Phylogenetic Analysis of the Species Theilovirus: Emerging Murine and Human Pathogens

Zhiguo Liang, 1 A. S. Manoj Kumar, 3 Morris S. Jones, 4 Nick J. Knowles, 5 and Howard L. Lipton 1,2*

Departments of Neurology and Rehabilitation Medicine 1 and Microbiology-Immunology 2 University of Illinois at Chicago, Chicago, Illinois; Biotechnology Division, Reliance Industries, Mumbai, India 3; Clinical Investigations Facility, David Grant USAF Medical Center, 101 Bodin Circle, Travis Air Force Base, California 4; and Institute for Animal Health, Pirbright Laboratory, Pirbright, Woking, Surrey GU24 0NF, United Kingdom 5

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The Cardiovirus genus of the family Picornaviridae includes two distinct species, Encephalomyocarditis virus (EMCV) and Theilovirus. We now report the complete nucleotide sequences of three Theiler’s murine encephalomyelitis virus (TMEV) strains (TO Yale, TOB15, and Vie415HTR) and of Vilyuiisk human encephalomyelitis virus (VHEV). This information, together with the recently reported sequences of divergent theiloviruses (Theiler’s-like rat virus [TRV] and Saffold viruses 1 and 2 [SAVF-1 and SAVF-2]), enables an updated phylogenetic analysis as well as a reexamination of several gene products important in the pathogenesis of this emerging group of viruses. In the light of the known neuropathism of TMEV and the new human SAVF-1 and SAVF-2, the resulting data suggest the existence of theiloviruses that cause human central nervous system infections. Our phylogenetic analyses point to the classification of presently known theiloviruses into five types: TMEV, VHEV, TRV, SAVF-1, and SAVF-2.

Encephalomyocarditis virus (EMCV) and Theilovirus are two distinct species in the Cardiovirus genus of the family Picornaviridae (52). The EMCVs comprise a single serotype and have a wide host range (64), while the Theilovirus species, until recently, included two serotypes, Theiler’s murine encephalomyelitis virus (TMEV) and Vilyuiisk human encephalomyelitis virus (VHEV), which appear to have much narrower host ranges than EMCV. The nucleotides of the RNA genomes of EMCV and TMEV are ~58% identical, and the amino acids of their polyproteins are ~50% identical. The amino acids of the capsid regions of TMEV and EMCV show the highest level of identity (~62%), resulting in their cross-reactivity in serological tests measuring broad antigenicity (complement fixation [CF] and enzyme-linked immunosorbent assays) but not in virus neutralization (VN) assays.

TMEVs were originally isolated from mice (54) and later from rats (18). Serological studies indicate that the feral house mouse Mus musculus is the natural host for TMEV (10, 29). In the early 1930s, TMEVs were isolated from colony-bred mice that developed spontaneous paralysis (54, 55). Based on the flaccid paralysis observed, indicative of the involvement of anterior horn cells or motor neurons, and on the revealed pathological changes of degenerating anterior horn cells, accompanied by microglial proliferation, these viruses were originally referred to as mouse polioviruses (41). In contrast to the strict motor neuron-trophic nature of human poliovirus in mice (21) and humans, TMEVs target both the anterior (motor) and posterior (sensory) neurons in the gray matter of the spinal cord (2, 27, 50). However, TMEVs are enteric pathogens that cause primarily asymptomatic infections of the digestive tract in colony-bred (nonbarrier) mice, and the spread of the virus to the mouse central nervous system (CNS) is rare.

TMEV isolates are grouped according to high or low neurovirulence. Highly neurovirulent TMEVs produce a rapidly fatal encephalitis in mice after intracerebral inoculation, while the low-neurovirulence strains, also referred to as persistent or Theiler’s original (TO) viruses, produce a biphasic infection and disease process, consisting of early poliomyelitis and late demyelinating disease (26, 27). A salient feature of the pathogenesis is the persistence of TMEV in the mouse CNS (6, 27, 55). The low-neurovirulence TMEVs have been widely studied because infection in mice provides a relevant experimental animal model for multiple sclerosis (4, 19, 40), a chronic inflammatory, demyelinating disease of possible viral etiology.

More than 50 years ago, VHEV was isolated by the inoculation of mice with nasopharyngeal secretions, serum samples, feces, cerebrospinal fluid (CSF) specimens, and brain specimens from the Yakut-Evenk population, indigenous rural people in Siberia that had a chronic form of encephalitis (16, 49). Antigenic, biophysical, and molecular characterization showed that VHEV was most closely related to TMEV, despite divergence in their capsid protein sequences (28, 46). However, it remains to be proven whether VHEV causes Vilyuiisk encephalitis (48).

In 2003, a virus herein designated Theiler’s-like rat virus (TRV), a genetically divergent theilovirus, was isolated from sentinel rats housed with TMEV-seropositive rats in Japan and sequenced (39). This virus has not yet been associated with disease in rats but has raised the possibility of additional clades of undiscovered theiloviruses. In fact, two new theiloviruses have recently been described. The first, named Saffold virus (SAVF), was isolated in California in 1981 from a fecal sample from an 8-month-old infant with fever of undetermined origin (20). The second, described as a Saffold-like virus, was isolated...
from a nasopharyngeal sample collected from a 23-month-old child in Canada in 2006 (1). Our present analysis of the complete genome sequences of these two viruses, referred to herein as SAFV-1 and SAFV-2, respectively, indicates that they belong to the species Theilovirus but are distinct from TMEV, VHEV, and TRV. Theiloviruses similar in sequence to SAFV were identified in a study of flu-like respiratory infections of unknown cause in humans (Don Ganem, personal communication) and in stool samples from individuals with acute gastrointestinal disease of unknown cause (Morris Jones, unpublished data).

We now report the complete nucleotide sequences of three TMEV strains (TO Yale, TOB15, and Vie 415HTR) and VHEV which, in addition to the recently reported sequences of divergent theiloviruses, has allowed updated phylogenetic analyses and a reexamination of several gene products important in the pathogenesis of these viruses. The known neurotropism of TMEV, along with the emergence of these new isolates, predicts the existence of other human theiloviruses that infect the human CNS. We also propose the classification of presently known theiloviruses into five types: TMEV, VHEV, TRV, SAFV-1, and SAFV-2.

### Materials and Methods

**Cells and viruses.** BHK-21 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s minimum essential medium (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine, 7.5% tryptose phosphate, and 10% fetal bovine serum. Vie 415HTR and the V-1 strain of VHEV were from Jordi Casals (Yale University, New Haven, CT), TOB15 was from Ben Mandel (New York City Health Department), and TO Yale was from the ATCC (also see Table 2). For infection, virus was adsorbed for 45 min at 20°C to monolayer cultures, which were washed twice with phosphate-buffered saline containing 1 mM CaCl2 and 0.5 mM MgCl2 and incubated in complete medium containing 2% fetal bovine serum at 37°C until the observation of the initial signs of cytopathology, when total RNA was extracted.

**Reverse transcription-PCR and DNA sequencing.** Total RNA, isolated from infected BHK-21 cell monolayers by using TRizol (Invitrogen), was reverse transcribed (2 μg of RNA in a 20-μl reaction mixture) with AffinityScript reverse transcriptase (Stratagene, Garden Grove, CA) in the presence of specific primers (10 μM). Two microliters of each cDNA sample was PCR amplified in a 50-μl reaction mixture by using specific forward and reverse DNA primers to obtain the nucleotide sequence. Gel-purified PCR products were sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Shelton, CT). Table 1 lists the primers used for sequencing.

**Phylogenetic analyses.** Sequences were aligned using BioEdit 7.0.5.3 (17) and Clustal W (57). Distance matrices were produced using the Kimura two-parameter method (24) for nucleotide sequences and the Poisson correction method (36) for amino acid sequences as implemented in MEGA 4.0 (53). These matrices were used to construct phylogenetic (neighbor-joining) trees with MEGA 4.0. Bootstrap resampling (1,000 pseudoreplicates) was used to assess branching confidence. Genome similarity plots were generated from aligned sequences using SimPlot version 3.5.1 (30).

**Nucleotide sequence accession numbers.** The complete nucleotide sequences of TMEV strains TO Yale, TOB15, and Vie 415HTR and VHEV determined in this study have been deposited in GenBank under accession numbers EU723238, EU718732, EU718733, and EU723237, respectively.

## RESULTS

### Phylogenetic analysis of complete genomes

The complete genomes of three previously sequenced TMEVs (38, 42, 43), three new TMEV sequences, and the sequences of four divergent theiloviruses, TRV (39), SAFV-1 (20), SAFV-2 (1), and VHEV, now enable a more comprehensive phylogenetic analysis of the Theilovirus species viruses listed in Table 2. The three new TMEV sequences include those of the low-neurovirulence Vie 415HTR, TOB15, and TO Yale strains. Partial sequences primarily of the 5′ untranslated region (UTR) and P1 coding region of the TO Yale strain have been reported previously (35). A previously reported partial sequence of a TMEV isolated from suckling rats and designated MHG (45) turned out to be that of the TO Yale strain when a more

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Table 2. Origins of theiloviruses included in the phylogenetic analyses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Place of isolation</th>
<th>Year</th>
<th>Accession no.</th>
<th>Reference</th>
<th>Specimen(s) of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEV</td>
<td>TO4</td>
<td>Rockefeller Institute, NYC</td>
<td>1937</td>
<td>M80885, U33045</td>
<td>55</td>
<td>Mouse brain</td>
</tr>
<tr>
<td></td>
<td>FA</td>
<td>Rockefeller Institute, NYC</td>
<td>1937</td>
<td>M80883, U32924</td>
<td>56</td>
<td>Mouse brain</td>
</tr>
<tr>
<td></td>
<td>GDVII</td>
<td>Rockefeller Institute, NYC</td>
<td>1937</td>
<td>M20562, X56019</td>
<td>56</td>
<td>Mouse brain</td>
</tr>
<tr>
<td></td>
<td>TO Yale</td>
<td>Yale Arbovirus Lab, New Haven, CT</td>
<td>1943</td>
<td>M80880, U33047, EU723238</td>
<td>33</td>
<td>Mouse feces</td>
</tr>
<tr>
<td></td>
<td>DA</td>
<td>Harvard University, Boston, MA</td>
<td>1948</td>
<td>M20301</td>
<td>8</td>
<td>Mouse brain</td>
</tr>
<tr>
<td></td>
<td>TOB15</td>
<td>NYC Health Department</td>
<td>1953</td>
<td>EU718732</td>
<td>13</td>
<td>Mouse feces</td>
</tr>
<tr>
<td>VHEV</td>
<td>V-1</td>
<td>Yakutsk, Russia</td>
<td>1955</td>
<td>M80888, M94868, EU723237</td>
<td>48</td>
<td>Human brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMEV</td>
<td>BeAn 8386</td>
<td>Belem, Brazil</td>
<td>1957</td>
<td>M16020</td>
<td>47</td>
<td>Feral mouse specimen</td>
</tr>
<tr>
<td></td>
<td>Vie 415HTR</td>
<td>Yale Arbovirus Lab, New Haven, CT</td>
<td>1962</td>
<td>EU718733</td>
<td>5</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>Wistar Institute, Philadelphia, PA</td>
<td>1977</td>
<td>M80889, U33046</td>
<td>59</td>
<td>Specimens from newborn mice</td>
</tr>
<tr>
<td>TRV</td>
<td>NGS910</td>
<td>Nagasaki University, Nagasaki, Japan</td>
<td>1991</td>
<td>AB9090161</td>
<td>39</td>
<td>Rat feces</td>
</tr>
<tr>
<td>SAFV-2</td>
<td>California-81</td>
<td>San Diego, CA</td>
<td>1981</td>
<td>NC009448</td>
<td>20</td>
<td>Human feces</td>
</tr>
<tr>
<td>SAFV-2</td>
<td>Can112051-06</td>
<td>Quebec, Canada</td>
<td>2006</td>
<td>AM922293</td>
<td>1</td>
<td>Human NP aspirate</td>
</tr>
</tbody>
</table>

* a Mice had spontaneous paralysis.
* b Two other sequences of BeAn 8386-derived viruses are also available: those of M2 (accession no. AF030574) and S2 (accession no. DQ401688).
* c NP, nasopharyngeal.
* d NYC, New York City.

Extensive sequence was obtained. This identification was confirmed by sequencing a recently obtained ATCC stock of M14H. We have also completed the sequencing of VHEV; previously, only the 5’ UTR (from nucleotide 137) and L-P1 coding regions (to nucleotide 3857) were sequenced (46).

Figure 1 shows the degrees of similarity of the nucleotide sequences encoding the polyproteins of nine theiloviruses and four EMCVs to that of the GDVII strain. TRV, SAFV-1, and SAFV-2 were divergent from TMEV but not as divergent as EMCV. The TMEV strains differed by ~10% in the nucleotides encoding the leader (L) protein and at most by ~15% in the regions encoding the P2 and P3 nonstructural proteins. In P1, VHEV was as divergent as TRV from the TMEV lineage; however, the similarity of the nucleotides encoding the VHEV nonstructural proteins to the corresponding sequences of the mouse TMEV strains, particularly Vie 415HTR, suggests that VHEV may in fact be a recombinant between a TMEV with a breakpoint near the 1D-2A protein junction and a previously unidentified theilovirus.

**Phylogenetic analysis of UTRs.** The 5’ UTRs of EMCV and theiloviruses clearly differ, despite the similar internal ribosome entry sites. The phylogenetic analysis supports the division into the two cardiovirus species, with TRV and SAFV-1 grouping with TMEV and VHEV (Fig. 2A); the 3’ UTR analysis suggests the same relationships (Fig. 2F). Based on both 5’ and 3’ UTRs, SAFV-1 clusters with TRV. Neither region in SAFV-2 has been sequenced.

**Phylogenetic analysis of the L and P1 capsid-encoding regions.** Phylogenetic analysis of the nucleotides of the L protein- and P1 capsid-encoding regions of the viruses, including the TO4, WW, and FA strains, revealed four clades for the L protein, TMEV and VHEV, TRV, SAFV-1, and SAFV-2 (Fig. 2B), and five clades for the P1 region, TMEV, VHEV, TRV, SAFV-1, and SAFV-2 (Fig. 2C). Both nucleotide- and amino-acid-level comparisons of the individual gene regions corresponding to VP4 (protein 1A), VP2 (protein 1B), VP3 (protein 1C), and VP1 (protein 1D) support the relationships observed in the P1 region (data not shown). However, in VP4 and VP2, the SAFVs differ from other theiloviruses to the same extent to which they differ from EMCV. In 1963, Casals (5) found that VHEV was highly divergent from other TMEVs by CF. Within the TMEV lineage, six distinct sublineages were observed: GDVII and FA; TO4, DA, and WW; TO Yale and TOB15; BeAn 8386, S2, and M2; Ask-1; and Vie 415HTR. S2 and M2 are laboratory derivatives of BeAn 8386 and are therefore expected to be nearly identical; however, laboratory contamination has been suggested as the explanation for the close relationship between WW and TO4 (35). Around the time WW virus was isolated in 1977, other investigators were using TO4 virus in experiments at the Wistar Institute (15). TO4 virus was also included in the serological characterization of DA virus that was isolated 15 years after TO4 (8). Therefore, DA and WW viruses may have been laboratory contaminants derived from the TO4 strain.

VP1 amino acid identities are all above 87.6% within the TMEVs and 94.9% within the EMCVs, while the levels of identity between EMCV and TMEV are 47.4 to 51.1%. TRV is at most 75.5% identical to TMEV and 48.4% identical to EMCV, while SAFV-1 and SAFV-2 are at most 58.1 and 60% related to TMEV and 49.1 and 50.7% related to EMCV, respectively.

**Phylogenetic analysis of the P2 and P3 nonstructural protein coding regions.** Analyses of the P2 and P3 regions, examined in their complete form (Fig. 2D and E) or at the level of individual genes, support the identification of three clades of theiloviruses: TMEV/VHEV, TRV, and SAFV-1/SAFV-2 (data not shown). The congruent phylogenies within the three groups suggest that there has been no recombination among groups. Incongruent phylogenies within the TMEV/VHEV group suggest that there may have been recombination among members of this group. Because of limited numbers of TRVs and SAFVs, it is impossible to comment on recombination within these groups.
**BeAn 8386 virus is a recombinant TMEV.** Nucleotide analyses of the 5′ and 3′ regions of the VP1-encoding sequence revealed an incongruity in the position of BeAn 8386 on the resultant tree (Fig. 3). Based on the 5′ half of the VP1 gene sequence, BeAn 8386 clustered with TOB15 and TO Yale, while based on the 3′ half, it clustered with DA, TO4, and WW (Fig. 3A). A single breakpoint within the VP1 capsid-encoding region was evident (Fig. 1); however, BeAn 8386 was most closely related to TOB15 from the 2A region (Fig. 1) to the 3′ end of the genome.

**TMEV and EMCV L proteins are distinct, whereas SAFV-1 and SAFV-2 L proteins are hybrids.** Picornaviruses in the genus *Cardiovirus* express an L protein at the N terminus of the viral polyprotein that affects the trafficking of interferon-regulatory proteins to the nucleus (9). Recently, the cardiovirus L protein was shown to bind to Ran GTPase and block the nuclear export of new mRNAs (44). The L protein has three domains: an N-terminal atypical (CHCC) zinc finger, an acidic domain, and a C-terminal Ser/Thr-rich domain (Fig. 4) (34). EMCV L protein is phosphorylated on residue Thr 47 (65) and possibly on Tyr 41 (12). An alignment of the amino acids of L protein revealed that the Ser/Thr-rich domain was deleted in EMCV, carried mutations of Ser and Thr residues as well as deletions of five residues in SAFVs, and was mutated at a single Ser residue in TOB15 and TRV (Fig. 4). Thr 47 and Tyr 41, which are potentially phosphorylated, were also conserved in the SAFV-1 and SAFV-2 L proteins. Thus, TRV and VHEV have TMEV-like L proteins, whereas SAFV-1 and SAFV-2 have a hybrid L protein with features of both *Theilovirus* and EMCV species.
FIG. 2. Phylogenetic relationships among cardioviruses based on the alignment of the nucleotide sequences of the 5′ UTRs; the L protein, P1, P2, and P3 coding regions; and the 3′ UTRs of the virus genomes. An unrooted tree was reconstructed using the neighbor-joining algorithm implemented in MEGA 4.0. Numbers at nodes represent the percentages of 1,000 bootstrap pseudoreplicates that contained the cluster distal to the node. Genetic distance is indicated by the bar at the lower left of each panel.
Variability in *Theilovirus* four major capsid surface loops, VP2 puffs A and B, the VP3 knob, and VP1 loops I and II. Four neutralizing immunogenic sites, identified in the capsid proteins of cardioviruses, are located predominately in the major capsid loops (25). Phylogenetic analysis based on aligned amino acid sequences of VP2 puffs A and B, the VP3 knob, and VP1 loops I and II of seven TMEVs showed limited variability of residues in the major capsid surface loops (Fig. 5), which are exposed to the selective pressure of antiviral antibodies. In contrast, VHEV and TRV showed greater variability of the surface loop residues, and VP1 loops I and II of both strains of TMEV showed limited variability of residues in the major capsid surface loops (Fig. 5), which are exposed to the selective pressure of antiviral antibodies. In contrast, VHEV and TRV showed greater variability of the surface loop residues, and VP1 loops I and II of both SAFV-1 and SAFV-2 showed no relationship to those of TMEV, VHEV, and TRV (Fig. 5). The low but not the high-neurovirulence TMEVs use α2,3 N-linked sialic acid as a co-receptor (62), and the crystal structure of DA virus in a complex with sialyllactose has identified three VP2 amino acids on puff B (Q^{2161}, A^{2163}, and G^{2174}) and one VP3 residue (Q^{3232}), all within a positively charged area on the viral surface, that make contact with sialic acid through noncovalent hydrogen bonds (63). Q^{3232} is at the C terminus of VP3 and part of the VP3-VP1 cleavage dipeptide recognized by proteinase 3Cpro, so it is likely to be highly conserved. Since these four residues were found previously to be conserved in all TMEV strains known at the time and since the largest difference in the root-mean-square deviation between the Ca coordinates of viruses of the two neurovirulence groups is in VP2 puff B (EF loops), the capsid conformation of this region is believed to be responsible for sialic acid binding. While the three VP2 puff B amino acids in TMEV strains TOB15 and Vie 415HTR were conserved, only two of the three residues in TRV and none in SAFV-1 and SAFV-2 were conserved, and the fourth residue was mutated in SAFV-2 to H^{3232} (Fig. 5), suggesting that
neither TRV nor SAFVs are likely to use sialic acid as a coreceptor, unless a different capsid conformation suffices.

**Conservation of CD4⁺ and CD8⁺ T-cell epitopes.** In the BeAn 8386 and DA strains, dominant CD4⁺ T-cell epitopes (peptides of 12 to 14 amino acids [aa] in length) have been mapped in each of the major capsid proteins (14, 60, 61), and dominant CD8⁺ T-cell epitopes (peptides of 8 to 9 aa in length) have been identified in VP2 for H-2b (C57BL/6) mice (3, 11) and in VP3 for H-2s (SJL) mice (22). Since all but one length) have been identified in VP2 for H-2b (C57BL/6) mice coreceptor, unless a different capsid conformation suffices.

**Zinc finger** (yellow), acidic (blue), and Ser/Thr-rich (gray) domains. The C-terminal Ser/Thr-rich domain of the L protein is partially deleted in SAFV and SAFV-2. Dashes indicate amino acid deletions, and periods indicate identity to the first sequence (at the top).

**DISCUSSION**

Phylogenetic analysis of the newly described cardioviruses SAFV-1 and SAFV-2 demonstrates that they belong to the species *Theilovirus*; however, these two viruses are related but distinct, particularly in the capsid region (Fig. 1 and 2) (1, 20). For example, VP1 amino acids of SAFVs differ by 23%. In human enteroviruses, differences in VP1 sequences serve to distinguish serotypes, and serotype boundaries have been set at differences of about 25% for nucleotide sequences and 12% for amino acids (37). By analogy, five types of theiloviruses, TMEV, VHEV, TRV, SAFV-1, and SAFV-2, that are expected to be serologically distinct can be distinguished, although cross VN assays of theiloviruses have been performed only for TMEV and VHEV. In contrast, an analysis of the noncapsid regions indicates only three types of theiloviruses, TMEV and VHEV, TRV, and SAFV-1 and SAFV-2, indicating a role for past recombination events in the evolution of these viruses. Frequent recombination in the evolution of other picornaviruses is well-established (51).

Abed and Boivin (1) also identified two additional Saffold-like viruses in children with upper respiratory tract infections, but only a 469-nucleotide region corresponding to proteins 2A to 2C was sequenced. Their amino acid sequences were identical and related to those of SAFV-1 and SAFV-2 by 97.2 and 96.6%, respectively; however, their capsid sequences may be much less closely related. Although SAFV-1 and SAFV-2 have been amplified and isolated, respectively, from clinical specimens, they have not yet been proven to cause disease in humans. Nonetheless, this remains a distinct possibility. At the least, the establishment of causation will require serological analysis demonstrating a fourfold or greater rise in virus-specific antibodies in convalescent- compared to acute-phase sera.

Two other cardioviruses may belong to the *Theilovirus* species. Syr-Daria Valley fever virus (SDVFV), recovered from the CSF of a patient with acute aseptic meningitis in Kazakhstan, appears to be another human theilovirus because it is cross-reactive with EMCV by CF but not by VN assays. SDVFV is responsible for tick-borne infection occurring in the river valleys of Kazakhstan and was first isolated from the blood of a patient in the summer of 1973 (32). The virus has also been isolated from *Hyalomma asiaticum* ticks in areas where SDVFV infection is enzootic, as well as from *Derma-
centor daghestanicus
ticks, and all documented cases of infection in humans included a history of tick bite. Electron microscopy analysis of the Kaz-3 strain revealed featureless 25-
to 27-nm virions typical of picornaviruses (23, 32). In 1970, Sikhote-Alin virus (SAV) was isolated from
Ixodes persulcatus
ticks taken from wild boars (Sus scrofa) in the Primorje region of the USSR (31). SAV is labile at pH 3.0 but stable at pHs
between 5.0 and 7.0, heating at 50°C inactivates the virus, and thermostability is increased in the presence of 1 M MgCl2, all
characteristics of cardioviruses. SAV was shown previously to be pathogenic for suckling and 2- to 3-week-old mice but not
for adult mice, rats, hamsters, guinea pigs, rabbits, or chicken embryos, and it failed to show cytopathic effects in several
different types of cell cultures (31). Although SAV and SDVFV are both serologically related to EMCV by CF, all
three viruses are distinct by VN. SAV and SDVFV have not been examined using molecular biological techniques, and their true relationships to the cardioviruses remain unclear.

It is plausible based on the recent discovery of new human theiloviruses that VHEV arose from human cases of Vilyuisk
encephalitis rather than emerging during virus isolation as a contaminant upon the inoculation of mice, as has been posited
previously. The isolation of VHEV from 14% of 43 brain and CSF specimens but from 3% of 181 nasopharyngeal, blood,
and fecal specimens is consistent with a role for this virus in CNS infection (48). VHEV was also isolated from voles in the
same area where Vilyuisk encephalitis is endemic; therefore, recombination between TMEV and a still unknown theilovi-
rus, either from humans or another rodent species, may be possible. Examining this possibility will require additional se-
quence data from human and/or rodent theiloviruses. Finally, although Vilyuisk encephalitis occurs in a remote and rela-

FIG. 5. Alignment of amino acids of the major surface structures in the theilovirus species: VP2 puffs A (aa 2137 to 2158) and B (aa 2162 to 2187), the VP3 knob (aa 357 to 366), and VP1 loops I (aa 172 to 193) and II (aa 195 to 197) (48). The alignment shows the increasing divergence of sequences of VHEV, TRV, and SAFV, respectively, from those of TMEV. Cardiovirus-neutralizing immunogenic sites are present in VP2 puffs A (with the VP2 first corner, not shown) and B, VP1 loop II, and the VP3 knob (25), while three amino acids in VP2 puff B, denoted by arrows, make contact through noncovalent hydrogen bonds with 2,3-linked sialic acid, the coreceptor for the low-neurovirulence TMEV. Symbols are as defined in the legend to Fig. 4.
tively inaccessible part of the world, further studies of the affected indigenous population will be needed to resolve the issue of causation.

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