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EPIDEMIOLOGY OF HANTAVIRUS INFECTIONS IN THE UNITED STATES

FINAL REPORT

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81

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) During this contract we have successfully detected the first human cases of HFRS in the United States, and have begun to characterize the acute symptoms and findings of the illness. Infection rate was approximately 2.4/1000 among individuals from Baltimore without specific risk factors for exposure, but was much higher in certain populations. Risk of exposure to rodents was highest among inner city, older males who owned dogs. Hantaviral infection was significantly associated with hypertension and hypertensive renal disease, suggesting infection may require long-term, medically intensive therapy in some cases. Polymerase chain reaction (PCR) methodology has been adapted for use with hantaviruses, and has been utilized to identify virus in naturally infected animals. This provides a method for rapid surveillance and diagnosis. Restriction fragment length polymorphism techniques have been applied to PCR products to permit identification of hantaviral strains. Details of the research conducted during this contract can be found in the midterm report dated 8 July 1991.				
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FOREWORD

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During the past quarter most administrative efforts have focused on preparation and completion of the mid-term report. This report was delivered to USAMRIID on 8 July 1991. Research administrative efforts focused on obtaining permission to obtain research samples from Detroit, MI. Letters were sent to the Department of Public Works for the city to obtain permission from the appropriate unions. To date no reply has been received. A notification of intent to terminate this contract, by the Army, was obtained in late August. Therefore it was decided not to pursue this effort any further at this time. Remaining administrative efforts during this time have focused on preparing a phase-out proposal to terminate this contract.

Numbers summarized in this report represent data collected since the mid-term report. Readers are referred to that report to obtain overall sample sizes, prevalences, and rates.

During this past quarter research efforts have involved the characterization of acute HFRS in Baltimore, MD, examination of the association between past infection and chronic disease, examination of the Detroit dialysis population, and the development of PCR for rapid diagnosis of hantaviral infection and its application to naturally infected animals.

A total of 52 patients from Johns Hopkins Hospital (JHH) were screened for exposure to hantaviruses, by ELISA. None were found to be positive. In addition, another 64 patients from the dialysis populations at JHH, Bon Secours, and Francis Scott Key Hospitals were screened for hantaviral antibodies. Four of these patients were positive by ELISA. All seropositive patients have primary diagnoses of hypertensive renal disease. Thus, infection with hantaviruses is associated with 8.6% of the cases of patients with end stage renal disease due to hypertension, the major cause of renal disease in Baltimore.

Four seroconversions to hantaviruses have now been documented at JHH. Three of the four individuals showed evidence of illness marked by the acute onset of nausea, vomiting, fever, epigastric pain, proteinuria, hypotension, and variable renal and hepatic involvement. The fourth individual appeared to be asymptotically infected. In all cases local Norway rats were implicated as the source of the infection. Two of the patients developed chronic renal disease following infection and one of these required hemodialysis.

Thus, the results of these studies indicate that hantaviruses do cause previously unrecognized disease in this country, and while the domestic rat strains do not cause severe, acute disease, they probably do not represent vaccine candidates, as has been suggested elsewhere. This is

particularly true because of their association with chronic hypertensive renal disease, and hypertension. While this study is the first to document the epidemiological association, earlier work by Rubini with KHF patients (Hantaan virus) and Lahdevirta with NE patients (Puumala virus) suggested such a pattern might exist. Lahdevirta's data in particular appears to have been overlooked, as he observed that 75% of his followup patients were hypertensive and 30% had decreased renal function. Taken together, these results indicate that long-term renal sequelae and hypertension are a common result of hantaviral infection regardless of the particular infecting strain.

These findings have obvious implications for U.S. servicemen infected with hantaviruses while serving in the military. Surveys of the Baltimore Veteran's Medical Center show that an average of 2.6% of the patients in the hypertensive unit have had previous hantaviral infections, and this increases to more than 5% in some age classes.

Data from the Detroit, MI dialysis units indicates a seroprevalence rate of 3.7% in that population, by ELISA. However, none of these sera show neutralizing antibodies to the hantaviruses used in the plaque reduction neutralization assays. This suggests either a high rate of false positives by ELISA in this population, or that another hantavirus may circulate in the Detroit region. Because of the administrative difficulties described above, and the early termination of this contract the possibility of another circulating hantavirus cannot be pursued. Reviews of medical records indicate that all ELISA positive patients in this population have received multiple (2-50) blood transfusions. Thus, the seropositives may represent patients with nonspecific antibodies that cross-react with the antigens used in the ELISA. The rate of multiple transfusions in the entire dialysis population is currently unknown, but is being reviewed. If false positives are being produced by transfusions, these data should be considered in further serological studies. We also have observed that patients with SLE tend to produce false positive results when tested with the current ELISA technology.

Research also continued on the use of polymerase chain reaction (PCR) technology for the detection of hantaviruses. Most work focused on the detection of hantaviruses due to natural infections, and using restriction fragment length polymorphisms to identify the infecting virus. This moves the research beyond the detection of tissue culture adapted viruses into the area of rapid diagnosis.

For this work, infection in a reservoir species was examined. Rats were captured from three alleys known to be enzootic for BRV, and tested for serological evidence of infection by either indirect immunofluorescent antibody (IFA) or enzyme-linked immunosorbent (ELISA) assays.

Both fresh and frozen (up to 3 yrs) tissues were examined. Typically, only kidney, lung and spleen were available as frozen specimens, but salivary gland, bladder,

liver, whole blood, urine, and oropharyngeal swabs were collected from recent captures. Frozen tissues from rats with a range of antibody titers were selected for processing as virus isolation has been most easily achieved using seropositive animals. Fresh samples were tested by PCR without prior knowledge of their serological status.

RNA extraction from tissues was a single-step method using the acid guanidinium-phenol-chloroform (AGPC) method. Within a laminar flow biosafety hood, a 2-3 mm³ piece of tissue was minced and subsequently homogenized with 0.5 ml of denaturing solution (solution D) consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, by repeated expression of the sample against the side of a 1.5 ml tube using a positive displacement pipettor. Whole blood (approximately 50-100 μ l) was treated as solid tissue, while urines (200-1500 μ l) were centrifuged 20 min. in a microfuge, about 75% of the fluid decanted, and pellets resuspended in 200 μ l of K buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 100 μ g/ml 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 the homogenized tissue 50 μ l of 2M NaOAc, pH 4.0, 0.5 ml of phenol (water saturated), and 0.1 ml of chloroform-isoamyl alcohol mixture (49:1) were sequentially added. Samples were allowed to cool on ice for 15 min. and then centrifuged at 10,000 X 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 X g (20 min.) and the pellet dissolved in 0.3 ml of solution D and precipitated with 1 vol of isopropanol at -20°C for 1 hr. The precipitate was centrifuged at 4°C for 10 min. in an Eppendorf centrifuge and the RNA pellet resuspended in 75% ethanol, sedimented, vacuum dried (15 min.), and resuspended in 50 μ l of DEPC treated 1.0% Laureth-12 at 65°C for 10 min. Two μ l of this material was then used in a RNA PCR protocol as recommended by Cetus (Perkin Elmer Cetus, Norwalk, CT; instructions included with GeneAmp RNA PCR Kit).

Two 20-base oligomer primers (HTN-S4 and HTN-S6) were chosen by comparing published sequences of the S-segments of Hantaan 76-118, SR-11 and Prospect Hill virus and selecting regions of near homology. Inosine was inserted in positions with nucleotide mismatches during synthesis, as described previously. The length of the regions of these three viruses that were targeted for amplification was 281 nucleotide pairs (np).

First strand synthesis of cDNA was accomplished according to the manufacturers protocols. Reactions were performed in 20 μ l volumes containing 1X reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 5 mM MgCl₂, each of the four dNTPs at a concentration of 1mM, RNase inhibitor (1U/ μ l), reverse transcriptase (2.5 U/ μ l) HPLC-purified oligonucleotide primer

HTN-S4 (1 μ l of 15 μ M stock), and 2 μ l of cytoplasmic RNA from tissues. Reaction mixtures were overlaid with light mineral oil and incubated at 42°C for 15 min. and then placed at 99°C for 5 min before cooling to 4°C. To amplify cDNA target sequences, 80 μ l of a PCR master mix (2.5 U of Amplitaq DNA polymerase, 1 μ l of oligonucleotide primer HTN-S6 (15 μ M stock), 4 μ l MgCl₂ (to bring final concentration to 2mM) and 8 μ l of 10X buffer). Samples were briefly centrifuged and subjected to 30 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Cetus). Amplification of target DNA used a thermal cycle of 30 sec at 95°C for denaturing the DNA, 30 sec at 50°C for annealing of primers and 1 min. at 72°C for sequence extension.

A 10 μ l volume of the PCR reaction product was electrophoresed on 4% gels (3% NuSieve + 1% SeaKem, FMC Bioproducts, Rockland ME), the gels stained with ethidium bromide, and examined for bands of the appropriate size. Products were transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene NH) by the method of Southern.

Following transfer, membranes were exposed to ultraviolet light for 2 min. and air-dried. Filters were incubated for 1 hr. at 42°C in a prehybridization solution (3X SSPE (20X SSPE; 3.6 M NaCl, 200 Mm NaH₂PO₄ [Ph 7.4], 20 Mm EDTA [Ph 7.4]), 5X Denhardt's solution (10X Denhardt's; 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), 0.5% sodium dodecyl sulfate (SDS), and 0.25 mg/ml tRNA). Filters were hybridized with 5 ng/ml of a ³²P-labeled PCR-amplified hantavirus product produced with a Hantaan virus (strain 76-118) template. This probe detected cDNA of most strains of hantavirus, including seven of rat origin. For hybridization, the labeled 76-118 probe (sufficient volume to obtain 1-5 X 10⁶ cpm per filter) was denatured by boiling for 2-3 minutes. The probe was placed on ice for 3 minutes and then added to the hybridization mixture and incubated overnight at 43°C. Filters were washed at room temperature (5 min.) with 1X SSPE containing 0.1% SDS and then at 55°C for 20-30 minutes with shaking. Autoradiograms were prepared using Kodak X-omat XAR-5 film with intensifying screens at -70° for exposure periods of 3 to 6 hrs.

One to five μ l of PCR reaction products, that had previously been shown to hybridize with the Hantaan probe, were digested for one hr. with 10 U of *AluI* or *RsaI* in a total reaction volume of 15 μ l using appropriate buffer and temperature conditions. Agarose gel electrophoresis of the digestion reaction was performed as described above. Restriction length fragment polymorphism (RFLP) patterns for these enzymes had previously been determined and the rat viruses from the United States and Hantaan virus could be distinguished on the basis of these patterns. Digestion of rat-associated hantaviruses with *AluI* yields observed fragments of 157 and 117 np, but cuts Hantaan into fragments of 159, 83 and 52 np. The domestic rat isolate from Houston has an identical RFLP pattern to all other rat viruses when cut with *AluI* but is distinguished from the Baltimore isolate

and Hantaan in that it is cleaved by *RsaI*.

To confirm that amplification products were consistent with the established RFLP pattern of BRV, we included duplicate samples of Houston virus RNA (provided by Dr. Richard Lofts, USAMRIID, Frederick, MD, USA) as a control template for each reverse transcriptase reaction. These products served as positive controls for reverse transcription and, with HTN and BRV RNA, as controls for contamination or specificity in 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 (TEB) which, for each rat processed, consisted of duplicate tubes of solution D carried through from the RNA extraction to PCR amplification.

Twenty one rats were processed including 13 with antibody titers to BRV indicating past or current infection. Twelve rats were previously frozen and nine were processed fresh.

Overall, cDNA bands of the predicted size were demonstrable in 10/13 seropositive rats (Figure 1), while no evidence of PCR products was detected in any of the seronegative rats (Figure 1). In animals in which viral RNA was detectable, frequently not all tissues sampled were positive (Figure 1).

Of the various tissues tested kidney produced the most positive tests (9/13 seropositive rats) and usually resulted in the most intense bands (Figure 1). Lung was PCR positive in 5/13 and spleen in 4/7 seropositive rats. No viral RNA was detected in oropharyngeal swabs or whole blood.

Samples from eight rats yielded bands of sufficient intensity for RFLP analysis (Figure 2). All bands were cut into fragments consistent with the pattern previously obtained from BRV, and were distinguishable from Hantaan and Houston virus (Figure 2).

These findings demonstrate the application of PCR methods to the detection and characterization of hantaviral RNA isolated directly from wild caught Norway rats. The results are in general agreement with serological findings on the same rats and indicate viral persistence in tissues, as has been the pattern previously shown for experimental infections with HTN virus in *A. agrarius*, PUU virus in *C. glareolus*, and SEO virus in *R. norvegicus*. These data show the potential application of PCR for field studies of reservoir hosts and hold promise for diagnostic applications to humans. The ability to obtain quantities of hantaviral cDNA, without resorting to amplification by cell culture, will also provide important controls on introducing genetic change through the multi-generation culture adaptation process.

The differential ability to amplify RNA from various tissues leaves several questions open. In suckling mice infected by HTN virus, PCR readily detected viral RNA in brain, heart, lung, liver, and spleen by day eight. In that study kidney was only irregularly sampled, so conclusions concerning the relative merits of this organ, as demonstrated

in Baltimore rats, as a target for PCR would be premature. Lung has been the traditional source tissue for culturing hantaviruses, but little is known about relative viral titers in different tissues. Isolation, being theoretically capable of detecting a single intact virion, is potentially a more sensitive detection technique than PCR, which preliminary sensitivity tests indicate can detect as few as 16 infected cells.

The failure to demonstrate viral RNA in oropharyngeal swabs and whole blood, and the irregular detection of RNA in urine, may indicate our inability to precipitate low yields of RNA from the sample rather than lack of virus. Virus is known to be present in saliva and urine of persistently infected rodents and has been isolated from human urine during acute HFRS. Viremia, however, appears to be only transient in infected rodents during early stages of infection, and preceding widespread tissue dissemination of infection.

The ability of PCR to detect naturally occurring hantaviral infections indicates, as we initially proposed, the technology will be useful in rapid diagnosis of infection in reservoir species, which can be applied to surveillance, and we anticipate rapid diagnosis in the identification of human disease.

In the future, we will focus on closing down the project, due to the termination of the contract. Of ongoing research projects, only the PCR rapid diagnosis portion of this contract will be continued through 30 September 1991. Major efforts through 31 December 1991 will involve the completion of manuscripts involving; the first descriptions of acute HFRS in the United States, examination of seroepidemiological patterns in selected populations of Baltimore, description of PCR methods for the detection of hantavirus specific sequences from the S segment genome and characterization by RFLP analysis, and the use of PCR to detect hantavirus in naturally infected animals. We also hope to present conclusions from this study at the meeting of the American Society of Tropical Medicine and Hygiene in Boston, in December.

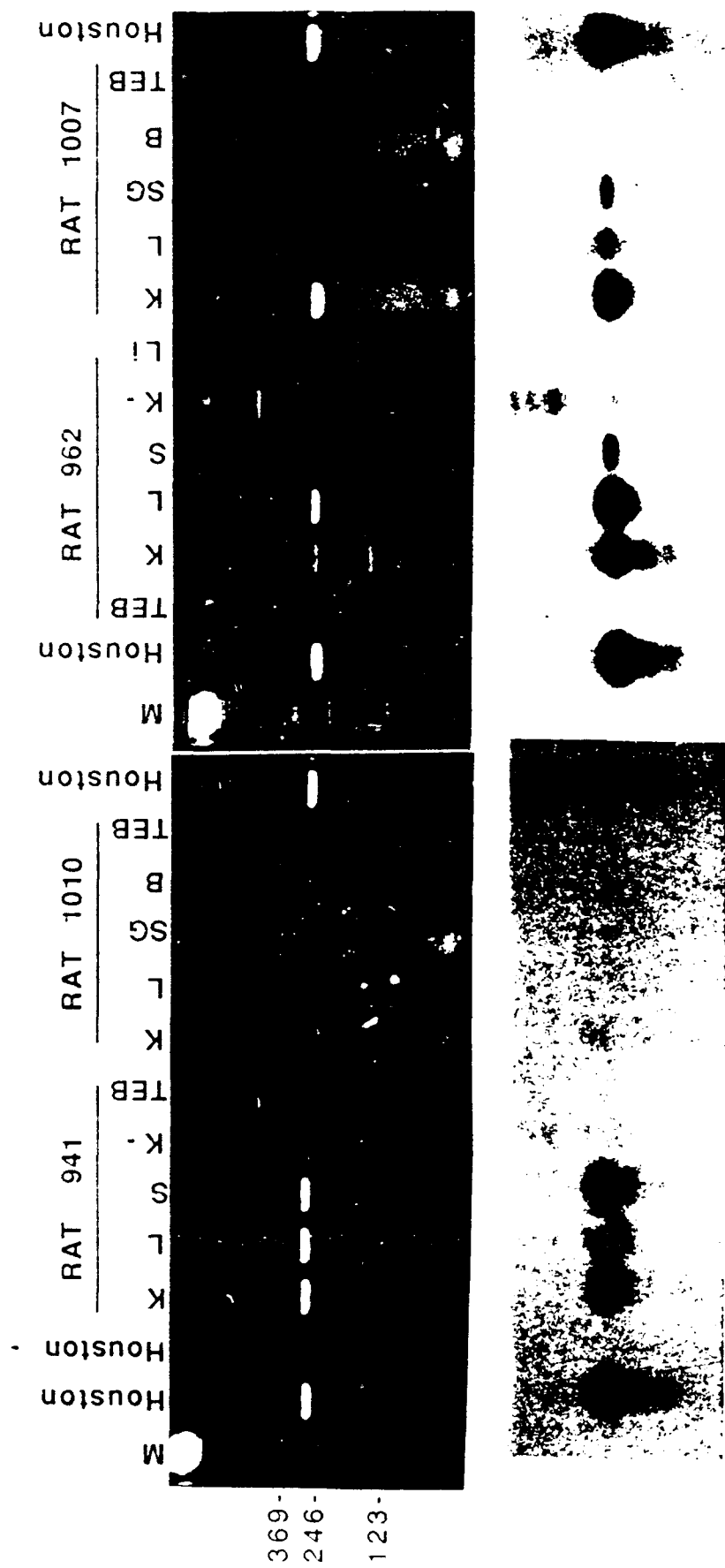
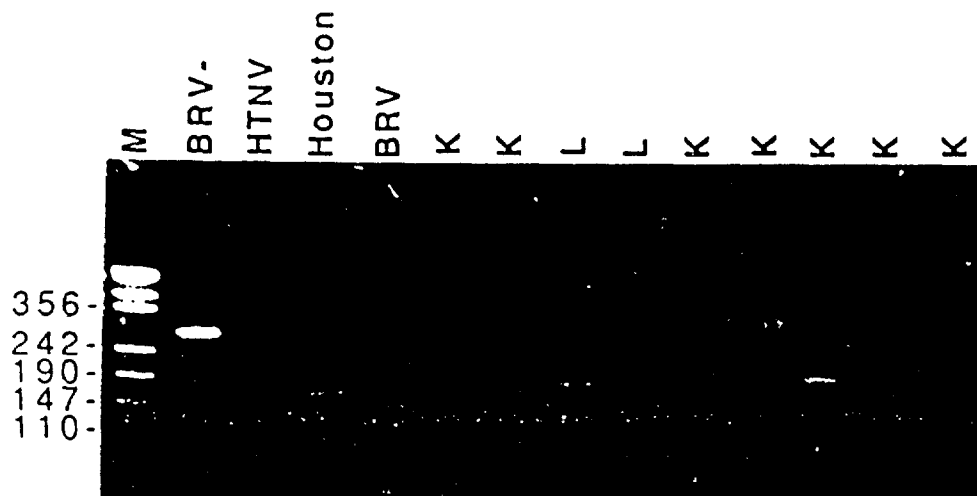


Figure 1. Reverse transcriptase-directed PCR amplification of hantaviral S genome segment RNA directly from wild caught rat tissues. Top panel-ethidium bromide staining of a 281 np cDNA fragment. M=123 bp ladder, Houston=RNA from cell culture adapted Houston rat virus, (-)=no reverse transcriptase added, K=kidney, L=lung, S=spleen, SG=salivary gland, Li=liver, B=bladder, TEB=tissue extraction blank. Lower panel-hybridization of top panel with PCR produced ³²p labeled Hantaan virus probe following Southern transfer of cDNA to nylon membrane and 24 hr exposure. Rats 941, 962 and 1007 were serologically and PCR positive. Rat 1010 was serologically and PCR negative. Not all tissues were positive by PCR.

A. *Alu* I



B. *Rsa* I



Figure 2. Restriction enzyme digests of PCR amplified products from wild-caught Baltimore rats. The restriction enzyme patterns demonstrate that the RNA amplified from Baltimore rats (Figure 1) is consistent with our previous established pattern for Baltimore rat virus (Arthur et al., submitted), and distinguishable from Hantaan or Houston RNA by *Alu* I or *Rsa* I, respectively.