JOURNAL OF CLINICAL MICROBIOLOGY, May 1997, p. 1090–1096 Vol. 35, No. 5
0095-1137/97/$04.00+0

Puumala Hantavirus Genome in Patients with Nephropathia Epidemica: Correlation of PCR Positivity with HLA Haplotype and Link to Viral Sequences in Local Rodents

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Received 13 July 1996/Returned for modification 31 December 1996/Accepted 4 February 1997

Reverse transcription-PCR was used to analyze specimens from 20 Finnish nephropathia epidemica (NE) patients hospitalized during the period from October 1994 to January 1995. Blood and/or urine sediment specimens from seven patients were found to be positive for the genome sequences of Puumala hantavirus (PUU). PCR positivity of the specimens from the patients correlated well with the HLA-DRB1*0301 and HLA B8 alleles, which previously were shown to associate with severe courses of NE. Genetic analysis of the partial M- and/or S-segment sequences obtained from three severely ill NE patients revealed three PUU strains related to but distinct from previously reported strains from Finland. The M-segment sequence of PUU from bank voles trapped near the probable site of infection for one of the patients showed 98.2% identity to that of the PUU strain obtained from the patient, suggesting a link between wild-type PUU from the natural focus and the NE case. The S-segment sequences from the patient and the bank voles, however, showed substantially lower identity (95.8%). As this difference in diversity for M and S genes (1.8 and 4.2%) is atypical for PUU genetic drift, one possibility is that the strain acquired at the putative place of infection is a reassortant one.

Nephropathia epidemica (NE) is a relatively mild form of hemorrhagic fever with renal syndrome. It presents as a generalized infection with signs of multiorgan involvement characterized by fever, myalgia, headache, nausea, and acute renal insufficiency. NE is sometimes associated with other symptoms, such as acute myopia, hepatitis, encephalitis, and pneumonia. Mortality in NE is 0.1 to 0.2%, as severe hemorrhages or hypotensive shock complicates NE rarely, significantly less than in the eastern form of hemorrhagic fever with renal syndrome, caused by Hantaan virus. Nevertheless, the acute febrile illness of NE manifesting as renal failure frequently requires hospitalization and requires transient dialysis treatment in 5% of the patients (5, 23, 26, 41).

The clinical picture of NE was first described in detail in 1971 (23), and detection of the causative agent, Puumala virus (PUU), in tissues of bank vole (*Clethrionomys glareolus*), the natural host for this hantavirus, was reported in 1980 (6). The genome of PUU, similar to the genomes of other members of the *Hantavirus* genus (family *Bunyaviridae*), consists of large (L), medium (M), and small (S) RNA segments of negative polarity encoding, respectively, a viral RNA polymerase, a glycoprotein precursor for two surface glycoproteins (G1 and G2), and a nucleocapsid protein (N) (11, 31, 39, 40, 42).

PUU is endemic to the Nordic countries and the European part of Russia but also occurs in the Baltic countries, central Europe, and the Balkans (5, 30). The distribution of NE reflects well that of its natural host, *C. glareolus*. Within the area where it is found the bank vole shows quite variable population dynamics (12–14, 17). The occurrence of NE in humans depends strongly on the local pattern of population dynamics of the bank vole (5, 18, 29). In northern Europe, the bank vole exhibits drastic multiannual population cycles which are reflected by human epidemics; on the other hand, in central Europe the pattern of population dynamics of bank voles is more stable and depends mainly on climatic conditions, which is also reflected in the human epidemiology. Person-to-person spread of PUU does not occur. Humans are believed to acquire PUU from infectious aerosols of animals' excreta, most probably by inhalation, during activities such as woodcutting, planting, gardening, and cleaning of summer cottages. Although an epidemiological link between PUU infection in humans and the natural virus host looks obvious, the transmission routes of infection as well as risk factors still await careful investigations.

The knowledge of the genetic properties of PUU strains obtained from patients' samples is very limited. Only the S- and the M-segment sequences of strains K27 and P360 from two Bashkirian (Bashkiria is an autonomous republic in the Russian federation) NE patients (47) and of strain Paris 90-113 (4) from one French patient have been reported. Besides, these strains had undergone serial passages in Vero E6 cells and therefore could not be considered identical to those in patients (25). On the other hand, amplification of PUU nucleotide sequences directly from clinical specimens in a reverse transcription-PCR (RT-PCR) appears to be very difficult to achieve, although a few attempts have been made. Short S-segment sequences (nucleotides [nt] 819 to 988) were obtained from 5 of 50 whole-blood specimens from Bashkiria (22). These sequences were 96.5 to 99.2% identical to the related region of PUU strain CG18-20 (isolated from *C. glareolus*), also from Bashkiria (the sequences of the P360 and K27 strains

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TABLE 1. Primers used for RT-PCR

Segment and primer	Sequence (5′→3′)	Polarity	Position (nt)	Reference
S				
S2	TTCTGCAGTAGTAGTAGACTCCTTGAAAAG	+	1–22	35
Sa31	TCATTTGA(A/G)GA(G/T)ATCAATGGCAT	+	532–554	This paper
PUU2	CCAGGCACACCAGCACAGGA	+	799–818	22
Sa5	GCTGTGCC(A/C)ACAGTCTTAGATGCC	−	1083–1106	This paper
PUU5	CCCATTCCAACATAAACAGTAGG	−	1197–1219	21
M				
A1	AATCCATCTGAGGCTACACCGTCT	+	1793–1816	20
B1	AACCCGGCAAATGAACAAGAA	+	2147–2167	20
HG2R1	TGAAACTAGCTGGGGTTG	−	2347–2364	46
C1	CCCCCTGATTGTCCTGGTGTAG	+	2369–2390	20
B2	TTGTTGGAGAGGACCGAGGAAT	−	2611–2632	20
C2	CCAACTCCTGAACCCCATGC	−	3011–3030	20

were 99.3 to 99.7% identical to those of CG18-20). Pilaski and coauthors (32) reported partial sequences of the M (nt 2488 to 3319) and the S (nt 173 to 1238) segments determined by analysis of a urine specimen from one severe NE case in Germany. No sequences of wild-type PUU strains from Germany have been reported so far; therefore, no comparisons with such strains have been done.

The present study was designed to (i) determine and analyze, for the first time, PUU nucleotide sequences of both the M and S segments from Finnish NE patients and (ii) investigate whether a human case of NE could be traced to a particular natural focus of PUU infection.

MATERIALS AND METHODS

Patients. Twenty acutely ill NE patients, 12 males and eight females aged 21 to 74 (mean, 45) years, all treated at Tampere University Hospital between August 1994 and January 1995, were included in the study. All followed a typical clinical course of NE, although clinical severity differed among the patients. All but three patients had a possible rodent contact in the recent past (e.g., in gardening or in cleaning summer cottages); three of the patients were farmers or farm technicians. Blood and urine samples were taken with the patients' informed consent 3 to 17 (mean, 7.0) days after the onset of symptoms. In each case the diagnosis was confirmed with an acute-phase serum by detecting the typical granular staining pattern in an immunofluorescent-antibody test using PUU-infected Vero E6 cells as the antigen (43) and/or low-avidity immunoglobulin G antibodies to PUU (15).

Clinical specimens. Peripheral blood mononuclear cells (PBMC) were isolated in Leucoprep tubes (Becton Dickinson, Franklin Lakes, N.J.) as recommended by the manufacturer. Briefly, 7 to 9 ml of blood anticoagulated with either lithium heparin or sodium citrate was centrifuged (1,500 × *g*, 30 min), and the PBMC layer was collected and washed twice with RPMI 1640 medium. PBMC were pelleted and suspended in 1 ml of guanidinium thiocyanate solution (9). Urine sediment (UR) was prepared by low-speed (2,000 × *g*) centrifugation of 50 ml of freshly collected urine. For RNA isolation, UR was suspended in 1 ml of guanidinium thiocyanate solution.

HLA typing. HLA typing was performed as described previously (27). Briefly, HLA A, B, and Cw alleles were determined by the standard microlymphocytotoxicity test. The DRB1 alleles were determined by group-specific DNA amplification followed by restriction enzyme digestion (45).

Rodents. Animal experimentation guidelines approved by the American Society of Mammalogists (1) were followed in animal studies. Bank voles (*C. glareolus*) were trapped on 6 to 8 November 1995 around the houses where patients 1 and 9 temporarily live (summer cottages). The year (1994) when the human cases occurred was a peak year for the rodent population in the area, and the following year, when rodent trapping was done, was locally a low to moderate rodent year. Trapped voles were sacrificed under anesthesia, and the lung tissues were removed and kept at −70°C. Small pieces (2 to 4 mm) of frozen tissues were used for immunoblotting and for RNA extraction.

Immunoblotting. Rodent tissue specimens were analyzed for the presence of PUU N antigen as described previously (33). Briefly, specimens were homogenized by sonication in Laemmli sample buffer, separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide (10%) gel, and immunoblotted with rabbit polyclonal antibodies raised against PUU recombinant antigen expressed as a glutathione *S*-transferase fusion protein (43). The secondary antibody was a swine anti-rabbit horseradish peroxidase conjugate (Dakopatts, Copenhagen, Denmark). The membranes were blocked and washed, and the bands were stained with *o*-phenylenediamine dihydrochloride.

RT-PCR amplification. RNA was extracted from 15 PBMC and six UR preparations of samples obtained from NE patients and extracted from five mechanically ground lung tissues from bank voles by the guanidinium thiocyanate-phenol-chloroform method (9). The RNA was denatured by incubation at 95°C for 3 min and subsequently placed on ice, before RT-PCR. The entire S segment was amplified with a single primer, S2 (sequences of primers are listed in Table 1), as follows. One hundred picomoles of primer, 5 U of Rous-associated virus 2 reverse transcriptase (Amersham International, Buckinghamshire, United Kingdom), 10 U of placental ribonuclease inhibitor, and 2 U of *Taq* polymerase in 100 μl of PCR buffer (Perkin-Elmer Cetus Instruments, Norwalk, Conn.) with 4 mM $MgCl_2$ were incubated at 42°C for 1 h, incubated at 95°C for 2 min, 60°C for 1 min, 72°C for 2 min, 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, and subsequently incubated at lower annealing temperatures of 56°C and 54°C, followed by 30 cycles at 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min. After exhaustion of the polymerase for 15 min at 72°C, the samples were kept at 4°C.

RT-PCR of the partial M segment (nt 1793 to 3030) was performed with primers A1 and C2 under the following conditions: 42°C for 1 h, 95°C for 2 min, and then 40 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Five microliters of this amplification mixture was added to 45 μl of PCR buffer and 1 U of *Taq* polymerase with 25 pmol each of either primers A1 and HG2R1 for nt 1793 to 2363, primers B1 and B2 for nt 2147 to 2632, or primers C1 and C2 for nt 2369 to 3030 and amplified for 30 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min. For the partial S-segment sequence (nt 799 to 1139), the outer primer pair Sa31 and PUU5 was used under the conditions described above for partial-M-segment PCR (except for the use of an annealing temperature of 53°C and an elongation period of 1 min at 72°C). Nested PCR was performed with primers PUU2 and Sa5, as described above except for the use of an annealing temperature of 59°C. Different rooms and sets of pipettes were used for handling of samples, for amplification and electrophoresis, and for mixing of PCR reagents.

Direct sequencing of RT-PCR amplicons. Amplified products were analyzed by electrophoresis in 2% agarose gels in Tris-acetate buffer and by staining with ethidium bromide and were purified with a Jetsorb kit (Genomed, Oeynhausen, Germany) according to the manufacturer's recommendations. Dideoxynucleotide sequencing analysis (37) was performed with an FS polymerase kit (Perkin-Elmer) according to the manufacturer's instructions on an ABI 310 automatic sequencing apparatus (Perkin-Elmer).

Cloning and sequencing of RT-PCR amplicons. Amplicons prepared with RNA from bank voles and corresponding to the entire S segment or to the partial M segment (nt 1793 to 3030) were purified from the low-melting-point 1% agarose gel (Wizard PCR preps kit; Promega, Madison, Wis.) and cloned with a pGEM-T kit (Promega), and recombinant plasmids were analyzed by sequencing with Sequenase version 2.0 (United States Biochemicals, Cleveland, Ohio) according to the manufacturer's recommendations.

Computer analyses. SeqApp and DNAsis programs for the Macintosh were used for manipulation of the sequence data. Phylogenetic analyses were performed on Macintosh Power PC 6200 with the PHYLIP program package (10). Two hundred bootstrap replicates of the sequence data were obtained (Seqboot). Distance matrices were calculated with Kimura's two-parameter model (Dnadist; ratio, 2.0). The matrices were analyzed by the Fitch-Margoliash tree fitting algorithm (Fitch) with the global arrangements option set. The bootstrap support percentages of particular branching points were calculated from these trees (Consense).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers Z69985-Z69990 and Z70201.

TABLE 2. Results of RT-PCR and HLA tests on NE patients[a]

Group (specimen)	Patient (gender, age)	No. of days[b]	RT-PCR result (segment[s])	HLA test result for allele	
				B8	DRB1*0301
1 (PBMC)	1[c] (F, 50)	9	+ (S)	+	+
	2 (M, 26)	3	−	−	−
	3 (M, 27)	7	−	−	−
	4 (F, 29)	12	−	−	−
	5[d] (M, 51)	17	−	−	−
	6 (M, 49)	6	−	ND[g]	ND
	7 (F, 54)	9	−	−	−
	8 (F, 45)	8	−	+	+
	9[e] (M, 45)	9	+ (S, M)	+	+
	10[f] (M, 46)	4	+ (S, M)	+	+
	16 (F, 74)	8	−	−	−
	17 (F, 47)	4	−	ND	ND
	18 (M, 66)	16	−	−	−
	19 (F, 44)	11	−	−	−
	20 (M, 64)	4	−	−	−
2 (UR)	10[f] (M, 46)	4	+ (M)	+	+
	11 (M, 37)	8	+ (S)	−	+
	12 (M, 25)	7	+ (S)	ND	ND
	13 (M, 55)	9	−	−	−
	14 (F, 21)	8	+ (S, M)	−	−
	15 (M, 47)	7	+ (S, M)	+	+

[a] In each case the diagnosis was confirmed from an acute-phase serum by detecting the typical granular staining pattern in an immunofluorescent-antibody test using PUU-infected Vero E6 cells as the antigen (43) and/or low-avidity immunoglobulin G antibodies to PUU (15).
[b] The number of days from onset of symptoms to sampling.
[c] Transiently hypotensive patient treated by hemodialysis five times.
[d] Treated by hemodialysis once.
[e] Symptoms included shock; treated by hemodialysis four times.
[f] Symptoms included hematemesis and shock; treated by hemodialysis once.
[g] ND, not done.

RESULTS

RT-PCR analysis of clinical specimens. The results of the RT-PCR analysis are summarized in Table 2. Based on our previous, although limited, experience, it was assumed that the sensitivity of detection of the partial S-segment sequences would be higher than that of the M-segment sequences. Thus, most of the specimens were first checked for the presence of the S-segment nucleotide sequences, and then the positive ones were analyzed for the presence of the M-segment sequences (group 1 in Table 2). Three of 15 PBMC preparations were positive for the S-segment sequences, and two of them (from patients 9 and 10) were also positive for the M-segment sequences. We believed that PBMC would be a better source of virus RNA for the RT-PCR test than UR. Therefore, most of the specimens analyzed were PBMC specimens. For five patients, however, only UR preparations were available for RT-PCR. Four of them were positive for the S-segment sequences, and two were also positive for the M-segment sequences (group 2 in Table 2). Because for one of the patients (no. 10) PBMC preparations were positive for both S- and M-segment sequences and a limited amount of UR was also available, only M segment RT-PCR was performed with that UR in order to obtain a longer product for the sequencing analysis.

Altogether, seven patients (no. 1, 9 through 12, 14, and 15) were RT-PCR positive. No PCR products were detected in two negative control samples, PBMC from patients with unrelated pathology.

Analysis of the association between RT-PCR positivity and HLA haplotypes of NE patients. Six of the seven PCR-positive and 11 of the 13 PCR-negative patients were typed for HLA alleles. Earlier we had shown that an HLA haplotype including HLA alleles B8 and DRB1*0301 was associated with severe NE with shock or prolonged uremia requiring hemodialysis (27). Therefore we focused our comparison on HLA B8 and DRB1*0301 positivity and PCR positivity. Five of six PCR-positive patients (83%) had the HLA DRB1*0301 haplotype, and only one of them had a B allele other than B8. On the other hand, only one of the 11 PCR-negative patients (9%) that were tested had the HLA B8 and DRB1*0301 alleles. The association of PCR positivity with both HLA B8 positivity and HLA DRB1*0301 positivity was clearly statistically significant ($P = 0.0276$ and $P = 0.0054$, respectively, by Fisher's exact test).

The PCR-positive and PCR-negative groups were also compared with respect to the following clinical and laboratory parameters: minimum diuresis, milliliters per 24 h; change in body weight; maximum serum creatinine, millimoles per liter; maximum blood leukocytes, 10^9 cells per liter; minimum blood thrombocytes, 10^9 cells per liter; need for dialysis; and duration of hospital treatment. According to all these parameters, PCR-positive patients had a more severe disease than PCR-negative patients, but according to the Mann-Whitney U test, the only parameter showing a statistically significant difference was the maximum leukocyte count ($P = 0.022$).

Sequence and genetic analyses of human PUU strains. Sequence analyses were performed for three patients (no. 1, 9, and 10) who suffered severe courses of NE and required hemodialysis. Paired sequence comparisons (Table 3) and phylogenetic analyses showed that the three patients were infected with PUU strains related to but distinct from all previously reported ones. Not surprisingly, the human strains, which were designated NE1, NE9, and NE10, were more similar to PUU strains from Finland than to those from other parts of Europe: while the range of sequence variability among Finnish PUU strains was 2 to 10%, that between Finnish strains and other strains from Europe was 15 to 23%. Also, on the phylogenetic trees based on nucleotide sequences of the S or the M segments (Fig. 1), the NE strains were placed within a cluster formed by Finnish PUU strains (probability, 99 to 100%).

To compare partial S and M sequences of human PUU strains with those of bank voles (*C. glareolus*) in putative places of infection, we made an attempt to trace the source of infection for patients 1 and 9. Trapping of bank voles was organized in two localities in November 1995. Trapping in an area around Teisko, 20 km north of Tampere (near the summer cottage of patient 1), yielded no bank voles. Another trapping area was situated in Virrat (80 km north of Tampere), where patient 9 temporarily lives. Trapping in a forest edge close to his summer cottage yielded 22 bank voles, but all were negative for PUU N antigen by immunoblotting. Trapping at a forest site 1.5 km from the house (continuous forest) yielded seven rodents, five of which (an old female and four youngsters, most probably her offspring) were N-antigen positive. Entire S-segment and partial (nt 1793 to 3030) M-segment sequences were detected by RT-PCR in the RNA preparations from the five N-antigen-positive bank voles. The sequences (identical for all five voles) belonged to a new type of PUU strain, which was designated PUU/Virrat/25Cg/95, or Virrat for short.

The M-segment nucleotide sequence of PUU strain NE9 (detected in the patient 9 specimen) was very close (98.2% identity) to that of strain Virrat (Table 3), suggesting a genetic linkage between the wild-type rodent PUU and NE9. Surprisingly, the S-segment sequences of these two strains were less

TABLE 3. Percentage of identity between nucleotide sequences of the strains obtained from the NE patients and those of known PUU strains

Segment and strain	% Identity with strain[a]:										
	NE1	NE9	NE10	Virr[b]	Sotk	Evo	Puu	Vind	Berk	Paris	1820
S[c]											
NE1	100	92.4	94.1	93.0	**97.6**	92.4	94.1	82.9	81.2	78.2	81.2
NE9		100	91.0	**95.8**	91.6	91.6	90.6	82.5	82.5	81.1	85.4
NE10			100	92.4	**94.7**	92.0	93.3	82.7	81.3	80.0	83.6
M[d]											
NE9		100	94.1	**98.2**	89.9	NA[e]	NA	81.6	77.2	81.1	82.8
NE10			100	**95.9**	89.6	NA	NA	80.8	78.0	83.1	84.2

[a] Numbers corresponding to the closest sequence are in boldface. Abbreviations: Virr, PUU/Virrat/25Cg/95; Sotk, PUU/Sotkamo (43); Evo, PUU/Evo/12Cg/93; Puu, PUU/Puumala/1324Cg/79 (34); Vind, PUU/Vindeln/L20Cg/83 (20); Berk, PUU/Berkel (32); Paris, PUU/Paris 90-13 (4); and 1820, PUU/Bashkiria/1820Cg (11, 39).
[b] Obtained from a putative place of infection for patient 9.
[c] Comprised of nt 819 to 1037 (nt 831 to 1037 for NE9).
[d] Comprised of nt 2168 to 2610 (nt 2484 to 2610 for Berk).
[e] NA, sequence data are not available.

related (95.8% identity). On phylogenetic trees (Fig. 1) strains NE9 and Virrat were situated very close to each other and formed a separate branch with bootstrap probabilities that, again, were much higher for the M tree than for the S tree (94 and 68%, respectively).

Human PUU strain NE1, according to its S sequence, was close to the Finnish PUU prototype strain Sotkamo (Table 3 and Fig. 1). Unfortunately, our attempts both to prepare M amplicons from specimens from patient 1 and to trap bank voles in a putative site of infection were unsuccessful. Human strain NE10 was on both phylogenetic trees situated between the Virrat-NE9 branch and the Sotkamo strain. It is of interest that according to its S sequence, this strain is closer to the Sotkamo strain than to the Virrat or NE9 strain, while its M sequence is more similar to that of Virrat. The source of infection for patient 10 thus remains unknown.

DISCUSSION

PCR detection of PUU genomic sequences in clinical specimens: correlation with the genetic status of patients. Accumulation of genetic data on human PUU strains is essential for understanding the nature of factors that determine the pathogenicity of the virus and influence the process of infection. To avoid changes in the virus that might be induced by an isolation procedure, genome sequences should be detected directly in clinical specimens. But so far, only two reports have been published on the topic (22, 32), and the sequences of both S and M segments of PUU have been determined for one patient only (32). The low concentration of PUU RNA in human specimens makes direct sequence analysis quite difficult. Our data showed that about one-third of the PBMC and/or UR specimens from acute-phase NE patients are PUU genome positive. This value is higher than the percent PCR positivity observed earlier for the whole-blood specimens (22). Interestingly, the frequency of PCR-positive findings in six UR specimens was even higher than that in PBMC specimens (Table 2). Whether UR is a better source of PUU genomic sequences in NE cases needs to be confirmed with a larger number of specimens.

PCR positivity, which reflects virus genome levels in the body fluids during PUU infection, was clearly associated with the HLA types B8 and DRB1*0301 of NE patients. Thus, our data represent the first case in which the virus level during an acute infection was proved to be connected to certain HLA haplotypes. The HLA alleles B8 and DRB1*0301 are in linkage disequilibrium (2), and they belong to a haplotype which was previously shown to be associated with a severe course of NE (27). The mechanisms of these associations are not clear but emphasize the role of host immune response in the pathogenesis of severe NE. The antiviral response either may be dysfunctional and fail to clear the virus, or may not only fail to clear the virus but also function with inappropriate efficacy that mimics autoimmune reactions and thus contributes to the pathogenesis. The array of possible responses is large, as the whole major histocompatibility complex region contains more than 50 genes, including, in addition to HLA genes that are involved in antigen presentation, complement components and tumor necrosis factor (7). Thus, anti-PUU and other immunological host responses during acute NE in patients with HLA B8 and DRB1*0301 haplotypes await further characterization.

The clinical course of NE in PCR-positive patients appeared more severe than that of the average disease in the PCR-negative patients. Thus, of the 13 PCR-negative patients, only one needed hemodialysis, whereas of the seven PCR-positive patients, three needed hemodialysis. However, due to the small numbers, we were not able to correlate most clinical parameters to PCR positivity with statistical significance, and thus more data should be accumulated.

Comparison of PUU sequences from NE patients with those from infected bank voles. The nucleotide sequences of three human PUU strains, NE1, NE9, and NE10, were closely related to those of the previously reported Finnish PUU strains which originated from bank voles, namely strains Sotkamo, Evo, and Puumala (Table 3). All strains from Finland form a separate cluster on the phylogenetic trees (Fig. 1) and, therefore, share an ancient ancestor. These data are in good agreement with the geographic clustering of PUU genetic variants that we have found previously (34, 35).

A putative epidemiological linkage of PUU genomic sequences from NE patient 9 and from *C. glareolus* trapped 1 year later in the natural focus around Virrat was suggested by analysis of nucleotide sequences of the M genome segments (Table 3 and Fig. 1). The two sequences were 98.2% identical within the region studied. A similar level of identity (99%) has been reported for the complete M sequences determined with specimens from a hantavirus pulmonary syndrome (HPS) patient and a wild-type New York hantavirus from a white-footed mouse in the northeastern United States (19). Nichol et al. (28) have reported 99 to 100% identity between partial M-segment sequences of Sin Nombre hantavirus (SN) derived

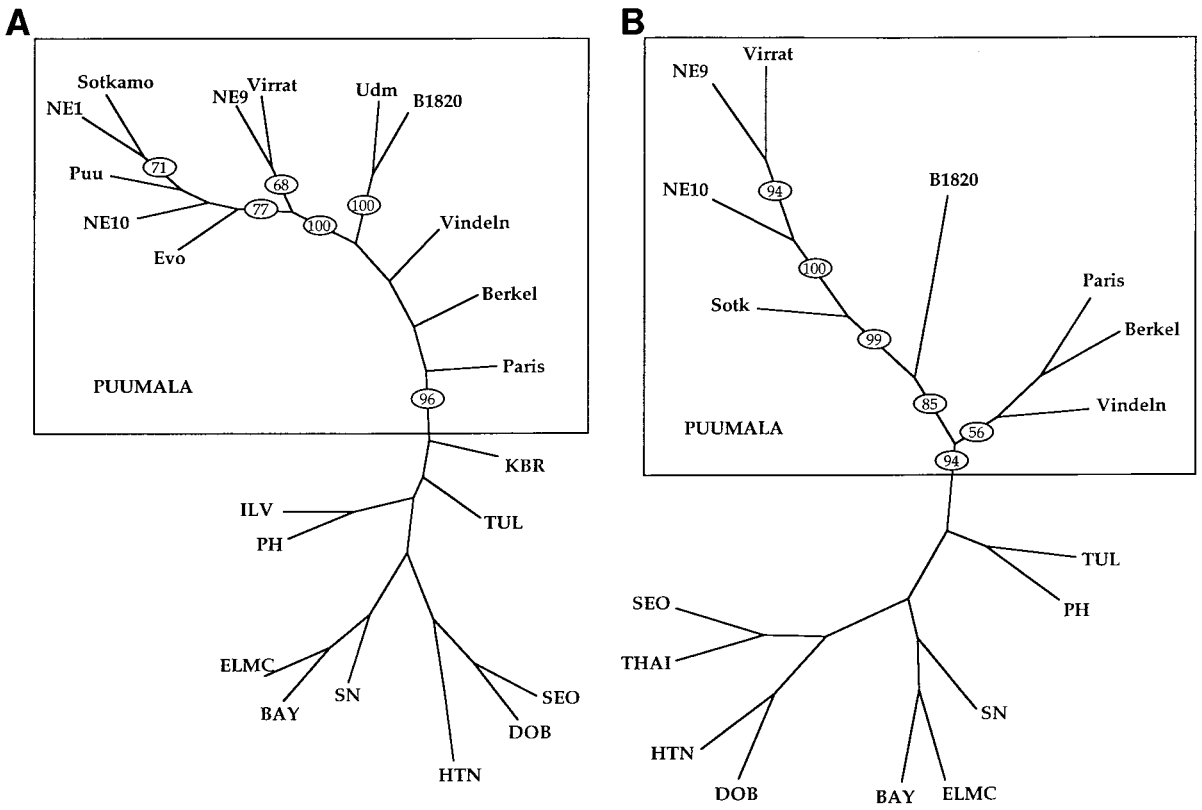

FIG. 1. Phylogenetic trees (consensus) of hantaviruses based on S-segment (nt 819-1037) (A) and M-segment (nt 2168 to 2610) (B) nucleotide sequences. Viruses are designated as follows: Sotkamo, PUU strain Sotkamo (GenBank accession numbers X61035 and X61034 for S and M segments, respectively); Virrat, PUU strain Virrat/25Cg/95 (Z69985 and Z70201); NE1, PUU strain NE1 (Z69986 for the S segment); NE9, PUU strain NE9 (Z69987 and Z69988); NE10, PUU strain NE10 (Z69989 and Z69990); Puu, PUU strain Puumala/1324Cg/79 (46942 for the S segment); Evo, PUU strain Evo/12Cg/93 (Z30702 for the S segment); Udm, PUU strain Udmurtia/894Cg/91 (Z21497 for the S segment); Vindeln, PUU strain Vindeln/L20Cg/83 (Z48586 and Z49214); B1820, PUU strain Bashkiria/CG1820 (M32750 and M29979); Berkel, PUU strain Berkel (L36943 and L36944); Paris, PUU strain Paris 90-113 (U22423 and U22418); TUL/M02V, Tula virus strain Moravia/5302V/95 (Z69991 and Z69992); PH, Prospect Hill virus strain PH-1 (Z49098 and Z55129); ILV, Isla Vista virus strain MC-SB-1 (U31534 for the S segment); KBR, Khabarovsk strain Mf43 (U35254 and U35255); SN, SN strain H10 (L25784 and L25783); BAY, Bayou virus strain Louisiana (L36929 and L36930); ELMC, El Moro Canyon virus strain RM-97 (U11427 and U26828); SEO, Seoul virus strain SR-11 (M34882 and M34881); HTN, Hantaan virus strain 76118 (M14627 and M14627); DOB, Dobrava virus (L41916 and L33685); and THAI, Thailand virus strain 749 (L08756 for the M segment).

from three HPS patients and those derived from deer mice trapped at or near the patients' residences in the Four Corners region of the southwestern United States. However, the region of comparison described by Nichol et al. (139 nt) was >3-fold shorter than the PUU M sequences we compared. It should also be taken into consideration that in our case we were searching within a rather large recreation area where the contact might have happened a year ago. Thus, we expected to find a bank vole strain(s) as similar to the human one as strains circulated within the same rodent population are similar to each other (34). And this scenario, indeed, worked for the M-segment sequences.

However, the S-segment sequences of PUU strains NE9 and Virrat were much less related (4.2% of nucleotides different) than the M-segment sequences (1.8%). Such a difference is atypical for hantaviruses in general (16, 24) and for PUU genetic drift in particular (3, 20, 24, and our unpublished observations). In our opinion, the most likely explanation for these findings is a cocirculation in bank voles in the Virrat focus of two types of PUU strains, NE9-like strains and Virrat-like ones, which differed much more in their S segments than in their M segments. A similar situation was reported by Li and coworkers (24) for two isolates of SN hantavirus from California: these isolates had M segments that differed by only 1% from each other but S segments that differed by 13%. Based on the data, the authors suggested a reassortment in nature among SN genetic variants. Another group of researchers (16, 36) came to the conclusion that reassortment occurred between SN viral genomes in deer mouse populations in Nevada and California based on phylogenetic analyses and pairwise comparisons of sequence identities. They described a cocirculation of two main genetic sublineages of SN, designated S1M1L1 and S2M2L2 according to their genome constellations, as well a number of reassortants (e.g., S1M2L1). Remarkably, all three strains (from HPS patients) described by Henderson et al. had the same reassortant genotype, S2M1L2, despite being from different geographic locations. Along these lines, a reassortant nature of the PUU strain acquired by patient 9 from a local bank vole seems possible. Interestingly, for the human strain NE10 we also cannot exclude a reassortant nature: the strain was most closely related to the Sotkamo strain in its S gene sequence but to the Virrat strain in its M sequence. Whether the putative wild-type PUU strains which infected the patients possessed a greater virulence than the Virrat or Sotkamo strain, similar to what was proposed for reassortant SN (16, 38), remains unknown.

An alternative possibility to explain the S- and M-segment differences would be a simultaneous or sequential infection of a patient by two distinct wild-type PUU strains following by reassortment between their genomes and elimination of both

parental viruses. The second scenario, however, looks less realistic than the first, as occasional contacts of humans with infested urine or feces are, obviously, less frequent that contacts of rodents inhabiting the same territory. Analyses of a greater number of rodent samples from Virrat and other foci as well as clinical specimens from NE patients will be necessary to clarify this point.

Although human epidemics caused by PUU in northern Europe follow the local cycle of the natural host of the virus (5, 18, 29), risk factors for the infection and, in particular, practical ways of transmission are poorly understood. A recent serological survey revealed an overall PUU antibody seroprevalence of 11% in European mammalogists, and the corresponding value for Finnish rodent specialists was 50%, suggesting a high risk of contracting the infection after contact with PUU natural hosts in areas to which PUU is endemic (44). When interviewed, patient 9 said that he is very active in picking berries and collecting mushrooms. He had been walking in the forests, all around the area within 3 km from his house, including the area where positive bank voles were trapped 1 year later. One cannot exclude the possibility that PUU infection in this case was connected to the patient's habits. Special safety precautions for activities in rodent-infested areas have been recommended in the United States, where SN causes HPS with a mortality rate of 50% (8), but no analogous guidelines exist for Nordic countries in view of the relatively low mortality rate for PUU-caused NE. However, the facts that dozens of NE patients require transient hemodialysis treatment and several lethal cases have been reported suggest the necessity of similar guidelines that should be based, above all, on accurate mapping of natural PUU foci.

In summary, our data are consistent with the view that both the genetic characteristics of the virus and the genetic susceptibility of patients can contribute to the outcome of hantavirus-human interaction.

ACKNOWLEDGMENTS

We thank Raija Leveelahti, Leena Kostamovaara and Anja Virtanen for expert technical assistance.

This work was supported by grants from the Medical Research Council of the Academy of Finland, the Medical Research fund of Tampere University Hospital, and the Sigrid Jusélius Foundation, Helsinki, Finland.

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