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A Novel Hantavirus Associated with an Outbreak of Fatal Respiratory Disease in the Southwestern United States: Evolutionary Relationships to Known Hantaviruses

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Four Corners hantavirus (FCV) is the tentative name of the suspected etiologic agent of the newly identified hantavirus-associated respiratory distress syndrome (HARDS). The identification in HARDS patients of serum immunoglobulin M and immunoglobulin G antibodies that cross-reacted with Hantaan, Seoul, and Puumala virus antigens first suggested that FCV is a hantavirus. Limited nucleotide sequence data from the FCV glycoprotein-2 (G2) confirmed that FCV is a hantavirus and showed that it is most closely related to Prospect Hill and Puumala viruses. We have molecularly cloned approximately 95% of the sequences of the M and S segments of the FCV genome encoding the envelope glycoproteins and nucleocapsid protein N from the lungs of a patient with HARDS. The nucleotide sequence has been determined for 2,632 bases. The nucleotide sequence data show that FCV is a new member of the Puumala virus and Prospect Hill virus division of the hantavirus genus. Phylogenetic tree analyses indicate that the M and S segments have evolved in parallel. Therefore, the novel pathogenic activity of FCV is not likely to be the result of recent reassortment of segments from less pathogenic viruses.

The hantaviruses are a genus of serologically related, negative-sense RNA viruses of the family *Bunyaviridae*. The hantavirus genome is composed of the three segments L (encoding the transcriptase), M (encoding the envelope glycoproteins 1 and 2), and S (encoding the nucleocapsid protein, N). When rodent hantaviruses are transmitted from their natural hosts to humans, they cause any of a variety of clinical syndromes that most often include hemorrhagic fever or nephropathy (17). In May 1993, an outbreak of unexplained adult respiratory distress syndrome (UARDS) was identified in rural residents of the Four Corners region of the southwestern United States (2). The syndrome was often preceded by a prodrome of fever, chills, and myalgia lasting 1 to 4 days. The prodrome was followed by abrupt and rapidly progressive dyspnea, oxygen desaturation, and hemodynamic lability. Death from respiratory insufficiency and/or hemodynamic collapse ensued in more than 50% of patients. At autopsy, patients had large pleural effusions and the lungs were grossly edematous. Histologically, pulmonary alveolar spaces were filled with edema fluid and scant hyaline membranes. The pulmonary interstitium had a lymphoid infiltrate.

In June 1993, immunoglobulin M and immunoglobulin G antibodies with reactivity to the known hantaviruses, Puumala, Hantaan, and Seoul viruses, were identified in serum samples of many patients with UARDS. Limited nucleotide sequence data indicated that the putative etiologic agent was a novel hantavirus related to the Puumala virus and Prospect Hill virus (PHV) (2–5, 12). For clarity, we are using the term Four Corners virus (FCV) for the new agent, although that name may change later. FCV was also found to be prevalent among deer mice (*Peromyscus maniculatus*) trapped in the vicinity of the homes of patients with UARDS (2–5, 12). The syndrome, currently called hantavirus-associated adult respiratory distress syndrome (HARDS), has also been identified among patients who died of similar syndromes in late 1992 (6).

Using primers designed to hybridize to conserved regions of previously characterized hantaviruses, we amplified and cloned a 1,275-nucleotide (nt) portion of cDNA from the M segment encoding most of the FCV glycoprotein 1 (G1), a 2,028-nt portion of M segment extending from the 3′ end of G1 through most of G2, and an 1,131-nt portion of the S segment encoding the nucleocapsid protein N. The viral sequences were obtained from the lung tissue of a patient designated with the random identifier 3H226, a resident of western New Mexico who died of a syndrome with clinical and pathologic findings typical of HARDS. For the purpose of phylogenetic comparison, a smaller (1,225-nt) portion of the FCV M segment from patient MHAR from southern Colorado was also cloned. We have determined the complete nucleotide sequences of the FCV insert of the 3H226 1,275-nt G1 amplimer, the 3H226 1,131-nt N gene amplimer, and a 366-nt portion of the 3H226 and MHAR G2 gene. The 366-nt portion of the G2 gene is homologous to a region that has been used extensively in previous hantavirus phylogenetic studies (15, 16).

MATERIALS AND METHODS

Patient samples. Specimens 3H226 and MHAR were from patients from western New Mexico and Colorado, respectively, who died after illnesses characterized by clinical and pathologic features typical of HARDS (2–4). Specimen M209A was taken from a presumably uninfected 4-year-old girl whose lung tissue was obtained after her death in an automobile accident. In each case, 1-cm^3 sections of lung tissue, obtained at autopsy, were frozen promptly at −70°C in the absence of fixative.

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Total lung RNA was prepared in guanidinium isothiocyanate-citrate-sarcosyl solution by standard methods (1).

Design of primers and reverse transcriptase PCR. Nucleotide sequences of Puumala virus and PHV M and S segments were aligned (GenBank accession numbers X61035, L08755, X55129, and X55128), and regions with more than 85% identity over ~20 bp were used to design primers for amplification. Regions encoding amino acids specified by a single triplet (methionine, tryptophan) were favored. In each reverse transcription-amplification procedure, a nested PCR strategy was used. Reverse transcription was carried out in the presence of a primer pair (outer primers) separated by at least 1 kb of viral RNA template, and the cDNA product was subjected to DNA amplification with the same primer pair. A second primer pair (inner primers), internal to the first, was used to produce the final PCR product. With the MHAR allele 1,225-nt M segment product, the same antisense primer was used for both outer and inner PCRs. For the amplification of the 2,028-nt M segment product from specimen 3H226, the sense strand primers were designed from sequences obtained directly from the 3′ end of the 1,275-nt G1 cDNA clone of FCV rather than from sequences of related hantaviruses.

Table 1 lists outer and inner primers used to amplify cDNAs used in this study. Total RNA (3 μg) was added to a cocktail containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dATP, dCTP, dGTP, and dTTP; 7 mM 2-mercaptoethanol; 10 pmol of each outer primer; 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim); and 2.5 U of *Taq* polymerase (Perkin-Elmer) in a volume of 100 μl. After a reverse transcription step for 1 h at 42°C, cDNA was subjected to 8 cycles of amplification at 94, 37, and 72°C for 1, 1, and 3 min, respectively, followed by 32 cycles at 94, 42, and 72°C for the same cycling times. Amplifications with the inner primer sets were performed under temperature-cycling conditions essentially identical to those used for the outer primer sets. The reaction components were the same, except that 2-mercaptoethanol and reverse transcriptase were not included and 50 pmol of each inner primer was added. A 3-μl sample of the product of the outer primer pair was amplified by inner primers in a final volume of 100 μl.

A band was considered to be a candidate product of FCV if it was of the predicted molecular weight on agarose gel electrophoresis and if it was produced after amplification of 3H226 and MHAR cDNA but not from the M209A negative control lung cDNA. DNA amplified by internal primers was excised from a gel and cloned into a "T-tailed" plasmid, pCRII, and the nucleotide sequence of the insert was determined by using the Sequenase (U.S. Biochemicals) dideoxy method as recommended by the manufacturer (9). Complete nucleotide sequencing of both strands of the cDNAs was carried out by generating a nested set of unidirectional deletions in the cDNA inserts by the method of Henikoff (8). In some cases, primers complementary to FCV cDNA sequences were used in sequencing reactions. We considered a sequence to be derived from FCV if it had clear nucleotide and amino acid similarity to previously described hantaviruses and retained the appropriate open reading frame predicted from alignments with other hantaviruses.

Nucleotide phylogenetic tree analysis. FCV sequences obtained from the 1,275-nt G1 gene amplimer, the 1,131-nt N gene amplimer, and the 366-nt portion of the 2,028-nt G1/G2 amplimer were aligned by using the multiple aligned sequence editor (MASE [7]). Trees were constructed by using various options of the PAUP 3.1 program (14), as described in the legend to Fig. 2.

Nucleotide sequence accession numbers. The sequences of

TABLE 1. Primers used in reverse transcription and PCR of FCV RNA templates

Specimen[a]	Segment	Nucleotide sequence		Predicted amplimer size (nt)
		Outer primers	Inner primers	
3H226	M	5′ TAGTAGTAGACTCCGCAAGAAGA; CTGACAAC(CT)TCACA(GA)ACCATTTCT	5′ ACCATGGAATGTCCTCATACTGTA; 5′ AGGGTTTTAGTAAGAAT(TG)AC(CT)	1,275
2H226	S	5′ TAGTAGACTTCGT(AG)AA(GA)AGCTACTA; 5′ GGCTCTTGGTT(AT)GATATCTCTT	5′ GGTGGACCC(AG)GATGACGTTAACAA; 5′ TAGCTCGGATCCAT(AG)TCATCACC	1,131
3H226	M	5′ GTTCAAAAATTCAGAGGCTCAGAA; 5′ ACTGC(CA)AC(TA)ACCATCCAATTACCA	5′ CAACTTCATGTGCCAAAGAGT; 5′ CCATTCCCC(AT)GA(TC)TTTTTAA	2,028
MHAR	S	5′ TGT(GT)TACTGTAATGG(CG)ATGAAGAA; 5′ GCAAATGCACATTTCTTTCTAAA	5′ ACATTCTGTTTTGGCTGG; 5′ GCAAATGCACATTTCTTTCTAA	1,225

[a] All specimens were obtained from lung tissue.

FIG. 1. Partial predicted amino acid sequence of the putative N protein of FCV, aligned with those predicted for Puumala virus and PHV. Amino acid coordinates are relative to the first methionine in the open reading frame for N. Asterisks indicate identity between aligned residues, whereas colons indicate conservative changes.

the 1,275-nt (G1) M segment and 1,131-nt S segment amplimers, exclusive of primers, have been assigned GenBank accession numbers U02471 and U02474, respectively. The 366-nt region of 3H226 and MHAR alleles of the glycoprotein G2 gene corresponding to PHV M segment coordinates 1996 through 2361 have been assigned GenBank accession numbers U02473 and U02472, respectively.

RESULTS

The HARDS hantavirus is most closely related to the Puumala-PHV subgroup of the *Hantavirus* genus. The predicted amino acid sequence of FCV N protein is aligned with those of Puumala virus and PHV in Fig. 1. The various regions of the FCV genome differ in the extent of their relatedness to other members of the *Hantavirus* genus. The portion of the predicted FCV N protein we considered (the region beginning with the amino acid corresponding to PHV residue 42 and ending with residue 397) is well conserved throughout, with the exception of a central region corresponding to PHV residues 233 through 305. The amino acid alignment in that domain is poor. By contrast, similarities in the amino-terminal portion (amino acids 37 through 447 of PHV) of the G1 protein are poor in several regions, such as residues 42 to 60 and 71 to 100 (data not shown). The poor alignment of the FCV G1 protein sequence with those of closely related hantaviruses is also reflected in protein sequence differences (Table 2). Smaller variability is observed in comparisons of N proteins and G2 proteins.

Phylogenetic tree analyses of the sequences of the 366-nt G2 gene (M segment) clones and the 1,131-nt N gene (S segment) clone are depicted in Fig. 2. The phylogenetic tree analysis of the FCV sequence contained in the 1,275-nt G1 gene clone produced a similar branching order to that found with G2 and N sequences (results not shown). Parsimony analyses showed that PHV and Puumala virus are the closest relatives to FCV among previously described hantaviruses. Examination of protein sequence trees and differences (Table 2) and examination of only the second nucleotide position of each codon (Fig. 2, inset) did not produce consistently closer alignment to either PHV or Puumala virus. Although minor variations in the phylogenetic distance can be inferred, depending on whether the analysis is conducted on G1, G2, or N gene sequences, a similar branch point is predicted for divergence of FCV from the other hantaviruses regardless of which segment or coordinates are used.

TABLE 2. Percent differences in amino acid sequence between predicted proteins of HARDS-associated viruses FCV-3H226 and FCV-MHAR and other hantaviruses

Virus and segment	% Difference from:				
	3H226	MHAR	PHV	Puumala virus[a]	Hantaan virus[b]
MHAR[c] G2	4.67				
PHV					
S	26.61				
G1	42.14				
G2	30.94	29.91			
Puumala virus					
S	29.66	ND[d]	21.71		
G1	39.90	ND	30.26		
G2	28.97	28.97	23.34		
Hantaan virus					
S	38.84	ND	39.75	41.28	
G1	54.36	ND	46.47	45.05	
G2	47.66	47.66	46.73	48.60	
SRV[e]					
S	40.98	ND	40.06	40.67	20.18
G1	57.11	ND	48.05	46.88	31.75
G2	45.79	44.06	43.93	42.99	21.43

[a] For S segment, PUUSSEG was compared; for G1 and G2, CG18-20 was used.
[b] For S segment, HANSNC was compared; for G1 and G2, HanM was used.
[c] Only the G2 segment sequence was obtained from sample MHAR.
[d] ND, not determined.
[e] SRV, Seoul/Sapporo rat virus group. For S segment, SRVAGSS was compared; for G1, SRVAGSM was compared; and for G2, SR11 was used.

DISCUSSION

Hantaviruses of cricetid rodents form a phylogenetic branch distinct from those of the murid rodents. Our results support results of previous studies that suggested that FCV is a new member of the Puumala virus-PHV subgroup of hantaviruses (2–5, 12). Puumala virus, PHV, and FCV are parasites of the cricetid and microtine rodents *Clethyronymus glareolus*, *Microtus pennsylvanius*, and *P. maniculatus*, respectively. These hantaviruses are genetically closely related to one another and are easily distinguished serologically (10) and genetically (15, 16) from the Hantaan, Belgrade, and Seoul hantaviruses of the murid rodents *Apodemus agrarius*, *Apodemus flavicollis*, and *Rattus norvegicus*, respectively.

Whether one considers two regions at distant sites of the M segment (G1 and G2) or the N protein of the S segment, a similar phylogenetic branch point is derived for FCV. That branch point is ancestral to the divergence of PHV and

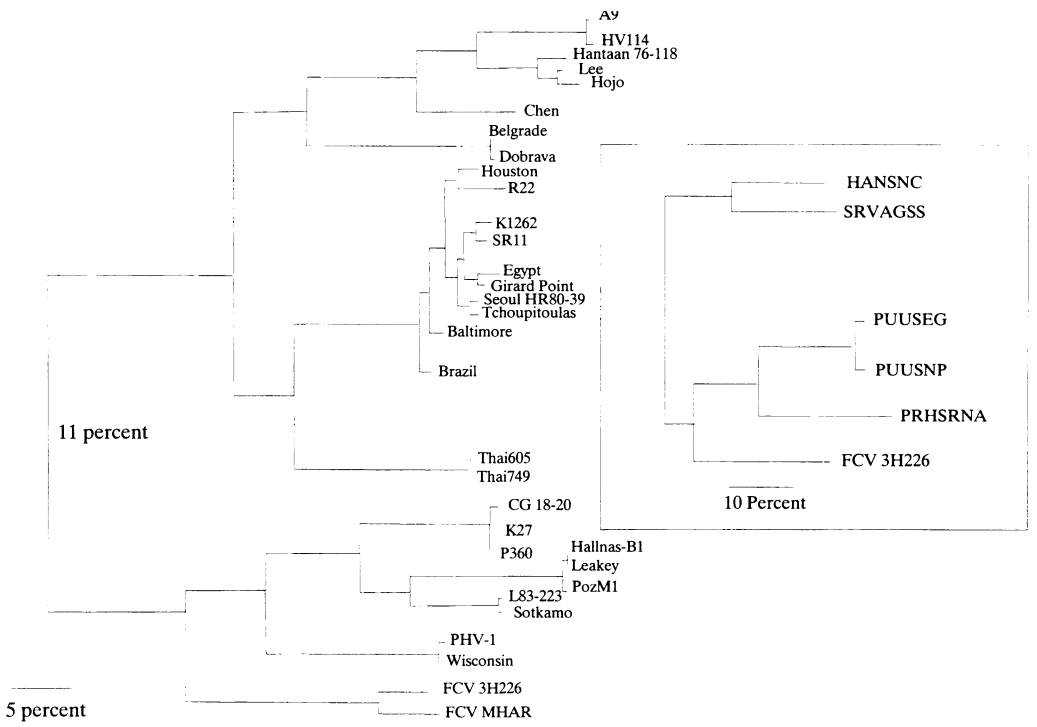

FIG. 2. Parsimony tree analysis comparing HARDS-related virus G2 sequences (alleles FCV-3H226 and FCV-MHAR) with other hantavirus sequences (15, 16). The MULPARS option of PAUP version 3.1 (14) was used to generate this minimal-length tree from 321 total sites, of which 212 displayed variation. Many equally minimal-length trees were obtained; the one shown was supported as the majority rule consensus tree in 30 replicates. The horizontal branch lengths are proportional to the number of single nucleotide changes, which can be read as a percentage by using the scale bar. Vertical distances are for clarity only. The tree was rooted at the midpoint of the greatest patristic distance. When tree analysis was performed on sequences from the G1 gene, a similar branch order was determined for FCV relative to Puumala virus, PHV, Hantaan virus, and Seoul virus (data not shown). The inset shows a parsimony tree analysis comparison of the HARDS-related virus FCV-3H226 S segment nucleotide sequence with other hantavirus sequences. The MULPARS option of PAUP version 3.1 (14) was used to generate this minimal tree from 227 total sites, which were second-base positions in the coding sequences. Of the 227 sites, 111 were variable. The tree is congruent with a larger tree based on all sites in the coding sequence (not shown).

Puumala virus and aligns FCV most closely with the PHV-Puumala virus branch of the hantaviruses. These data indicate that FCV M and S segments have evolved in parallel and that FCV did not arise through the recent reassortment of M and S segments from preexisting hantaviruses. Since PHV is the prototype New World hantavirus and the only hantavirus other than FCV known to be of New World origin, the PHV-FCV branch of the hantavirus tree may represent the first of a cluster of New World hantaviruses. Whether members of this branch will also be distinguished by new pathogenic properties such as an association with HARDS remains to be seen (6). The possibility that PHV itself causes disease has not been excluded (18).

New clinical syndrome associated with hantaviruses. Although it has been recognized that hantavirus infection can produce lung disease (11, 17), pulmonary edema in the absence of renal disease or hemorrhage has not been associated with infection by this class of agents. Clinical evidence of pulmonary disease appears to be unusual in patients infected with Puumala virus (13). The syndrome caused by FCV is distinctive in both the severity and frequency of pulmonary disease and in the nearly complete absence of proteinuria and renal failure.

The distinctiveness of the clinical syndrome caused by FCV supports the finding that it is genetically distinct from previously described hantaviruses. Serologically it is most closely related to Puumala virus, PHV, and Seoul virus (2–4). Previous analyses of the partial sequence of the G2 transmembrane glycoprotein from HARDS virus cDNA support a close relationship with both Puumala virus and PHV and a distant relationship to Hantaan and Seoul viruses (5, 6, 12).

The recognition of a novel, Puumala virus-like hantavirus with greatly enhanced and qualitatively different pathogenic potential raises new questions about which viral determinants are important in the genesis of injury to different organ systems. Apparently, determinants of pulmonary injury can be dissociated from those responsible for renal injury. Thus, Puumala virus causes both renal and pulmonary disease in humans but FCV appears to completely spare the kidneys while producing severe lung disease. It may be possible to substantially narrow the field of candidate sequence determinants for injury to specific organ systems through comparison of sequences derived from Puumala virus- and FCV-like viruses implicated in the different human diseases now known to be caused by members of this viral group. The discovery of a HARDS-associated virus with significant sequence differences from FCV may assist in this effort (6).

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