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A Newly Recognized Virus Associated with a Fatal Case of Hantavirus Pulmonary Syndrome in Louisiana

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Genetic analysis of virus detected in autopsy tissues of a fatal hantavirus pulmonary syndrome-like case in Louisiana revealed the presence of a previously unrecognized hantavirus. Nucleotide sequence analysis of PCR fragments of the complete S and M segments of the virus amplified from RNA extracted from the tissues showed the virus to be novel, differing from the closest related hantavirus, Sin Nombre virus, by approximately 30%. Both genome segments were unique, and there was no evidence of genetic reassortment with previously characterized hantaviruses. The primary rodent reservoir of Sin Nombre virus, the deer mouse *Peromyscus maniculatus*, is absent from Louisiana. Thus, the virus detected in Louisiana, referred to here as Bayou virus, must possess a different rodent reservoir.

Hantaviruses are rodent-borne, negative-strand RNA viruses that represent a distinct genus of the family *Bunyaviridae*. Their genomes consist of three segments (L, M, and S), which encode the virus polymerase protein (L), the glycoproteins G1 and G2, and the nucleocapsid protein (N), respectively (1, 14, 16, 17, 27, 32). Each of the eight well-characterized hantaviruses is associated with a different primary rodent reservoir: Hantaan (HTN) virus with *Apodemus agrarius* (stripped field mouse), Dobrava (DOB) virus with *Apodemus flavicollis* (yellow-necked field mouse), Seoul (SEO) virus with *Rattus norvegicus* (Norway rat), Thailand (Thai) virus with *Bandicota indica* (bandicoot), Puumala (PUU) virus with *Clethrionomys glareolus* (bank vole), Tula virus with *Microtus arvalis* (European common vole), and Prospect Hill (PH) virus with *Microtus pennsylvanicus* (meadow vole). HTN, SEO, and PUU viruses are causative agents of hemorrhagic fever with renal syndrome in Asia and Europe (24). However, PH virus, the only hantavirus isolated from rodents indigenous to North America, was not known to be associated with human disease. An eighth novel hantavirus, Sin Nombre (SN) virus (initially referred to as Four Corners or Muerto Canyon virus), was recently identified as a cause of a relatively uncommon but frequently fatal respiratory illness throughout much of the western United States (13, 25, 35). This disease has been termed hantavirus pulmonary syndrome (HPS) (6, 12). Serologic and genetic analyses of infected rodents strongly implicated *Peromyscus maniculatus* (deer mouse) as the primary rodent reservoir for this virus (8, 25). During continued disease surveillance, several additional cases of illness compatible with HPS have been identified outside of the known range of *P. maniculatus*, including an HPS-like fatality in Louisiana (22). Investigation of this case revealed not only a compatible illness but also evidence of hantavirus RNA and antigens in autopsy tissues (22). The complete S and M nucleotide sequence analysis presented here shows that this virus, provisionally named Bayou (BAY) virus, represents another newly recognized member of the *Hantavirus* genus capable of causing severe respiratory illness.

RNA extraction, PCR amplification, primer design, and sequence analysis. RNA was extracted from autopsied frozen lung and brain tissues as described previously (25). PCR products were synthesized by nested reverse transcriptase-PCR (*Taq* polymerase; Boehringer GmbH, Mannheim, Germany) and sequenced directly by the dyedeoxy cycle sequencing technique (Applied Biosystems, Foster City, Calif.) (25). In general, because of the small sample size and suboptimal tissue condition, no product DNA bands were obtained from any single-round amplification reactions. The nested reverse transcriptase-PCR amplification of a 185-bp fragment of the virus genetic material from the autopsy tissue initially used a nested set of PUU/PH- and SN-specific primers as described previously (22, 35). Additional primers used for analysis of the complete M and S nucleotide sequences were initially based on regions predicted to be conserved among known hantavirus sequences as described previously (35). The specially designed primer GGTGGTTGTGGTAGTAGTAGACTCC was used for the amplification of 3′ and 5′ terminal fragments. This primer contains the terminal sequence TAGTAGTAGACTCC, which is highly conserved among hantaviruses, and 11 additional nucleotides to provide strong specific binding with intermediate templates during the PCR amplification. The exact sequences of 14 nucleotides of both the 3′ and 5′ termini of the S and M segments were verified by ligation of the ends of genomic RNA segments, reverse transcription, PCR amplification, and sequencing through the site of ligation (31). The sequence alignments and comparisons were performed on a DEC computer using LINEUP, PILEUP, and GAP programs of the GCG software package (Genetics Computer Group, Madison, Wis.).

S segment RNA and encoded protein. BAY virus detected directly in the Louisiana case lung autopsy tissue was found to possess a S RNA genome segment 1,958 nucleotides in length. The open reading frame (ORF) encoding the nucleocapsid protein starts at position 43 and ends at position 1326. The length of the predicted protein, 428 amino acids, is identical to that of SN virus. A potential overlapping second ORF has been noted in the S segments of PUU viruses, PH virus (26), and SN virus (35) and is present in BAY virus in exactly the same

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TABLE 1. Percentage nucleotide or deduced amino acid sequence identity between Bayou virus and other hantaviruses

Sequence	% identity[a]								
	HTN 76-118	SEO SR-11	Thai[b]	PUU			Tula	PH	SN
				cg1820	Sotkamo	Udm[c]			
S segment	62.4 (8)	58.8 (10)	ND	66.4 (10)	65.5 (9)	65.5 (9)	64.7 (7)	68.4 (7)	69.0 (11)
N protein	64.8 (2)	63.4 (2)	ND	74.5 (1)	73.4 (1)	74.3 (1)	76.6 (1)	77.3 (1)	86.9 (0)
M segment	59.6 (10)	59.1 (10)	59.0 (10)	64.0 (5)	64.5 (6)	ND	ND	64.5 (6)	71.5 (4)
GPC protein	55.5 (5)	54.8 (7)	55.2 (8)	65.6 (4)	65.2 (5)	ND	ND	66.6 (4)	81.0 (3)

[a] The number of gaps introduced to optimize sequence alignment is shown in parentheses. ND, not done.
[b] Thai sequence is from reference 42, L08756. For all other sequences, see Fig. 1 legend.
[c] Udmurtia strain of PUU virus.

position as in SN virus (with initiation codon at position 122 and coding capacity for 63 amino acids). As has been discussed previously (35), the remarkable conservation of the position of the second ORF in the S segment of BAY virus and the statistically significant decrease of the third base substitution frequency in this region strongly suggest this ORF is functional.

Comparison of the nucleotide sequence of the BAY virus S segment with previously characterized hantaviruses shows the highest degree of identity (69%) with SN virus (Table 1). The deduced N protein amino acid sequences of these two newly recognized North American hantaviruses are relatively conserved, differing by only 13% overall. However, a more variable domain can be identified at amino acid positions 233 to 300, a region which displays only 57% amino acid identity between BAY and SN viruses and might serve as a spacer or hinge region separating two major functional domains of N protein on the basis of similar arrangements seen in some other nucleic acid binding proteins (4, 20, 23, 28). It has already been observed that the carboxyl termini and, to a lesser extent, the amino termini are the most conserved areas of the N proteins (36), suggesting a significant role of these regions in the encapsidation and/or replication processes. The 95-amino-acid C-terminal conserved region of the N protein of HTN virus strain 76-118 and PUU virus strain CG 18-20, expressed in *Escherichia coli*, were recently found to be sufficient for the efficient nonspecific binding of RNA and DNA, especially double-stranded molecules (18). The corresponding C terminus of the BAY virus N protein is also conserved and contains a lysine- and arginine-rich amino acid sequence which bears some similarity to putative RNA-binding motifs found in virus and eucaryotic RNA-binding proteins (5, 34). Recent data show that alteration and/or deletion of the last five C-terminal amino acids of the vesicular stomatitis virus N protein leads to an inability to bind vesicular stomatitis virus RNA (10). On the basis of these observations, one may speculate that an RNA binding site near the C terminus of the N protein may be a common feature of negative-strand RNA viruses. The function of the N-terminal conserved domain of hantavirus N proteins is unknown. However, it may be accessible on the surface of the N protein based on the observation of a major linear epitope in this region of the SN virus N protein (amino acids 17 to 59), allowing involvement of the region in interaction with other molecules (21).

BAY virus, like SN virus, has an unusually long S genome segment 5′ noncoding region which is 627 nucleotides in length for BAY virus and 728 nucleotides for SN virus (35). The size of the nontranslated region of the other characterized hantavirus S segments varies from 331 (PH virus) to 487 (PUU360) nucleotides (26, 43). Examination for the presence of any potential ORFs in this region revealed no evidence of any other small ORFs in the sense or ambisense orientation relative to the N protein ORF. More detailed nucleotide sequence analysis of this region revealed the presence of numerous imprecise repeats which could be generated by virus polymerase slippage events as seen in other negative-strand RNA virus mRNA noncoding regions (3). Some motifs resemble those of SN virus (35). However, comparison of nontranslated S segment regions of BAY and SN viruses showed no significant overall nucleotide sequence homology except limited regions of strong homology in the 5′ terminal 150 nucleotides of the virus genome. This includes the exact homology of the terminal 32 nucleotides and three other G/A-rich regions of significant homology, each approximately 25 nucleotides in length. Whether regions in the noncoding region possess specific sequence motifs or base composition (i.e., they are C deficient in the genomic RNA sense) important in specific interactions with other virus or cellular components remains to be determined. Recently, it was shown that HTN virus (strain 76-118) N protein mRNA transcription terminates at position 1470 (template sequence 3′-CCCCACCCAGUCA-5′), 223 nucleotides from the S segment template 5′ terminus (11). Similar sequences, 3′-CCC ACCCAAUCU-5′ and 3′-CCCACCAAAUUCU-5′, were found in the noncoding region of BAY virus S segment at positions 1409 and 1451, respectively. The sequence 3′-CCCAC CCAAGUUC-5′ can also be found at position 1406 in the same region of the SN virus S segment (35) and at position 1443 (3′-CCCACCCAGUUUA-5′) in Tula virus (29). However, only a shorter variant, 3′-CCCAAA-5′, repeated three times (positions 1450, 1473, and 1498), can be found in this region in PUU viruses (16, 38), and only 3′-CCCACCAAA-5′ can be found in SEO virus strain SR-11 (position 1537) (1). These all somewhat resemble the snowshoe hare virus (15) and Rift Valley fever virus (9) M segment template regions coding for the mRNA 3′ ends (3′-ACCCC-5′) and may function similarly as signals for mRNA termination.

M segment RNA and encoded proteins. The complete sequence of the BAY virus M segment was determined to be 3,677 nucleotides in length. The single ORF for the glycoprotein precursor (GPC) is located from position 52 to position 3475 and potentially encodes an unprocessed GPC 1,141 amino acids in length. The nucleotide sequence identities (Table 1) between the entire M segment of BAY virus and those of previously characterized hantaviruses range from approximately 59% (SEO virus SR-11) to 72% (SN virus).

Comparison with the deduced glycoprotein sequences of previously characterized hantaviruses reveals a number of common structural features, suggesting the overall structures of the glycoproteins are similar. The GPC of BAY virus possesses four potential N-glycosylation sites conserved among all hantavirus glycoproteins, three of which are situated in G1 (positions 141, 353, and 405) and one of which is situated in G2

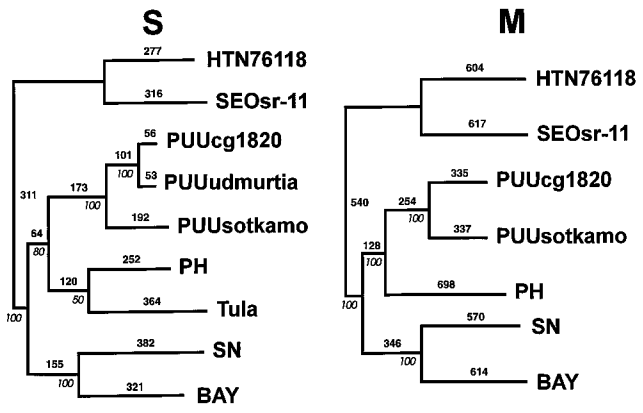

FIG. 1. Phylogenetic relationship of the S and M genomic segments of BAY virus to the equivalent segments of previously characterized hantaviruses. Phylogenetic analysis was carried out by the maximum parsimony method using PAUP version 3.1.1 run on an Apple Macintosh Quadra 800 computer (37). Trees were calculated by using the branch and bound option, and bootstrap confidence limits were obtained from 1,000 heuristic search replicates. A single most parsimonious tree was obtained in each case. Horizontal branch lengths are proportional to nucleotide step differences which are indicated above each branch. Bootstrap confidence limits are indicated in italics below appropriate branch points. Vertical distances are for visual presentation only. The complete M segment nucleotide sequences of the following viruses were used in M segment analyses: HTN strain 76-118 (33), M14627; SEO virus strain SR-11 (1), M34882; PUU strain Sotkamo (38), X61034; PUU strain CG18-20 (16), M29979; PH virus strain PH-1 (27), X55129; and SN virus (35), L25783. Nucleotide sequences used in S segment analyses included HTN strain 76-118 (32), M14626; SEO virus strain SR-11 (1), M34881; PUU strain CG18-20 (36), M32750; PUU strain Sotkamo (38), X61035; PUU strain Udmurtia (30), Z21497; Tula virus (29), Z30941-Z30945; PH virus (26), M34011; and SN virus (35), L25784.

(position 933). All 57 conserved cysteine residues and the potential transmembrane regions of G1 and G2 (33) are also located at identical positions. The five amino acids (WAASA) preceding the proposed site for cotranslational cleavage of the GPC are completely conserved in all the hantaviruses analyzed (14, 35). They were also found to be conserved and located at the same position in the BAY virus GPC amino acid sequence. Cleavage at this site would produce G1 and G2 proteins 654 (including signal peptide) and 487 amino acids in length, respectively. The putative signal peptide of G1 is 18 amino acids in length and extremely variable, sharing only 50% of amino acid sequence identity even with that of SN virus. However, the predicted cleavage site of the signal peptide is more conserved and has overall properties that fit most of the rules established for signal peptide cleavage sites (39, 40).

The G1 protein appears to be more variable (especially in the amino-terminal half) than the G2 protein, as seen in earlier hantavirus comparisons (1, 27, 35, 38). On alignment of amino acid sequences, small (1- to 3-amino-acid) deletions or insertions in the amino-terminal part of G1 can be seen, which have specific patterns for different hantaviruses. It has been reported that the major type-specific linear epitope of SN virus G1 protein at amino acid positions 58 to 88 (21) is completely conserved in SN virus G1 amino acid sequences from different geographic areas (19). Although the first 10 amino acids of the corresponding region of BAY virus are identical to those of SN virus, the remaining 21 amino acids share only 50% identity. Thus, it appears likely that BAY virus may possess a potentially useful type-specific epitope at amino acid positions 68 to 88.

Phylogenetic analysis of virus genome segments. The detailed phylogenetic relationship of BAY virus to the previously characterized hantaviruses was determined on the basis of the nucleotide sequence differences among the complete virus S and M segments. Maximum parsimony analyses of both S and M segment sequence differences resulted in a single most parsimonious tree for each. Bootstrap analysis indicated that the main branch points on both trees were well supported. Essentially identical tree topologies were obtained in both cases (Fig. 1). The nucleotide sequences were unique for both segments of BAY virus, each showing at least approximately 30% nucleotide sequence divergence from the respective segments of other well-characterized hantaviruses. The similar phylogenetic tree topology obtained by analysis of the sequence differences of S and M virus genomic segments shows clearly that the emergence of this virus does not involve a recent reassortment event between S or M segments of different hantaviruses. The BAY virus RNA segments display greatest sequence similarity to another newly recognized hantavirus associated with HPS, SN virus, isolated from a rodent (*P. maniculatus*) indigenous to North America (13). These two viruses represent a distinct clade on the hantavirus evolutionary tree (Fig. 1). The next closest relative of BAY virus, PH virus, also isolated from a rodent (*M. pennsylvanicus*) indigenous to North America, forms another separate branch and has not been associated with any human disease. Results of the recent investigation of SN virus infections, together with earlier reports, confirm the extensive presence of hantaviruses throughout North America maintained in murid rodents of the subfamilies *Arvicolinae* and *Sigmodontinae* (41) indigenous to North America (7, 8, 44). The rodent host of BAY virus is still unknown, but *P. maniculatus* is not present in Louisiana (2). We predict that it is likely to be another species of sigmodontine rodent indigenous to North America, on the basis of the position of BAY virus on the hantavirus phylogenetic tree and the rodent host of the closest relatives. Finally, it is proposed here that this virus be named Bayou virus, after a geographic feature close to the location of the current confirmed case in Louisiana.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article can be obtained from GenBank. The accession numbers for the S and M segment sequences of Bayou virus are L36929 and L36930, respectively.

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