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GROUPING OF HANTAVIRUSES BY SMALL (S) GENOME SEGMENT POLYMERASE CHAIN REACTION AND AMPLIFICATION OF VIRAL RNA FROM WILD-CAUGHT RATS

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Abstract. A single pair of consensus primers in the polymerase chain reaction (PCR) amplified a conserved region of the small genome segment of twenty hantavirus isolates. Isolates tested included representatives of the four recognized hantaviruses, Hantaan, Seoul, Puumala and Prospect Hill, as well as isolates from *Mus musculus* (Leakey), *Bandicota indica* (Thailand 749), and *Suncus murinus* (Thottapalayam). Viruses from the *Nairovirus* and *Phlebovirus* genera yielded negative results. The amplification products were 281-nucleotide pairs (np) in length, with the exception of Thottapalayam, which had an amplification product of approximately 320 np. Products of all isolates were detected by Southern hybridization with a ^{32}P-labeled Hantaan 76-118 amplicon, while an oligonucleotide probe to a conserved region of the amplified fragment failed to detect some isolates of Seoul and Puumala viruses. Restriction endonuclease analysis allowed three groupings: Hantaan-like viruses, Seoul-like viruses, and a diverse group of patterns for the other viruses. Differences were found within the Seoul-like virus group by this method, whereas the Hantaan-like viruses were shown to be similar. RNA extracted from tissues of seropositive and seronegative rats trapped in Baltimore showed the practical application of the test. Hantavirus-specific RNA was detected in 12 (92%) of 13 seropositive rats, but not in seronegative rats. This simple method for detecting and characterizing hantaviruses has potential for epidemiologic studies and for diagnosing human hantavirus infections.

Viruses of the genus *Hantavirus* (Bunyaviridae) are zoonotic agents with a global distribution. Virus serotypes are maintained primarily by rodent reservoirs, and they cause a spectrum of human disease across Eurasia referred to collectively as hemorrhagic fever with renal syndrome (HFRS).[1] Four distinct hantaviruses are recognized on the basis of serologic differences, and they include Hantaan, Puumala, Seoul, and Prospect Hill viruses. Each of these viruses is primarily associated with a single species of rodent (*Apodemus agrarius, Clethrionomys glareolus, Rattus norvegicus,* and *Microtus pennsylvanicus,* respectively). A fifth virus (Leakey virus) isolated from *Mus musculus* has been proposed as a new virus type of the *Hantavirus* genus,[2] and two additional viruses, Thailand 749 from *Bandicota indica* in Thailand[3] and Thottapalayam from *Suncus murinus* in India,[4] may eventually be recognized as new viruses. Hantaviruses have also been isolated from shrews and domestic cats, in addition to some ectoparasites, but the significance of these mammals or the role of arthropod vectors in the transmission of these agents is not clear.[1]

Clinical presentation, seroconversion, and virus isolation are accepted methods for the diagnosis of HFRS, but none of these is well suited for early and specific diagnosis. Clinical presentation of HFRS is variable,[5] and in geographic regions such as eastern Asia,[6] the Balkans,[7] and western Europe,[8] at least two distinct hantaviruses occur, and each can result in markedly different diseases. Even with severe cases of HFRS a diagnosis based solely on clinical manifestations may be accurate only in approximately 50% of cases.[9] Documentation of seroconversion requires acute and convalescent sera for IgG-based assays,[10] although a positive IgM test result has promise for early diagnosis.[11, 12] In most circumstances, viral isolation is not the method of choice in aiding diagnosis. Not only are hantaviruses

difficult to isolate from patient specimens[13], but the procedure for isolation and subsequent passage of virulent strains in cell culture or animal inoculation requires strict biocontainment. The facilities and training required for this level of biocontainment are available in relatively few laboratories in the world.

The limitations of existing methods for virus identification and characterization are also evident in studies to define the probable source of viral infection and the reservoir involved in transmission. Several comparative serologic techniques for analyzing convalescent serum have been used in epidemiologic studies.[8, 14] One widely accepted test differentiates serogroup-specific viruses on the basis of cross-neutralizing antibody titers.[10, 15] However, this assay requires the maintenance of a reference collection of viruses and antisera, the availability of a biohazard containment facility, and may not be sensitive enough to differentiate between closely related virus strains.

Recent advances in the polymerase chain reaction (PCR) technology that allow detection of viral RNA in patient specimens and that provide DNA fragments for restriction endonuclease analyses could lead to a significant improvement in the rapid diagnosis of HFRS and in the understanding of the epidemiology of transmission. The polymerase chain reaction has been adapted for amplification of the medium (M) segment of the tripartite hantavirus genome.[16] The purpose of this study was to identify consensus primers and probes capable of detecting the small (S) genome segment of all hantaviruses in a PCR-based assay, to evaluate the ability of restriction endonuclease analysis to differentiate individual viruses, and to apply these techniques to the study of naturally infected animals.

MATERIALS AND METHODS

Viruses

Twenty isolates of hantaviruses were used in this study. Each isolate, its country of origin, and the animal species from which each was isolated are shown in Table 1. Two isolates were from humans, seven from a single species of rat (*R. norvegicus*), six from three species of field or house mice (*Apodemus agrarius*, *A. flavicollis*, and *M. musculus*), three from two species of vole (*C. glareolus* and *Microtus pennsylvanicus*), and one each from a shrew (*S. murinus*) and a bandicoot (*B. indica*). The initial isolations of Girard Point, Houston, and Baltimore rat viruses were performed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). Wisconsin virus was isolated at USAMRIID and at the University of Wisconsin-Madison. The other hantaviruses were provided to USAMRIID by the WHO Collaborating Center for Virus Reference and Identification (Hemorrhagic Fever with Renal Syndrome) in Seoul, South Korea.

The isolates were propagated in the E6 clone of Vero cells (C1008; American Type Culture Collection, Rockville, MD) under growth conditions described by Schmaljohn and others.[17] Briefly, 150-cm^2 plastic tissue culture flasks were inoculated with 10^4 plaque-forming units of virus seed stock. Cells were maintained in growth medium consisting of Eagle's minimum essential medium (Earle's salts) that contained 10% fetal bovine serum, 100 units of penicillin, 100 μg/ml of streptomycin, 0.5 μg/ml of Fungizone® (Gibco, Grand Island, NY), and 60 μg/ml of tylosin tartrate (Sigma, St. Louis, MO). Virus was allowed to adsorb to the cell monolayers for 1 hr before adding additional media to give a final volume of 30 ml. Virus cultures were incubated for 14–21 days before harvesting for isolation of RNA.

Several viruses from the *Nairovirus* (Crimean-Congo hemorrhagic fever virus 10200 and Gangam virus) and *Phlebovirus* (Rift Valley fever virus 501) genera of the Bunyaviridae were tested to assess the specificity of the hantavirus PCR. The Nairoviruses and Rift Valley fever virus were propagated in SW-13 cells and Vero E6 cells, respectively, in a manner similar to that for the hantaviruses. RNA from these viruses was harvested and tested by PCR.

Rat tissues

Twenty-one rats (*R. norvegicus*) were collected and processed according to methods previously described.[18, 19] Rats were captured from three alleys known to be enzootic for Baltimore rat virus, and tested for serologic evidence of infection by either indirect immunofluorescent antibody assay (IFA) or enzyme-linked immunosorbent assay (ELISA), as previously described.[18, 20]

Both fresh and frozen (up to three years) tissues were examined, since samples obtained from

TABLE 1
Characteristics of hantaviruses tested by the polymerase chain reaction

Virus	Species from which the virus was isolated	Country of origin
Baltimore rat	*Rattus norvegicus*	United States
CG 3883	Human	USSR
Girard Point	*Rattus norvegicus*	United States
Greek (Porogia)	Human	Greece
Hantaan 76-118	*Apodemus agrarius*	Korea
Houston	*Rattus norvegicus*	United States
Jinhae 494	*Apodemus agrarius*	Korea
Jinhae 502	*Apodemus agrarius*	Korea
Leakey	*Mus musculus*	United States
Maaji	*Apodemus agrarius*	Korea
Puumala	*Clethrionomys glareolus*	Sweden
Seoul	*Rattus norvegicus*	Korea
Singapore	*Rattus norvegicus*	Singapore
SR-11	*Rattus norvegicus*	Japan
Tchopitoulas	*Rattus norvegicus*	United States
Thailand 749	*Bandicota indica*	Thailand
Thottapalayam	*Suncus murinus*	India
USSR 18-20	*Clethrionomys glareolus*	USSR
Wisconsin	*Microtus pennsylvanicus*	United States
Yugoslavia (Fojnica)	*Apodemus flavicollis*	Yugoslavia

1988 through May 1991 were stored as whole tissues or pieces of tissue at −70°C. Typically, only kidney, lung, and spleen were available as frozen specimens, but salivary gland, bladder, liver, whole blood, urine, and oropharyngeal swabs were also collected from recent captures. Frozen tissues from rats with a range of antibody titers were selected for processing, since hantaviruses establish persistent infections within their rodent hosts.[21] and virus isolation has been most easily achieved using seropositive animals.[18, 22] Fresh samples were removed aseptically and tested by PCR without prior knowledge of their serologic status.

RNA extraction

Total cellular RNA from each infected or uninfected flask of cells (150 cm^2) was extracted using a guanidine isothiocyanate (GIT) procedure and processed to give a final volume of 300 μl. Each flask of cells was washed three times with 10 ml of ice cold phosphate-buffered saline, and the cells were scraped into the last wash and pelleted for 10 min at 2,000 rpm and 4°C in a Beckman (Palo Alto, CA) RT6000 centrifuge. After decanting the supernatant, the cells were resuspended in 4 ml of ice cold GIT buffer (4 M guanidine isothiocyanate, 3 M sodium acetate, pH 5.4, 1% 2-mercaptoethanol) and mixed vigorously for 1 min, followed by several expulsions through a 21-gauge needle from a 5-ml syringe. The cell lysates were then overlaid onto a 4 ml cushion of CsCl buffer (5.7 M CsCl, 3 M sodium acetate, pH 5.4) in a Beckman SW41 ultracentrifuge tube and centrifuged at 32,000 rpm (175,000 × *g*) for 21 hr at 20°C. Supernatants were carefully removed so as not to disturb the RNA pellets, and the pellets were resuspended in successive aliquots of 200 μl and 100 μl of diethylpyrocarbonate (DEPC)–treated water, and combined into 1.5-ml microcentrifuge tubes. Resuspended RNA was then extracted once by vortexing with 1/10 volume of 10% sodium dodecyl sulfate (SDS) and one volume of Tris-EDTA–saturated phenol, followed by a second extraction with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). Next, the RNA was precipitated overnight at −30°C after adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol. When ready for use, the precipitated RNA samples were pelleted in a microcentrifuge for 15 min at 10,000 × *g*, the supernatants were decanted, and the pellets were allowed to air dry before resuspending the RNA in 300 μl of DEPC-treated water.

Extraction of RNA from rat tissues followed previously described techniques.[23] and consisted of a single-step method that used the acid guanidinium isothiocyanate-phenol-chloroform

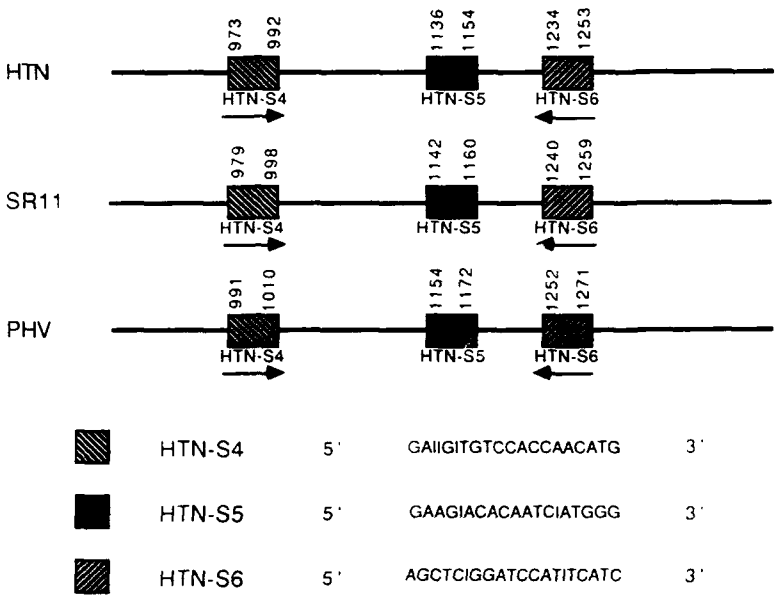

FIGURE 1. Consensus oligonucleotide primers (HTN-S4 and HTN-S6) used for amplification of hantaviruses by the polymerase chain reaction. The consensus probe HTN-S5 is also shown. The targeted sequences are on the small segment of the hantavirus genome and were selected using sequence data for Hantaan 76-118 (HTN),[24] Sapporo rat virus (SR-11),[25] and Prospect Hill virus (PHV).[26] Numbers indicate nucleotide positions.

method. Within a laminar flow biosafety hood, a 20–25-mm^3 piece of tissue was minced and subsequently homogenized in 0.5 ml of denaturing solution (solution D) containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol by repeated expulsion of the sample against the side of a 1.5-ml tube using a positive displacement pipetter. Whole blood (approximately 50–100 μl) was treated as solid tissue, while urine samples (200–1,500 μl) were centrifuged for 20 min in a microcentrifuge, approximately 75% of the fluid was decanted, and pellets were resuspended in 200 μl of a solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μg/ml of proteinase K. Oropharyngeal samples were collected with Dacroswabs® (Spectrum Laboratories, Los Angeles, CA) and were placed directly in 0.5 ml of solution D and firmly squeezed against the tube. To all samples, 50 μl of 2 M sodium acetate, pH 4.0, 0.5 ml of phenol, and 0.1 ml of chloroform:isoamyl alcohol (49:1) were sequentially added. Samples were cooled on ice for 15 min and centrifuged at 10,000 × *g* for 20 min at 4°C. The aqueous phase containing the RNA was transferred to a fresh tube, mixed with 0.5 ml of isopropanol, and placed at −20°C for at least 1 hr. Sedimentation of precipitated RNA was performed at 10,000 × *g* for 20 min, and the pellet was dissolved in 0.3 ml of solution D and precipitated with one volume of isopropanol at −20°C for 1 hr. The precipitate was centrifuged at 4°C for 10 min in a microcentrifuge and the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried for 15 min, and resuspended in 50 μl of DEPC-treated 1.0% Laureth 12 (Mazer Chemicals, Gurnee IL) at 65°C for 10 min.

Polymerase chain reaction

Selection of primers and probes. The nucleotide sequences of the S genome segments of Hantaan 76-118,[24] SR-11,[25] and Prospect Hill virus[26] were examined for regions of homology, and oligonucleotide sequences for primers and probes were selected, as shown in Figure 1. Each of the two 20-base oligomer primers (HTN-S4 and HTN-S6) was complementary to a region of genome where all three viruses have nearly perfect nucleotide sequence homology. During synthesis, inosine was inserted in positions with nucleotide mismatches (Figure 1). A 19-nucleotide sequence was selected from the region flanked by the primers for use as a consensus probe to identify amplified viral DNA in the PCR products

(Figure 1). The length of the regions of these three viruses that were targeted for amplification was 281-nucleotide pairs (np).

First-strand synthesis of cDNA. For propagated virus, annealing reactions were performed in 10-μl volumes of DEPC-treated water containing 0.01 μM of the HTN-S4 primer (Figure 1) and 1 μl of cytoplasmic RNA from infected cells. The reaction mixture was incubated at 95°C for 3 min, and then at 42°C for 3 min. For first-strand synthesis, the volume of the reaction mixture was increased to 20 μl and contained 10 U of human placental RNase inhibitor (RNasin®; Promega, Madison, WI), 1 × reverse transcriptase reaction buffer (0.25 M Tris-HCl, pH 8.3, 0.375 M KCl, 15 mM $MgCl_2$), 1 mM of each dNTP (Pharmacia LKB Biotechnology, Piscataway, NJ), 10 mM dithiothreitol, and 200 U of RNase H^- reverse transcriptase (SuperScript®; Life Technologies, Inc., Gaithersburg, MD). The reaction mixture was incubated at 42°C for 1 hr and at 95°C for 3 min to eliminate reverse transcriptase activity, and was finally quenched on ice.

Samples from wild-caught rats were processed using the GeneAmp® RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). The RNA PCR protocol, as recommended by the manufacturer, used 2 μl of tissue-extracted RNA per reaction.

Amplification. Target sequences were amplified in a total reaction volume of 100 μl containing 1 μl of first-strand cDNA, 200 μM of each of the four dNTPs, 1.0 μM of each high-performance liquid chromatography–purified oligonucleotide primer (HTN-S4 and HTN-S6), 2.5 units of polymerase (AmpliTaq®; Perkin Elmer Cetus), and 1 × reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 0.01% [wt/vol] gelatin). Reaction mixtures were overlaid with light mineral oil and subjected to 30 cycles of amplification on a DNA thermocycler (Perkin Elmer Cetus). Phase settings were 30 sec at 95°C, 30 sec at 42°C, and 30 sec at 72°C per cycle, followed by 10 min at 72°C.

Analysis of PCR reaction products

Agarose gel electrophoresis. A 10-μl volume of the PCR reaction product was electrophoresed on a 4% gel (3% NuSieve® plus 1% SeaKem®; FMC Bioproducts, Rockland ME), the gel was stained with ethidium bromide, and examined for bands of the appropriate size.

Hybridization. The specificity of amplified products was determined by Southern transfer hybridization from agarose gels.[27] Following transfer, the nylon membrane filters (Nytran®; Schleicher & Schuell, Keene, NH) was exposed to ultraviolet light for 2 min and air-dried. The filter was incubated for 1 hr at 42°C in a prehybridization solution containing 3 × SSPE (20 × SSPE = 3.6 M NaCl, 200 mM NaH_2PO_4, pH 7.4, 20 mM EDTA, pH 7.4), 5 × Denhardt's solution (10 × Denhardt's solution = 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), 0.5% SDS, and 0.25 mg/ml of tRNA. Five nanograms per milliliter of the HTN-S5 oligonucleotide probe (Figure 1), end-labeled with ^{32}P-ATP and T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, OH) to a specific activity of 2–5 × 10^8 cpm/μg, was added, and the filter was incubated for 1 hr at 42°C. The filter was then washed for 5 min at room temperature in 3 × SSPE, three times for 5 min in 1 × SSPE containing 0.1% SDS, and once for 10 min at 52°C in 5 × SSPE containing 0.1% SDS. Autoradiograms were prepared by exposing the filters to X-Omat XAR-5 film (Eastman Kodak, Rochester, NY) with intensifying screens for 2–24 hr at −70°C.

The oligonucleotide probe was removed from the filters by washing at 95°C, and the hybridization procedure was repeated with a ^{32}P-labeled PCR-amplified hantavirus product. The probe was prepared using 2 μl of a 1:100 dilution of Hantaan 76-118 amplified reaction mixture as a template in a modified amplification protocol. Conditions were as described above with the following modifications: ^{32}P-labeled dATP, dGTP, and dCTP (800 Ci/mmol) were substituted for unlabeled dNTPs at a final concentrations of 6.25 picomoles each, and the amount of Amplitaq® was reduced to 1 U. Oil was not used to overlay the reaction mixture and 25 cycles were performed. The labeled probe was separated from unincorporated dNTPs by column chromatography using G50 Sephadex (Pharmacia LKB Biotechnology). A volume of the labeled probe sufficient to obtain 1–5 × 10^6 cpm/filter was denatured by boiling for 2 min, quenched on ice for 3 min, and added to the hybridization mixture described previously for the oligonucleotide probe. After hybridization for 1 hr at 43°C, the filters were washed once at room temperature for 5 min with 1 × SSPE containing 0.1% SDS and then at 55°C for 20–30 min with shaking.

Autoradiograms were prepared using exposure periods of 1–6 hr.

Restriction endonucleases. Polymerase chain reaction products were analyzed for restriction fragment length polymorphism (RFLP) with the following endonucleases: *Alu* I, *Bam* HI, *Dde* I, *Eco* RI, *Hae* III, *Hha* I, *Mbo* I, *Mbo* II, *Rsa* I, and *Taq* I. Restriction endonuclease digests were incubated for 1 hr in 15-μl reaction volumes containing 10 units of enzyme. Buffer and temperature conditions were used according to the manufacturers' specifications, and reaction products were analyzed by agarose gel electrophoresis as described above.

The restriction fragment lengths were estimated by applying the method of least squares linear regression to the relative migrations of molecular weight markers (*Msp* I-digested pUC18) and restriction endonuclease fragments.

Controls

To confirm that PCR amplification products were consistent with the established RFLP pattern of Baltimore rat virus, we included duplicate samples of Houston virus RNA as a control template for each reverse transcriptase reaction. These products served as positive controls for reverse transcription, and with Hantaan and Baltimore rat virus RNA, as controls for contamination or specificity in the RFLP analyses. Other controls included in each set of reactions were Houston RNA without reverse transcriptase, processed Baltimore rat kidney without reverse transcriptase, and numerous tissue extraction blanks, which for each rat processed, consisted of duplicate tubes of solution D processed by the RNA extraction method to PCR amplification.

RESULTS

Amplification of RNA from propagated virus

Nucleic acid sequences from all 20 hantaviruses were amplified by PCR using the consensus HTN-S4 and HTN-S6 primers. Amplification occurred only when reverse transcriptase was present in the first-strand synthesis reaction, indicating that the products were produced from RNA templates. A single amplified product was seen on agarose gels for all strains (Figure 2A) except Thailand 749, which had one prominent band of predicted length and two minor bands (Figure 2A, lane 16). The lengths of the amplified fragments for 19 of the 20 isolates were consistent with the 281-np product predicted by sequence data for Hantaan 76-118,[24] SR-11,[25] and Prospect Hill virus.[26] Amplification of Thottapalayam produced a larger fragment of approximately 320 np (Figure 2A, lane 20). The relative quantity of amplified products was comparable for all but two isolates, Jinhae 502 and Puumala viruses (Figure 2A, lanes 4 and 18, respectively) amplified to a lesser extent. When other bunyaviruses were tested with the consensus primers, no fragments of a size consistent with the hantaviruses were seen on ethidium bromide–stained gels nor were hantavirus-specific signals observed in any hybridization studies.

Hybridization of PCR products with specific probes

Amplification of hantavirus sequences was confirmed by hybridization of the reaction products with ^{32}P-labeled oligonucleotide and PCR-generated probes. The ability of the HTN-S5 oligonucleotide probe to detect amplified products from isolates was variable, suggesting sequence diversity within this 19-nucleotide region. Despite the appearance of approximately equivalent amounts of amplified product on the gels, strong signals were seen with 10 viruses, weak to moderate signals with seven viruses, and no signals with three viruses (Figure 2B). The strongest signals occurred with isolates of Hantaan virus (Figure 2B, lanes 1–8). Within this group, Jinhae 502 produced the weakest signal, but this was possibly due to a smaller amount of DNA on the filter. Hybridization with the isolates of Seoul virus varied considerably; no signal for Baltimore rat isolate (Figure 2B, lane 11), weak signals for SR-11, Girard Point, Tchopitoulas, and Seoul (Figure 2B, lanes 10, 12, 13, and 15, respectively), a moderate signal for Singapore (Figure 2B, lane 9) and a strong signal for Houston (Figure 2B, lane 14). Thailand 749, the virus isolated from a bandicoot, produced a strong signal (Figure 2B, lane 16), but the probe also reacted with the other amplified bands seen on the agarose gel. These other bands probably represented products in which one of the PCR primers hybridized at and extended from its intended binding site, whereas the other primer extended from a non-specific site. The oligoprobe did not hybridize with the Eurasian vole strains Puumala and USSR 18-20 (Figure 2B, lanes 18 and 19,

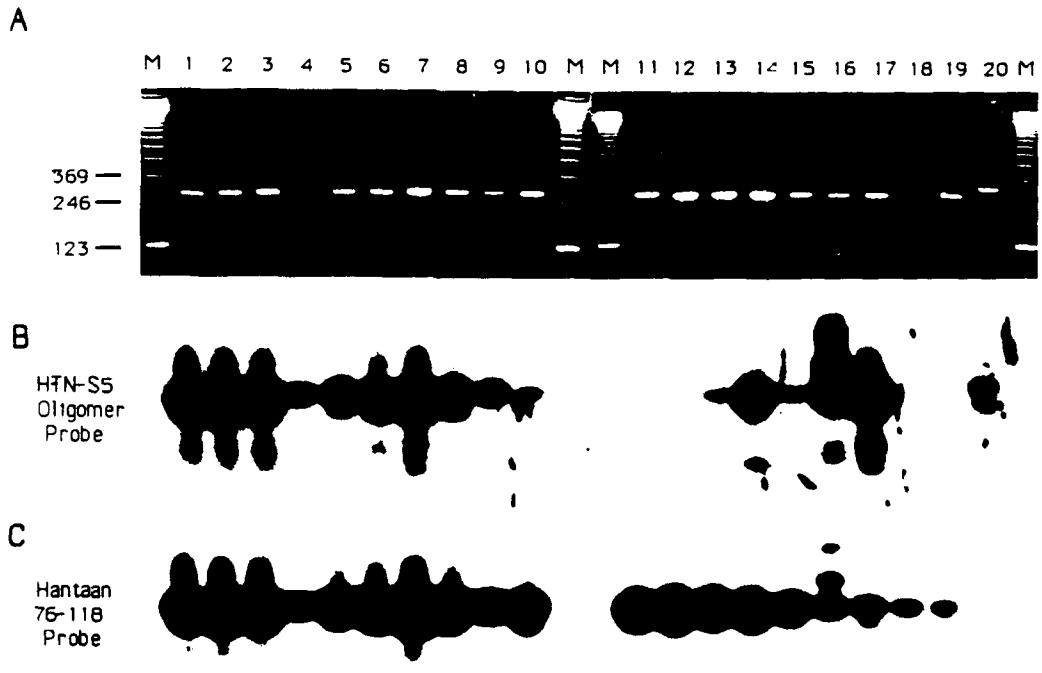

FIGURE 2. Analysis of polymerase chain reaction (PCR)–amplified products from 20 hantaviruses by **A**, agarose gel electrophoresis, **B**, Southern transfer hybridization with oligonucleotide and **C**, PCR-amplified probes. Lane M contains size markers (123-basepair ladder; Life Technologies, Inc., Bethesda, MD). Lane 1, Hantaan 76-118; lane 2, CG 3883; lane 3, Jinhae 494; lane 4, Jinhae 502; lane 5, Greek (Porogia); lane 6, Leakey; lane 7, Maaji; lane 8, Yugoslavia (Fojnica); lane 9, Singapore; lane 10, SR-11; lane 11, Baltimore rat; lane 12, Girard Point; lane 13, Tchopitoulas; lane 14, Houston; lane 15, Seoul; lane 16, Thailand 749; lane 17, Wisconsin; lane 18, Puumala; lane 19, USSR 18-20; lane 20, Thottapalayam. Hybridizations with oligoprobe HTN-S5 (**B**) and labeled Hantaan 76-118 amplification product (**C**) were performed sequentially on the same filter after the probe was removed by washing at 95°C. Autoradiographic exposures times were 24 hr (**B**) and 6 hr (**C**). Basepair values are shown on the left side of the gel.

respectively), but produced a strong signal with Wisconsin virus (Figure 2B, lane 17) isolated from a meadow vole in the United States. Moderate hybridization occurred with Thottapalayam (Figure 2B, lane 20), the shrew virus from India.

A second probe, made as a full-length PCR product and labeled during the amplification reaction, was tested for its ability to detect the amplified sequences from all of the hantavirus strains. The Hantaan 76-118 amplicon was used as template to make this probe. This probe was hybridized to the same filter that was tested with the HTN-S5 probe after the oligonucleotide was removed in a boiling water bath. The Hantaan 76-118 probe detected all amplified hantavirus fragments (Figure 2C). The strength of the signal was strong for all isolates of Hantaan and Seoul viruses, but less intense with the other hantaviruses. Moderate signals were observed with Thailand 749, Wisconsin, Puumala, and USSR 18-20 (Figure 2C, lanes 16, 17, 18, and 19, respectively). Thottapalayam had a weak but detectable signal (Figure 2C, lane 20).

Restriction endonuclease analysis

To further investigate sequence differences that might be useful for virus differentiation, the PCR products were analyzed by a panel of restriction endonucleases. The targeted regions from the known sequences of Hantaan 76-118, SR-11, and Prospect Hill viruses were examined by computer analysis to identify a candidate panel of enzymes. The RFLP patterns for each enzyme versus each virus were determined and the isolates were then grouped on the basis of pattern similarity.

The different groups of cleavage patterns pro-

duced by each of the 10 enzymes for all 20 virus strains, along with the estimated lengths of restriction fragments for each group, are summarized in Table 2. The RFLP designations, as defined in Table 2, are then presented for each virus in Table 3. Using the *Alu* I results as an example, seven groups of patterns were observed among the 19 viruses with amplified products of 281-np in length (Table 2 and Figure 3). Restriction endonuclease patterns were analyzed on ethidium bromide–stained agarose gels; therefore, the sum of fragment sizes did not always add up to 284 np since small fragments would not have been detected. Restriction fragment length estimations by the linear regression method were within 5 np according to the predictions made from viruses with known sequences, e.g., Hantaan 76-118 and SR-11. For example, the predicted fragment lengths of *Alu* I digestion of SR-11 are 153, 115, 11, and 2 np, whereas the observed fragment sizes are 157 and 117 np (Figure 3, lane 2). A similar analysis of data for the other enzymes produced comparable results. The only exception was with *Hae* III; sequence data indicated that Hantaan 76-118 has a *Hae* III site within the amplified region, but the amplified product was not cleaved by this enzyme.

TABLE 2

*Restriction endonuclease fragment patterns of the hantavirus S genome segment amplified by the polymerase chain reaction using consensus primers**

Group	*Ala* I	Group	*Hha* I
1	Not cleaved	1	Not cleaved
2	157, 117	2	213, 81
3	159, 83, 52		
4	115, 85, 75		
5	148, 92		
6	119, 96, 67		
7	130, 95, 68		
8	166, 146		
Group	*Bam* HI	Group	*Mbo* I
1	Not cleaved	1	Not cleaved
2	189, 91	2	211, 83
3	308	3	211, 59
		4	194, 92
		5	213, 75
		6	253, 74
Group	*Dde* I	Group	*Mboi* II
1	Not cleaved	1	Not cleaved
2	122(2), 48	2	235, 51
3	122, 164	3	157, 63(2)
4	98, 62, 48	4	139, 100
5	187, 102		
6	183, 157		
Group	*Eco* RI	Group	*Rsa* I
1	Not cleaved	1	Not cleaved
2	182, 90	2	175, 115
3	182, 97	3	227, 73
		4	168, 74, 63
Group	*Hae* III	Group	*Taq* I
1	Not cleaved	1	Not cleaved
2	227, 53	2	154, 134
		3	173, 119

* Values are the sizes of fragments in nucleotide pairs. Values in parentheses indicate there are two fragments with the same length.

Grouping of hantaviruses by RFLP

Analysis of the data in Table 3 revealed a pattern of profiles that separated the isolates into three groups: Hantaan-like, Seoul-like, and the remaining viruses. These groups were clearly differentiated with *Alu* I alone. *Dde* I and *Mbo* II similarly differentiated these groups, but with less consistency. The sequences of both *Apodemus* and the *Mus* viruses were highly conserved as indicated by identical profiles in five of the six isolates. The only exception was Jinhae 502, which produced patterns different from the other Hantaan isolates for six of the 10 restriction enzymes. Greek and CG 3883 viruses, which were isolated from humans, showed Hantaan-like profiles.

Sequences among the Seoul-like viruses also were well conserved; however, differences in the *Dde* I, *Mbo* II, *Rsa* I, and *Taq* I restriction endonuclease patterns produced unique profiles for several viruses. Singapore was an outlier among the Seoul virus group because it had a profile identical to Hantaan isolates. Although Houston, an isolate from the United States, had a profile similar to other Seoul-like viruses, it was distinctly different in that it was cleaved by both *Mbo* II and *Rsa* I, whereas the other Seoul-like isolates were not. Sequence differences were also detected by hybridization: Houston was the only Seoul-like virus that produced a strong signal with the HTN-S5 probe. SR-11 had one less *Dde* I cleavage site and Seoul does not have a *Taq* I site when compared with the other viruses. Baltimore, Girard Point, and Tchopitoulas rat viruses from the United States had the same profiles.

The remaining viruses had unique restriction endonuclease fragment profiles. Surprisingly,

TABLE 3

Restriction endonuclease analysis of polymerase chain reaction products of 20 hantaviruses

	Alu I	*Bam* HI	*Dde* I	*Eco* RI	*Hae* III	*Hha* I	*Mbo* I	*Mbo* II	*Rsa* I	*Taq* I
Girard Point	2*	1	2	1	1	1	1	1	1	2
Tchopitoulas	2	1	2	1	1	1	1	1	1	2
SR-11	2	1	3	1	1	1	1	1	1	2
Baltimore rat	2	1	2	1	1	1	1	1	1	2
Seoul	2	1	2	1	1	1	1	1	1	1
Houston	2	1	2	1	1	1	1	2	2	2
Singapore	3	1	4	1	1	1	1	3	1	1
76-118	3	1	4	1	1	1	1	3	1	1
CG 3883	3	1	4	1	1	1	1	3	1	1
Greek	3	1	4	1	1	1	1	3	1	1
Jinhae 494	3	1	4	1	1	1	1	3	1	1
Yugoslavia	3	1	4	1	1	1	1	3	1	1
Leakey	3	1	4	1	1	1	1	3	1	1
Maaji	3	1	4	1	1	1	1	3	1	1
Jinhae 502	4	1	C†	1	C	2	2	1	1	1
USSR 18-20	1	1	1	1	1	1	3	C	3	1
Puumala	5	1	5	2	2	1	3	C	1	3
Wisconsin	6	2	1	1	1	1	4	4	4	1
Thailand	7	1	1	3	2	1	5	C	1	C
Thottopalayma	8	3	6	1	1	1	6	C	1	1

* Values are the restriction fragment length polymorphism group no. as defined in Table 2.
† C = cleaved but incomplete pattern analysis due to weak band intensities.

Puumala and USSR 18-20, both isolated from *C. glareolus*, had different patterns for six of the 10 enzymes. Wisconsin, the only other vole virus tested, differed considerably from the Eurasian vole viruses, but was similar to the profile predicted for Prospect Hill virus. Wisconsin and Prospect Hill, both isolated from *Microtus pennsylvanicus* in the United States, had a only a single difference for one of the enzymes. Wisconsin was cleaved by *Hha* I, whereas the Prospect Hill virus sequence data indicates that there is no recognition site for this enzyme in the region amplified by the primers. Although the multiple bands amplified from Thailand 749 complicated the restriction endonuclease analysis, some sites in this virus and the Puumala virus appeared to be conserved. Unique restriction endonuclease patterns for all of the enzymes were seen with the Thottapalayam PCR product, which was consistent with its larger size (320 np).

Amplification of viral RNA from wild rat tissues

Of the 21 rats processed, 13 had antibody titers to Baltimore rat virus, indicating past or current infection (Table 4). Twelve rats were previously frozen and nine were processed fresh. Overall, amplified bands of the predicted size were demonstrable in 12 (92%) of 13 seropositive rats (Table 4 and Figure 4), while no evidence of PCR products was detected in eight seronegative rats (Table 4 and Figure 4A). In animals in which viral RNA was detectable, it was frequently observed that not all tissues sampled were positive (Table 4).

Of the various tissues tested, kidney showed the most positive test results (11 [85%] of 13 seropositive rats) and usually showed the most intense bands (Figure 4). All rats with high antibody titers (IFA $\geq$ 1:512 or ELISA optical density $\geq$ 0.40) were positive for viral RNA in the kidneys. Lung and spleen were positive in seven (54%) of 13 and four (50%) of eight seropositive rats, respectively. Liver and salivary gland were only occasionally positive. No viral RNA was detected in oropharyngeal fluid swabs, urine, or whole blood.

Samples from eight rats yielded bands of sufficient intensity for RFLP analysis (Figure 4B). All bands were digested into fragments by *Rsa* I and *Alu* I that were consistent with the pattern previously obtained from Baltimore rat virus, and were distinguishable from Hantaan and Houston virus (Table 3 and Figure 4B).

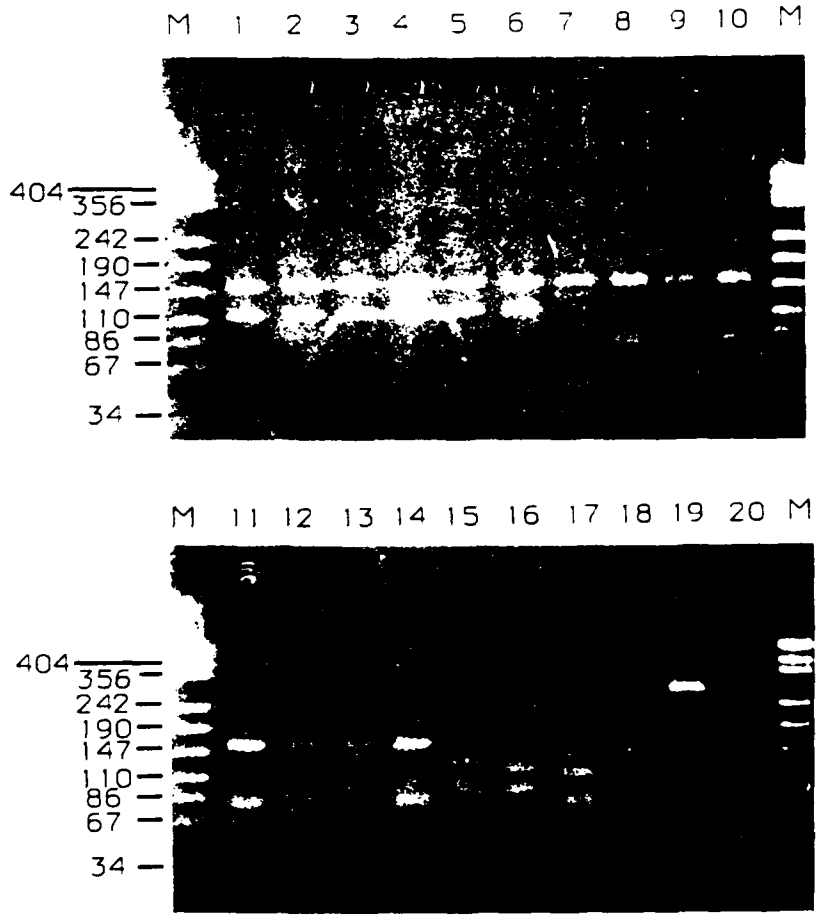

FIGURE 3. *Alu* I digests of polymerase chain reaction products from 20 hantaviruses. Lane M contains size markers (*Msp* I digest of pUC18). Lane 1, Seoul; lane 2, SR-11; lane 3, Baltimore rat; lane 4, Girard Point; lane 5, Tchopitoulas; lane 6, Houston; lane 7, Singapore; lane 8, Hantaan 76-118; lane 9, Greek (Porogia); lane 10, Jinhae 494; lane 11, CG 3883; lane 12, Yugoslavia (Fojnica); lane, 13 Leakey; lane 14, Maaji; lane 15, Thailand 749; lane 16, Wisconsin; lane 17, Jinhae 502; lane 18, Puumala; lane 19, USSR 18-20; lane 20, Thottapalayam. Basepair values are shown on the left side of each gel.

TABLE 4

*Detection of Seoul virus RNA in tissues and body fluids from 21 wild-caught Norway rats collected in Baltimore, Maryland (1988–1991)**

Reciprocal antibody titer†	No. of rats	Kidney	Lung	Spleen	Liver	Salivary gland	Bladder	Urine	Oropharyngeal fluid	Whole blood
≤16	8	0/8	0/6	0/2	0/1	0/2	0/2	0/2	0/2	0/2
≥32–≤256	2	0/2	1/2	0/2	1/2	—	—	—	—	—
≥512	11	11/11	6/11	4/6	1/6	1/5	1/6	0/3	0/5	0/5

* Rats are divided into groups with no antibody, low antibody titers, and high antibody titers. Values are the no. positive no. tested. — = not tested.

† Includes 16 animals tested by indirect immunofluorescent antibody assay and 5 animals tested by enzyme-linked immunosorbent assay. Animals tested by the latter method were placed in the ≥512 class by virtue of their extremely high optical density values.

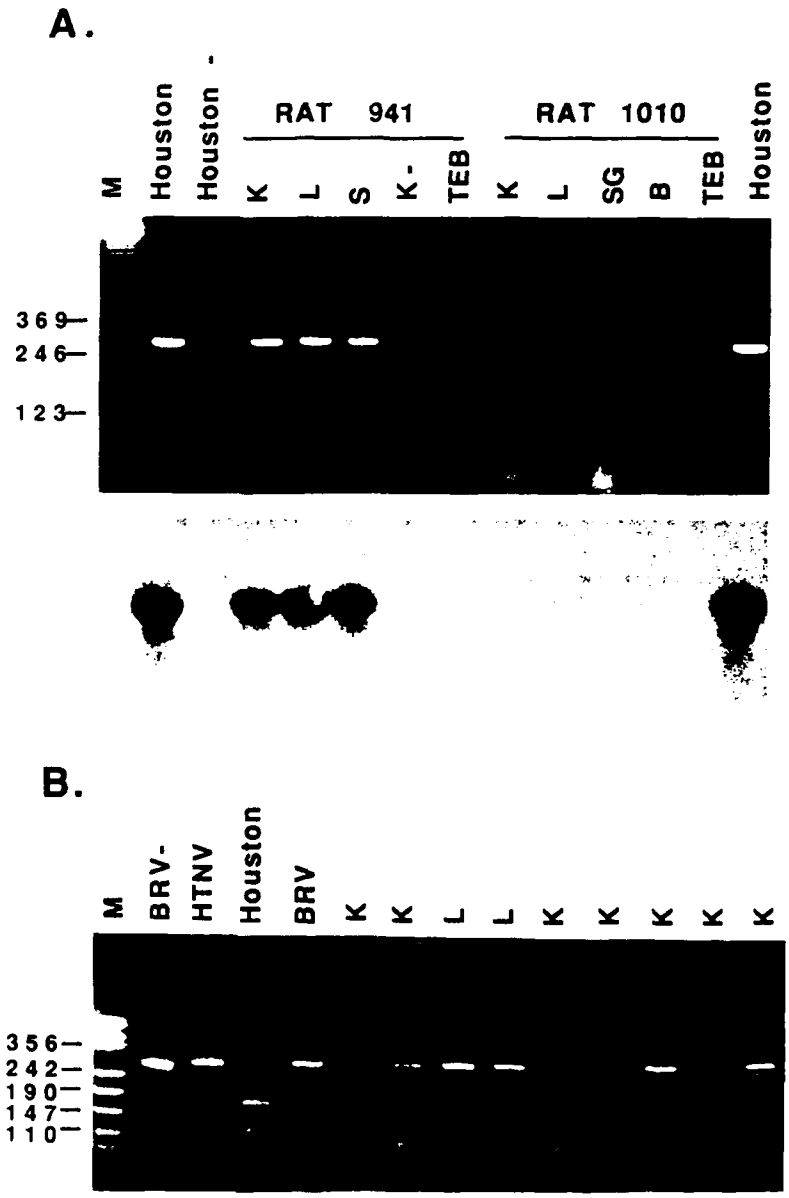

FIGURE 4. A. polymerase chain reaction (PCR) detection and **B.** restriction fragment length polymorphism (RFLP) analysis of hantaviruses from wild-caught rats. **A (upper panel).** analysis of kidney (K), lung (L), saliva (S), salivary gland (SG), and blood (B) from hantavirus-seropositive (rat 941) and seronegative (rat 1010) animals. Lane M contains size markers (123-basepair ladder; Life Technologies, Inc., Bethesda, MD). Purified Houston virus RNA and tissue extraction blanks (TEB) were used as positive and negative amplification controls, respectively. – denotes that reverse transcriptase was omitted from the reaction mixture. **A, (lower panel).** Southern transfer hybridization with PCR-amplified Hantaan 76-118 probe. The autoradiographic exposure time was 24 hr. **B.** RFLP analysis using an *Rsa* I digest of PCR-amplified sequences from eight wild-caught rats. Lane M contains size markers (*Msp* I digest of pUC18). Amplification products from Baltimore rat (BRV), Hantaan 76-118 (HTNV) and Houston viruses are shown for comparison. – denotes a reaction product that was not treated with *Rsa* I. Basepair values are shown on the left side of each gel.

DISCUSSION

In the present study, we identified a single primer set, based on comparative sequence alignment, that was capable of amplifying a fragment of S genome segment RNA from each of 20 different hantaviruses. The S segment of the hantavirus tripartite genome codes for the nucleocapsid protein and was targeted for amplification because nucleocapsid genes of genetically related viruses are frequently highly conserved. By aligning the genomes of hantaviruses for which sequence data was available and selecting highly conserved regions, we identified a single primer pair that amplified all hantaviruses tested. Since the primers did not have complete homology with all known viral sequences, inosine was incorporated in nonhomologous positions to enhance primer binding. To what extent this contributed to successful amplification with these primers was not evaluated. These primers appeared to be specific for hantaviruses since no amplification was observed with the other Bunyaviruses tested. Detection of amplified hantaviruses by hybridization with a consensus oligonucleotide inosine-containing probe failed to detect several of the rat viruses and the viruses from Eurasian voles. However, a PCR-generated probe that was derived from Hantaan 76-118 virus and encompassed the entire amplification target sequence detected PCR products from all viruses tested. The longer length and higher specific activity of this probe may explain its ability to detect those viruses that were not recognized by the oligonucleotide probe.

The polymerase chain reaction has been used previously by other investigators to amplify hantaviral RNA, but for the most part, these efforts have focused on the amplification of specific viruses. It has also been used in the molecular characterization of the S genome segment of Hallnas B1,[28] and the M segments of Hallnas B1 and Puumala[29] and DX[30] viruses. Xiao and others have detected Hantaan 76-118 in experimentally infected mice using Hantaan 76-118 specific M segment primer.[16] Two studies have used PCR to differentiate hantaviruses. Tang and others used two pairs of M-segment primers, one specific for Hantaan 76-118 and the other for R22, a Seoul-like virus, to differentiate these viruses.[31] A broadly reactive pair of consensus primers has been reported by Xiao and others[32] that amplified the M segment of all hantaviruses (41 strains), with the notable exception of Thottapalayam. The primers described in the present report are the first consensus primers described for the S segment of the genome. Furthermore, the region targeted for amplification by these primers may be more highly conserved than the M segment consensus primers because they amplify Thottapalayam. Thottapalayam is morphologically and antigenically related to the hantaviruses.[33] The classification of Thailand 749 as a hantaviruses is based on antigenic characteristics and is presently tentative. The amplification of the nucleocapsid genes of Thottapalayam and Thailand 749 by our primers is strong additional evidence that they are also hantaviruses. Confirmation of this awaits the analysis of nucleotide sequence data.

We have identified a set of restriction enzymes that, when used to digest the amplification products, can distinguish between hantaviruses and differentiated several isolates of Seoul-related virus. Analysis of PCR-amplified hantavirus sequences by restriction endonucleases to establish the characteristics of specific viruses and relatedness within this group of viruses appeared to be a valid alternative to immunologic methods for virus classification. Definitive serologic identification of a virus requires neutralization tests that are tedious and time-consuming to perform. The observed restriction endonuclease patterns, with the exception of the *Hae* III digestion pattern of Hantaan 76-118, matched those predicted by published nucleotide sequence data. With the exception of Jinhae 502, our grouping scheme showed that the hantaviruses from *Apodemus* that are associated with severe disease appear to be identical to prototype Hantaan virus. Therefore, it may be difficult to differentiate viruses within this group. Alternatively, some isolates of Seoul virus were differentiated by the panel of restriction enzymes. The greatest diversity was seen in the amplified region of other hantavirus isolates. Examination of additional hantaviruses by restriction endonuclease analysis of PCR-amplified sequences should permit the development of criteria for grouping all hantaviruses.

This method of genotypic characterization may be a useful means of validating the integrity of stock virus strains. For example, there have been conflicting reports regarding the antigenic relationship of Leakey virus, isolated from *M. mus-*

culus in the United States, to prototype Hantaan virus (Dalrymple J, unpublished data). In our experience, the amplified S segments of these viruses are identical. Similarly, Singapore virus isolated from a rat had a pattern identical to isolates of Hantaan virus. Further investigation of the same viruses obtained from several laboratories will be required to resolve these inconsistencies.

These preliminary findings confirmed the application of PCR methods to naturally infected reservoirs by demonstrating the detection and characterization of hantaviral RNA extracted directly from tissues collected from wild-caught Norway rats. The results, with a single exception, are in complete agreement with serologic findings on the same rats and indicate viral presence in tissues. Viral persistence, in the presence of a vigorous antibody response, has been the pattern previously demonstrated with experimental infections with Hantaan virus in *A. agrarius*,[21] Puumala virus in *C. glareolus*,[34] and Seoul virus in *R. norvegicus*.[35]

Our data suggest that the use of serologic analysis alone is sufficient to establish the status of Norway rats as hantaviral carriers, since we failed to amplify viral RNA from our limited sample of seronegative rats. The polymerase chain reaction may still be preferable in some situations because serologic analysis is prone to give false-positive findings. Passive acquisition of maternal antibody may also cloud interpretation of data from small animals with low antibody titers.[36] The one PCR-negative rat in our study had a relatively low IFA titer of 1:256.

Our differential ability to amplify RNA from various tissues of naturally infected rats (Table 4) leaves several questions open. In suckling mice infected by hantavirus, the PCR readily detected viral RNA in brain, heart, lung, liver, and spleen eight days after infection.[16] In that study, kidney was sampled irregularly, so conclusions concerning the relative merits of this organ, which was demonstrated in Baltimore rats, as a target for PCR would be premature. Lung has been the traditional tissue source for culturing hantaviruses,[18, 22, 37] but nothing is known about relative viral titers persisting in different tissues of naturally infected animals. The failure to demonstrate viral RNA in oropharyngeal swabs, whole blood, and urine may indicate our inability to precipitate low yields of RNA from the sample or the presence of inhibitors of transcriptase or polymerase activity, rather than the lack of virus. Virus in known to be present in the saliva and urine of persistently infected rodents,[21, 34] while viremia appears transiently during early stages of infection.[21] In the future, we hope to use additional procedures such as spiking samples with carrier RNA or use RNA capture beads to resolve these questions.

The ability to amplify a broad spectrum of hantaviruses by a single primer set will facilitate rapid sequence acquisition from this region of the genome for those viruses not yet sequenced. The additional sequence data will be useful in several respects. First, it may lead to the development of group- or virus-specific oligonucleotide probes. Although this approach appears feasible for most hantaviruses, the RFLP data for the Hantaan viruses suggest a high level of sequence conservation within this group, which may not allow virus-specific differentiation with oligonucleotide probes. Second, the genetic relatedness derived from sequence comparison of a large number of strains may provide insight as to how these viruses evolved and became distributed among their respective reservoirs. This approach is particularly attractive for amplifying sequences directly from tissues of infected animals and thereby eliminating the possibility of analyzing minor variants that have selective growth advantages in cell culture systems.

Finally, this application of PCR has potential as a rapid diagnostic test for examining specimens from humans. Although PCR methodologies are exquisitely sensitive and have an infinitely variable window of specificity, they are presently being used almost exclusively in research settings. The extensive processing that is required in some systems before the specimen is subjected to the amplification procedure and the steps involved in the analysis of products are labor intensive. Therefore, processing small numbers of samples at one time is expensive. Meticulous operator technique and laboratory facilities that permit compartmentalization of specimen processing, thermal cycling, and product analysis are required to avoid false-positive results that arise when small numbers of target nucleic acid sequences contaminate reaction mixtures. As more laboratories gain experience with PCR methodology and the methods for processing samples and analyzing amplification products are simplified, a larger number of laboratories will have the capability to diagnose

hantavirus infections. This will provide a greater opportunity to study hantavirus infections, since it will eliminate the need for high-level biosafety containment facilities currently required to propagate live virus that is used in some current serologically based assay systems.

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