream of all the SNVV ORFs (Table 2). Compared with the initiation motifs found in NYMV, MIDWV, and SbCNV,[3,17] SNVV shows high similarity with NYMV and MIDWV, but not with SbCNV. Despite that difference, all four viruses share a common characteristic where the initiation motif is located directly after the termination motif for the upstream gene, which is different from the paramyxovirus SV5 where there is always a 1 to 22 nucleic acid long spacer between the termination motif and the initiation motif for the following gene.[18]

To further characterize the function of the six ORFs encoded by SNVV, we have performed protein domain searches against each of these using the InterProScan webserver.[19] As expected, we find protein domains associated with RNA polymerases (IPR014023, IPR025786, IPR026890) for ORFVI (RdRP), and a Borna disease virus P40 nucleoprotein subdomain 2 (IPR015970) for ORFI (nucleoprotein) (Figure 3). Using COILS,[15] we found two putative coiled-coil domains in the P protein (ORF III) of SNVV. Similar domains were also predicted for the P proteins of NYMV and MIDWV, but not SbCNV. Interestingly, the specific location of this domain is approximately the same in SNVV, NYMW, and MIDWV. As shown in (Figure 4B), two peaks in the probability curves are identified, indicating the existence of two putative coiled-coil

TABLE 2

Conserved transcription initiation and termination motifs upstream and downstream of every SNVV ORF, and comparison of motifs across NYMV, MIDWV, and SbCNV[3,6]

	Initiation motif	Termination motif
ORFI	[missing end]	3′-AGAAAAGUUUUU-5′
ORFII	3′-CGUUGGUUGGU-5′	3′-AGGAAUGUUUUU-5′
ORFIII	3′-CGUUGGCAGGG-5′	3′-AGGAAAGUUUUU-5′
ORFIV	3′-GGUUGGUCGUG-5′	3′-AGAAAAGUUUUU-5′
ORFV	3′-CGUUGGGAGGU-5′	3′-AGGAAUGUUUUU-5′
ORFVI	3′-AGUUGGAGGGG-5′	3′-AGAAAUCUUUUU-5′
SNVV Motif		
NYMV Motif		
MIDWV Motif		
SbCNV Motif		

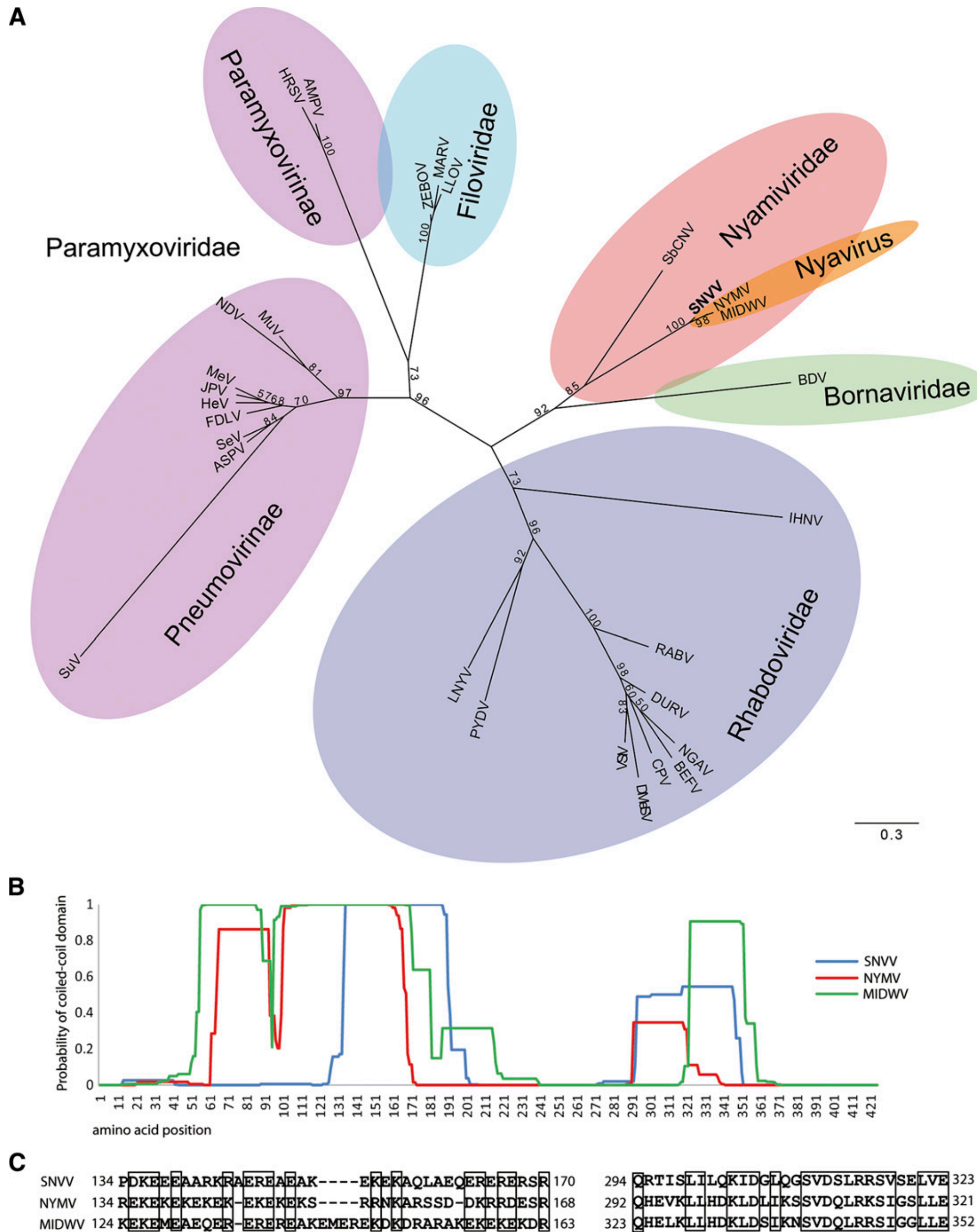

FIGURE 4. (**A**) Phylogenetic tree of RdRP sequences of the order *Mononegavirales*. The order *Mononegavirales* comprises five families, within which the family *Paramyxoviridae* is split into two subfamilies: *Pneumovirinae* and *Paramyxovirinae*. The Sierra Nevada virus (SNVV) branches within a clade of *Nyamiviridae* and branches closely, but as sister to Nyamanini virus (NYWV) and Midway virus (MIDWV). In comparison, the soybean cyst nematode virus 1 (SbCNV) is a distant, deep branching member of the *Nyamiviridae*. The accession numbers and full names of all the viruses in the tree are listed in Supplemental Table 1. (**B**) Coiled-coil domain prediction for open-reading frame (ORF) III in SNVV, NYMV, and MIDWV. The coiled-coil domain prediction was done based on a 28-residue window using the program of COILS[15]; there are two peaks shown along the curves, and both of them partially overlap with each other across the three viruses, suggesting a similar pattern of the coiled-coil domain distribution in their genomes. (**C**) Sequence alignment for the putative coiled-coil regions of ORF III in SNVV, NYMV, and MIDWV. Alignment of the sequences of N- and C- terminal coiled-coil domain across SNVV, NYMV, and MIDWV shows that 39% of the residues are conserved in the N-terminal domains, whereas 63% of the residues are conserved in the C-terminal domains.

domains in this protein. A coiled-coil domain is a type of structural motif in the amphipathic α-helix, and is formed by heptad repeats denoted $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$;[15] "a" and "d" often refer to hydrophobic amino acids at the interface of two helices, whereas "e" and "g" are solvent and polar residues.[20] The putative coiled-coil domains found in the N- and C-termini of SNVV, NYMV, and MIDWV show different characteristics in sequence composition. As shown in the sequence alignment in (Figure 4C), the N-terminal coiled-coil domains are of low conservation across the three viruses, whereas the C-terminal domains are better conserved where the "a" and "d" amino acid sites are always hydrophobic residues (i.e., isoleucine,

leucine, or valine). Most members of the *Paramyxoviridae* family, such as the respiratory syncytial virus,[21] rinderpest virus,[22] Sendai virus, parainfluenza virus, measles virus, Newcastle disease virus, and mumps virus,[23] have been reported to contain a coiled-coil domain in the P protein. None of these proteins, however, contain two domains, as predicted for the nyaviruses. Without a functional assay we cannot determine whether these predicted domains are real.

In summary, based on its genomic structure and phylogeny SNVV appears to be closely related to NYMV and MIDWV, pointing toward a third variant of the Nyavirus genus. However, antigen reactivity and virion size indicate that it may represent a closely related but distinct group. Although NYMV and MIDWV have been isolated from bird species and their ticks,[3,24–27] it is unclear whether SNVV can also infect birds. Based on its phylogenetic location it is likely that birds are also natural vertebrate hosts. This, however, will require confirmation by surveillance efforts.

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Note: Supplemental table appears at www.ajtmh.org.

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