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# Detection and Subsequent Sequencing of Puumala Virus from Human Specimens by PCR

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**A sensitive method based on PCR was developed for the detection of Puumala virus (PUU) in human samples. The assay was found to be specific for PUU-like strains and distinguished between these and hantaviruses of other serotypes. The detection limit was found to be $10^{-5}$ focus-forming units. Clinical samples were collected from patients with nephropathia epidemica in Sweden and western Russia. Five whole blood samples collected from patients in Russia with the acute phase of disease were found to be positive by the PCR. All samples were negative for PUU antigen when examined by enzyme-linked immunosorbent assay. Virus isolation on Vero E6 cells from several of the acute-phase samples, including the 5 PCR-positive samples, was not successful. The amplified samples were subjected to direct nucleic acid sequencing for confirmation of identity. The sequences differed from each other and were closely related to the Russian bank vole isolate CG-1820, thereby indicating the origin of nephropathia epidemica. The PCR was used for amplification and subsequent nucleotide sequencing of eight PUU-like isolates with different geographic origins. The Swedish strains were more closely related to the Finnish PUU prototype strain, Sotkamo, than to the Russian isolates. Interestingly, a Belgian isolate, CG-13891, differed markedly from all other PUU strains.**

Puumala virus (PUU), a member of the genus *Hantavirus* within the family *Bunyaviridae*, is associated with the human disease nephropathia epidemica (NE) (5, 32). NE belongs to a group of several diseases commonly referred to as hemorrhagic fever with renal syndrome, which is characterized by fever, renal failure, and in severe cases, hemorrhagic manifestations (52). NE occurs in Scandinavia, Finland, western Russia, and central Europe (19).

The hantaviruses are enveloped viruses with a tripartite (large, medium and small segment) negative-sense, single-stranded RNA genome packed in helical nucleocapsids (7). The genome encodes four structural proteins; the L segment encodes a large protein (L) presumed to be an RNA-dependent RNA polymerase (1, 39), the M segment encodes two glycosylated envelope proteins (G1 and G2), and the S segment encodes a nucleocapsid protein (N) (7).

Members of the genus *Hantavirus* can be divided into at least six serotypes (6). The representatives of each serotype are Hantaan, Seoul, PUU, Prospect Hill, Thailand, and Thottapalyam viruses, respectively. Hantaviruses are transmitted to humans by aerosolized excretions from infected rodents (18).

The possibility of a prolonged PUU infection in individuals convalescing from NE has been suggested in several studies. Sequentially drawn sera have been shown to have increasing antibody and neutralization titers against PUU during the first year after infection, and the titers were equally high even after more than 10 years (14, 33). The immunoglobulin G (IgG) subclass pattern and specific responses against the viral structural components also suggested a prolonged PUU infection in humans (23, 26). Viral persistence has been demonstrated in rodents with experimental Hantaan virus infection in *Apodemus agrarius* (18), Seoul virus in *Rattus norvegicus* (2, 43), and PUU in *Clethrionomys glareolus* (51).

To address the question of whether PUU antigen or RNA could be detected in samples from patients with NE, we used a sensitive immunoassay for the detection of viral antigen (25) and adapted the PCR (28, 37) for the detection of PUU RNA and subsequent nucleic acid sequencing of amplified fragments. The PCR was further used to amplify and partially sequence PUU strains from Sweden, Russia, Belgium, and Bosnia-Herzegovina.

Several investigators have developed PCR for hantavirus (2, 9, 11, 36, 50). The present study, however, reports a PCR applied on acute- and convalescent-phase samples from patients with NE and with subsequent nucleic acid sequencing of amplified products, thereby ensuring the accuracy of the study.

## MATERIALS AND METHODS

**Samples.** The presence of specific IgM antibodies to PUU in 10 patients admitted to the Sundsvall County Hospital during 1991 and 1992 was serologically confirmed by a μ-capture enzyme-linked immunosorbent assay (ELISA) (31). Blood samples were obtained from the patients on admittance to the hospital (2 to 6 days after the onset of disease). Additional serum samples were drawn from each patient approximately every 2 days during their hospitalization (4 to 13 days after the onset of disease) and 2, 3, and 6 weeks and 6 and 12 months after discharge. Urine samples were taken from each patient on admission to the hospital. Serum and whole blood samples in heparinized tubes were drawn from 14 separate patients convalescing from NE 6 to 18 months after the onset of disease. All samples were sent by mail to the Swedish Institute for Infectious Disease Control and were delivered 1 to 2 days later. The lymphocyte fraction was collected by Lymphoprep gradient separation (Nycomed Pharma A/S, Oslo, Norway) according to the manufacturer's instructions and was frozen in 10% dimethyl sulfoxide and 20% fetal calf serum in RPMI medium (Gibco, Paisley, Scotland). Fifty heparinized whole blood samples from patients with the acute phase of NE (2 to 11 days; average, 6 days) were collected in Ufa, western Russia. The samples were serologically confirmed to be positive for the presence

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TABLE 1. PUU oligonucleotide primer sequences and PCR products

| Primer | Nucleotides position no. | Polarity | Primer sequence (5′-3′) | Product |
|---|---|---|---|---|
| PUU 1 | 770–790 | Positive | AGT GCC CAT TCA TAA AGC CTG | 391 |
| PUU 4 | 1140–1160 | Negative | GTT CGA CGC AGG TAA GAT TGG | |
| PUU 2 | 799–818 | Positive | CCA GGC ACA CCA GCA CAG GA | 210 |
| PUU 3 | 989–1008 | Negative | TGT TGG GCA TCG GTC TG | |

of specific IgM antibodies to PUU by the μ-capture ELISA. All samples were stored at −70°C for a maximum of 2 years until they were examined by PCR.

**Viruses.** Supernatants from Vero E6 cells infected with PUU (Sotkamo) containing $1.25 \times 10^5$ focus-forming units (FFU)/ml were used for the development of the PCR and as a positive control. The other hantaviruses included in the study are listed in Table 1.

**Preparation of samples for PCR.** The samples were prepared for extraction of nucleic acids as follows. Patient sera were diluted 1:5 in water. Urine samples were clarified by centrifugation at 500 × *g* for 15 min. Lymphocytes were diluted to $10^6$ cells per ml. Heparinized whole blood was diluted 1:10. Supernatants from virus-infected Vero E6 cells were clarified by centrifugation as described above. Each sample (0.5 ml) was extracted once with phenol (pH 7.8), once with phenol-chloroform, and once with choroform and was finally precipitated with 2.5 volumes of ethanol–0.3 M sodium acetate at −20°C for 2 h or until it was used in the PCR. The precipitates were collected by centrifugation at 20,000 × *g* for 15 min 4°C and were dissolved in 50 μl of TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). The extractions were performed essentially as described by Maniatis et al. (27).

**Primers.** Four primers were selected from the S segment of the PUU Sotkamo sequence (47). The outer primer pair, PUU 1 and PUU 4, resulted in an amplification product of 319 bp, and the inner primer pair, PUU 2 and PUU 3, resulted in an amplification product of 210 bp (Table 2). Primers and annealing temperatures were selected with the aid of a computer program (OLIGO; National Biosciences Inc., Hamel, Minn.). The primers were synthesized by Scandinavian Gene Synthesis (Köping, Sweden).

**Reverse transcription.** Three microliters of the precipitated samples was added to 17 μl of the following mixture: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 7 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM (each) deoxynucleoside triphosphate (dNTP; Perkin-Elmer), 0.75 μM (each) primers PUU 1 and PUU 4, 10 U of placental RNase inhibitor (Gibco BRL, Gaithersburg, Md.), and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) in micro-reaction tubes (Perkin-Elmer Cetus Instruments, Norwalk, Conn.). The mixtures were overlaid with 100 μl of mineral oil (Perkin-Elmer) and were incubated for 60 min at 37°C and 5 min at 99°C and were subsequently cooled to 4°C.

**Amplification 1.** The cDNA generated by the reverse transcription described above was amplified by adding 80 μl of the following mixture to each tube: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Perkin-Elmer). The samples were amplified in a Perkin-Elmer Cetus thermocycler under the following conditions: 95°C for 1 min and then 30 cycles of 95°C for 1 min, 53°C for 45 s, and 72°C for 30 s, with 3 s of extension per cycle, and subsequently at 72°C for 10 min.

**Amplification 2.** A nested PCR strategy was used. Five microliters of the mixture from amplification 1 was added to 45 μl of the following mixture: 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 U of *Taq* polymerase (Perkin-Elmer), 1 mM (each) dNTP, and 0.75 μM (each) primer PUU 2 and PUU 3. The mixture was incubated at 95°C for 1 min and amplified for 26 cycles at 95°C for 1 min, 59°C for 45 s, and 72°C for 30 s, with 3 s of extension per cycle, and subsequently at 72°C for 10 min. Amplification reactions (10 μl of each sample) were analyzed by electrophoresis in 2% agarose gels in Tris-acetate buffer, and the reaction products were stained with ethidium bromide (27). The reverse transcription and the DNA amplifications were optimized by box titrations of primer, $MgCl_2$, reverse transcriptase, and *Taq* polymerase concentrations and by various annealing temperatures and times, extension times, and denaturing conditions.

**Controls used for PCR.** Nucleic acids from sera and lymphocytes from 10 patients with Ockelbo disease were extracted in parallel with the samples from patients with NE and were included in the original amplification experiments to exclude the possibility of nonspecific amplification of human DNA or other infectious agents. Ockelbo disease, characterized by rash, arthralgia, and fever, is caused by a positive-strand RNA alphavirus that is transmitted to humans from birds via mosquitoes and is endemic in central Sweden, Finland, and Karelian parts of Russia (15). Several PCR reagent controls and one positive control consisting of an RNA preparation from a PUU (Sotkamo)-infected cell supernatant, diluted $1{:}10^{-5}$, were included in all experiments. Different rooms and sets of pipets were used for the handling of samples, for amplification and electrophoresis, and for mixing of PCR reagents. All specimens were tested at least twice by PCR. The specificities of the amplified products were confirmed by direct sequencing.

**Direct dideoxy sequencing.** The PUU 2 and PUU 3 primers were radioactively labelled by incubating 10 pmol of primer with 8 U of T4 kinase (Amersham) and adenosine 5′ [γ-$^{33}$P]triphosphate (Amersham) in 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–7 mM DTT in a reaction volume of 10 μl for 30 min at 37°C and 5 min at 55°C. Dideoxy sequencing (38) in both directions was performed by using a kit (Hot tub; Amersham) essentially as described by the manufacturer. The sequencing reaction was performed under the following conditions: 84°C for 2 min and then 30 cycles of 84°C for 1 min, 48°C for 1 min, and 62°C for 1 min. Five microliters of each mixture was loaded onto denaturing 6% polyacrylamide-urea gels. The gels were run at 50°C with constant voltage (1,400 V). After gel electrophoresis, the gels were fixed in 10% methanol and 10% acetic acid for 30 min, dried on a vacuum gel dryer (Bio-Rad, Richmond, Calif.) to sequencing-grade filter papers (Bio-Rad), and autoradiographed to Hyperfilm βmax films (Amersham) for 24 to 72 h.

**Hantavirus antigen ELISA.** Urine and convalescent-phase lymphocyte preparations were analyzed for hantavirus antigen by ELISA as described previously (25). Briefly, the ELISA was constructed with protein G-purified bank vole monoclonal antibodies (MAbs) directed to different epitopes on the PUU nucleocapsid protein (24). The samples were diluted 1:2 in RIPA buffer (0.01 M

TABLE 2. Histories of hantavirus strains used in the study

| Virus strain | Species | Geographic origin | Reference or source | PCR reactivity |
|---|---|---|---|---|
| 83-223L | *Clethrionomys glareolus* (lung) | Sweden | 32 | + |
| 83-L20 | *Clethrionomys glareolus* (lung) | Sweden | Trapped in Hällnäs in 1983 | + |
| 83-S41 | *Clethrionomys glareolus* (lung) | Sweden | Trapped in Hällnäs in 1983 | + |
| Sotkamo | *Clethrionomys glareolus* (lung) | Finland | 4 | + |
| K27 | Human (blood) | Bashkiria, Russia | 40 | + |
| H45 | Human (blood) | Bashkiria, Russia | E. A. Tkachenko, Moscow | + |
| P360 | Human (lung) | Bashkiria, Russia | E. A. Tkachenko, Moscow | + |
| CG18-20 | *Clethrionomys glareolus* (lung) | Bashkiria, Russia | 44 | + |
| Kazan | *Clethrionomys glareolus* (lung) | Russia | 8 | + |
| CG-13891 | *Clethrionomys glareolus* (lung) | Belgium | 46 | + |
| Vranica | *Clethrionomys glareolus* (lung) | Bosnia-Herzegovina | 10 | + |
| Dobrava | *Apodemus flavicollis* (lung) | Slovenia | 3 | − |
| Prospect Hill | *Microtus pennsylvanicus* (lung) | Maryland | 21 | +/−[a] |
| Hantaan (76-118) | *Apodemus agrarius* (lung) | Korea | 17 | − |
| Seoul (80-39) | *Rattus norvegicus* (lung) | Korea | 20 | − |

[a] Amplified at a lower annealing temperature.

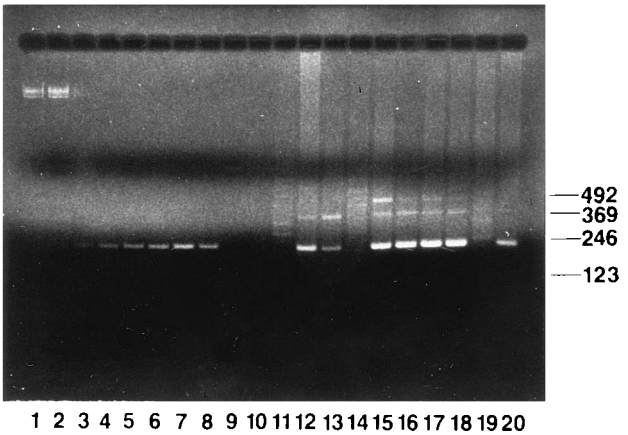


FIG. 1. Agarose gel electrophoresis of products from amplification of hantaviruses from infected Vero E6 cell supernatants. Lanes 2 to 8, 10-fold titration of strain 83-223L, from $10^{-9}$ (lane 1) to $10^{-3}$ (lane 7); lanes 9 to 10, negative controls; lanes 11 to 20, virus strains of different origins: lane 11, Hantaan; lane 12, Sotkamo; lane 13, CG-13891; lane 14, Seoul; lane 15, H-45; lane 16, K-27; lane 17, CG18-20; lane 18, P-360; lane 19, Prospect Hill; lane 20, 83-L20. The numbers on the right indicate the sizes of the DNA fragments (in base pairs).

Tris-HCl [pH 7.8], 2% Triton X-100, 0.15 M NaCl, 0.6 M KCl, 5 mM EDTA, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride) before analysis. Briefly, microtiter plates were coated with MAbs 2E12 and 5E1 (5 μg of each MAb per ml). After blocking of nonsaturated binding sites the samples were incubated for 2 h at 37°C; this was followed by incubation with biotin-labelled MAbs 1C12 and 3G5 (0.1 μg/ml) for 1 h at 37°C. Streptavidin-peroxidase (Sigma, St. Louis, Mo.) and then 3,3′,5,5′-tetramethylbenzidine (ICN Sigma) substrate were used to detect specific antibody binding.

**Virus isolation.** The acute-phase serum samples from the 10 sequentially bled Swedish patients and 15 of the Russian heparinized blood samples were inoculated onto confluent layers of Vero E6 cells (CRL 1586; American Type Culture Collection) in 25-$cm^2$ cell culture flasks (Nunc), cultivated in Eagle's minimal essential medium supplemented with 2% fetal calf serum–2 mM L-glutamine–60 μg of penicillin per ml–100 μg of streptomycin per ml. Every 2 weeks after inoculation the cells were removed from the flask with a cell scraper. One-tenth of the cell suspensions was transferred to fresh Vero E6 cell flasks. The remaining cells were pelleted by centrifugation, washed and acetone fixed to glass slides, and stained by immunofluoroscence as described previously (24). Briefly, a cocktail of PUU nucleocapsid-specific MAbs was incubated on the slides for 60 min at 37°C. After washing three times for 5 min each time in phosphate-buffered saline (PBS), fluorescein isothiocyanate-conjugated rabbit anti-mouse $F(ab')_2$ fragments (Dako, Glostrup, Denmark) diluted 1:40 in PBS were incubated and washed as described above. The experiment was terminated after 90 days.

## RESULTS

**Evaluation of PCR.** Two sets of primer pairs were chosen for the PCR and resulted in the amplification of the predicted 390-bp fragment with primers PUU 1 and PUU 4 after the first PCR with the PUU RNA preparation as the template and a 210-bp fragment with primers PUU 2 and PUU 3 after the nested PCR. No other bands were detectable. No bands were detected in any of the samples used as negative controls or in the PCR reagent controls. The sensitivity of the assay was determined by amplification of 10-fold dilutions of an RNA preparation from a cell supernatant containing $1.25 \times 10^5$ FFU of PUU per ml directly or mixed with a negative serum control diluted 1:5. It was repeatedly possible to visualize an amplified product at a dilution of $10^{-8}$, corresponding to $3.75 \times 10^{-5}$ FFU/ml (Fig. 1). The sensitivity of the assay in spiked sera was decreased 10-fold. Since it is known that the reverse transcription may be inhibited in sera (15) and that heparin, even in minute quantities, inhibits the *Taq* polymerase (13), the positive control was amplified in different dilutions of samples. It was found that dilutions of less than 1:5 for sera and 1:10 for heparinized sera and more than $10^6$ lymphocytes per ml inhibited the PCR.

TABLE 3. Nucleotide sequence differences in the amplified fragment from five human samples compared with the sequence of the CG18-20 isolate[a]

| Base in CG18-20 (position no.) | Sequence difference in: | | | | |
|---|---|---|---|---|---|
| | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
| G (864) | A | A | A | — | A |
| G (888) | A | —[b] | — | — | A |
| G (906) | A | A | — | — | A |
| G (939) | A | A | A | — | A |
| T (960) | C | C | — | — | C |
| T (981) | G | G | G | G | G |

[a] The sequence of isolate CG18-20 was from Stohwasser et al. (41).
[b] —, the same nucleotide as that in the sequence of CG18-20.

**Amplification of hantaviruses.** Eleven PUU-like isolates, Hantaan (76-118), Seoul (80-39), Dobrava, and Prospect Hill viruses, were examined by the PCR. The histories of the isolates are summarized in Table 2. All the PUU-like isolates were amplified and resulted in fragments with apparently identical sizes (Fig. 1). Hantaan, Seoul, and Dobrava viruses were not amplified by the PCR. By lowering the annealing temperatures to 45 and 50°C in PCR 1 and PCR 2, respectively, Prospect Hill virus could be amplified, resulting in a slightly larger fragment (data not shown).

All PCR products were sequenced by direct PCR sequencing. The amplified DNA was sequenced in both directions. The sequencing reactions were performed at least twice on separately amplified material.

**Analysis of human samples.** Urine, sera, lymphocytes, and whole blood samples were analyzed by the PCR. Five samples scored positive and could be analyzed by direct sequencing. These samples were all acute-phase whole blood samples from Russia drawn at 4, 6, 6, 7, and 9 days after the onset of disease, respectively. The obtained sequences were, with one exception, found to be distinguishable from each other but were closely related to the Russian strains CG18-20, K27, H45, and P360. The substituted bases, in comparison with the sequence of CG18-20, are illustrated in Table 3. The mutations were found to be nonrandomly distributed and silent. The sequence homologies of the acute-phase blood samples to CG18-20 were found to be 96.5, 99.4, 97.7, 98.2, and 96.5%, respectively.

The hantavirus antigen-ELISA was evaluated by titration of PUU Sotkamo in RIPA buffer-treated sera, urine, and lymphocyte preparations. It was found that the detection limit in urine and lymphocyte preparations was 50 FFU/100 μl of sample except in 25% of the urine samples, for which the sensitivities were reduced fourfold, and that sera could not be analyzed because of nonspecific reactions. It was, however, not possible to detect viral antigen in any of the acute-phase urine specimens or in the lymphocyte preparations drawn from patients with NE.

Virus isolation attempts were performed on the acute-phase sera from the 10 patients and 15 of the Russian whole blood samples, including the 5 samples scoring positive in the PCR. Six blind passages during 3 months on Vero E6 cells were performed, and the cells were examined for the presence of PUU on each passage. No viral antigen could be detected in any of the cells.

**Nucleotide sequence analysis of hantaviruses.** The sequences of the Russian isolates CG18-20, K27, P360, and H45 were found to be indistinguishable; that for K27, however, had

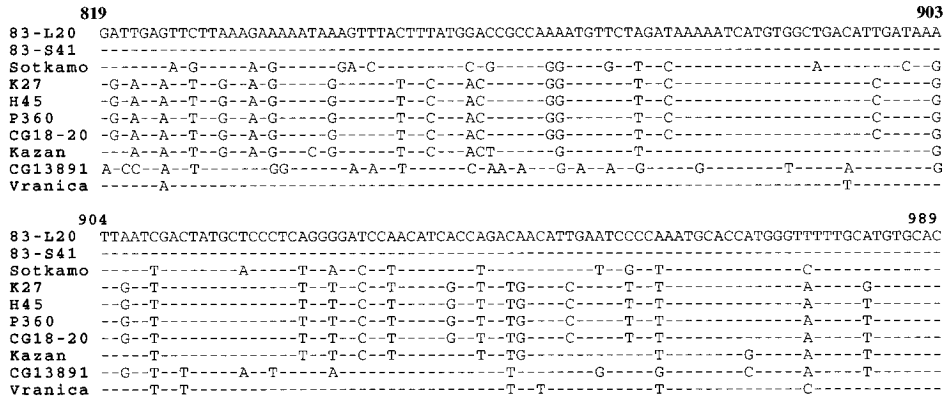


FIG. 2. Nucleotide sequence from positions 819 to 988 of amplified PUU-like strains compared with that of the Swedish PUU strain 83-L20. The hyphens indicate homology to the 83-L20 sequence.

a silent T-to-G change at position 981, not giving rise to any amino acid substitutions. Another Russian isolate, Kazan, passaged in bank voles, differed in 7.1% of the nucleotides from the other Russian isolates, but the predicted amino acid sequence was identical to those of the other Russian isolates. The sequences of Swedish isolates 83-S41 and 83-L20 were found to be identical to each other but were clearly distinct from those of the Finnish and Russian isolates, with nucleotide homologies of 83.5 and 80.6%, respectively.

The isolate Vranica, from Bosnia-Herzegovina, was found to be more closely related to the Swedish isolates than to the Russian isolates, with 95.3 and 81.2% nucleotide homology, corresponding to 0 and 10.7% substituted amino acids, respectively. The Belgian isolate CG-13891 differed markedly from all other PUU-like strains. The homologies to the Swedish, Finnish, and Russian isolates were 80.6, 77.6, and 76.5%, respectively, corresponding to 87.5, 89.3% and 85.7% amino acid sequence homology, respectively. The nucleotide sequences are depicted in Fig. 2.

The Swedish isolate 83-223L was sequenced and found to be identical to the Finnish prototype strain Sotkamo. However, an early passage of this isolate was different from that of Sotkamo but indistinguishable from that of the Swedish strain 83-L20, isolated in the same area in the same year. It is therefore clear that 83-223L has been mixed with Sotkamo at a relatively early stage. The sequences of Sotkamo, CG18-20, K27, and P360 that were obtained were compared with those published by other investigators (41, 47, 49). The results were found to be identical except for those for K27, in which the published T-to-C and G-to-A substitutions at positions 849 and 952, respectively, were not observed (Table 3).

## DISCUSSION

A sensitive PCR for the detection of PUU was developed and evaluated. By use of a nested strategy, the PCR was shown to amplify RNA from a dilution corresponding to $10^{-5}$ FFU. From these data, the number of amplifiable genomes can be estimated to be approximately 100,000 genomes per FFU. The relatively high genome/FFU ratio achieved in our system may reflect the difficulties of growing PUU in cell culture and may not be directly correlated to the detection limit of viral genomes in clinical material. The antigen ELISA was found to detect nucleocapsid protein from as few as 50 FFU, which was equal to the sensitivity achieved in immunoblots (25).

Only the predicted band of 210 bp could be detected on ethidium bromide-stained agarose gels after the nested PCR. The two primer pairs were found to be reactive with PUU-like strains of different geographical origins. The primer pairs did, however, distinguish between PUU-like strains and Seoul, Hantaan, and Prospect Hill viruses. Prospect Hill virus could be amplified at lower annealing temperatures, which is in accordance with the published sequence of this virus (34).

Five of the specimens scored positive by the PCR. All five specimens were whole blood samples collected from patients in Ufa, Bashkirtostan (European part of Russia), during the first 10 days after the onset of illness. Viral antigen could not be detected by the ELISA in any of the samples, and virus isolation attempts performed on several samples, including those which scored positive in the PCR, were unsuccessful.

It has been reported that the use of peripheral blood monocytes rather than plasma allows the isolation of Hantaan virus (53). It has also been shown that antigen can be detected in human B and T cells during the early stages of Hantaan virus infection (12). These data are concordant with our results, since PUU RNA was detected in whole blood preparations rather than in sera. Whether our finding of PUU RNA only in Russian samples indicates a difference in the pathogenesis of the Russian and Swedish virus strains or that whole blood samples were not available from the Swedish patients remains to be elucidated. We could not detect PUU RNA in the convalescent-phase sera or lymphocyte preparations. However, before the theory of viral persistence can be abandoned, additional samples such as lung and kidney tissue must be investigated. It can also not be excluded that PUU may have been present in the samples collected in Sweden, although in amounts below the detection limit of the PCR.

Nucleotide sequence analysis of the isolates from the five PCR-positive Russian patients showed that they were closely related to Russian PUU isolates from both patients and rodents. This provides additional evidence that the virus found in the rodents is responsible for the disease. Similar data have recently been generated for hantavirus pulmonary syndrome in the United States, where direct sequencing of samples from patients and rodents has proven the etiologic link (29). The present study is, to our knowledge, the first to show this for hemorrhagic fever with renal syndrome caused by a PUU-like isolate.

Almost all nucleotide changes observed were G-to-A transitions, which is the expected result, since transitions are the most frequent nucleotide substitution mutations (45). Similar frequencies of G-to-A transitions were observed in a study of

the nucleotide substitution rate in the influenza virus hemagglutinin gene (42).

Several PUU-like isolates from different parts of Europe were amplified, and the PCR fragments were subjected to direct sequencing. The sequences that were obtained were compared with the cloning vector sequence data available in the literature. The results were identical except for those for one isolate, K27, in which two nucleotide substitutions were observed when the isolate's sequence was compared with the published sequences. Direct sequencing of PCR-amplified hantavirus RNA has been compared with traditional cloning by others, and the two methods were found to be equivalent (35).

To base relationships on short sequences may be misleading, but it has been shown that such assumptions can indeed be made for hantaviruses (48). The phylogenetic distances obtained for the 210-bp fragments sequenced in the present study differ slightly from the distances obtained with the total S segments (data not shown), but the clustering patterns are not altered.

The sequence analysis of amplified fragments of PUU-like isolates revealed that the Swedish isolates are very closely related or identical to each other but are clearly different from the Finnish strain Sotkamo. The Russian isolates are closely related to each other, but they are not identical and are clearly distinguishable from the Finnish and Swedish isolates. The Belgian strain differs markedly from all sequenced hantaviruses and may represent a separate central European strain. The isolate Vranica from Bosnia-Herzegovina was very similar to the Swedish isolates and was identical to the Swedish isolates on the amino acid level. Vranica has been confirmed to be antigenically indistinguishable from the Swedish strains by using MAbs directed against both the nucleocapsid and glycoproteins (22a). It is necessary to investigate other strains from former Yugoslav republics to determine whether this homology represents a laboratory error. The deduced amino acid sequence relationship data are in line with earlier cross-neutralization data (30) and MAb reactivities (24) for the strains investigated.

Many investigators have pointed out the risk of obtaining false-positive results by PCR because of the abundance of previously amplified material, especially when low copy numbers are to be amplified (16, 22). By preparing the samples and PCR reagents in one laboratory and using another laboratory for amplification and the analysis of samples, this risk can be substantially reduced.

We believe that PCR and then direct sequencing, as described in this report, provide a useful tool for comparing different PUU-like isolates and studying the pathogenesis of disease in both humans and animals. By sequencing the amplified fragments, information on the relationships of different virus strains will be gained and the possibility of laboratory contamination can be excluded.

## ACKNOWLEDGMENT

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