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EPIDEMIOLOGY OF HANTAVIRUS INFECTIONS IN THE UNITED STATES

MIDTERM REPORT

JAMES E. CHILDS

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This report describes studies carried out in Baltimore and Detroit to investigate: 1) the prevalence and incidence of hantaviral infections in different populations of urban residents; 2) acute or chronic diseases associated with positive serologies; 3) risk factors associated with rodent exposure, hantaviral infection or infection with other rodent-borne zoonoses; 4) polymerase chain reaction (PCR) methods for the amplification of the S segment of the hantaviral genome; 5) using PCR to define the occurrence of hantaviruses in rodent and human tissues collected in Baltimore and stored by various techniques; 6) using S segment PCR to identify and gorup hantavirus serotypes by their annual reservoir to aid in the diagnosis and epidemiological investigation of hemorrhagic fever with renal syndrome (HFRS).

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INTRODUCTION

Hantaviruses (Family Bunyaviridae) are the etiological agents responsible for a spectrum of illnesses referred to collectively as hemorrhagic fever with renal syndrome (HFRS: Lee and Dalrymple, 1989). The viruses are distributed worldwide and rodents are the primary reservoirs (Yanagihara and Gajdusek, 1987; LeDuc, 1987), although carnivores and insectivores may also be infected (Tang et al., 1985; Zhao-zhuang et al., 1985). HFRS varies in severity depending on the particular virus involved. The primary clinical differences occur in the degree of hemorrhaging, hypotension, oliguria, and case mortality. Despite the widespread geographic range of hantaviruses, and the severity of HFRS, only the recent achievements of virus isolation (Lee et al., 1978) and cell culture adaptation (French et al., 1981) leading to the development of serodiagnostic tests have permitted evaluation of the importance of HFRS worldwide. Broad serosurveys of potential reservoir species (see LeDuc, 1987 and Yanagihara and Gajdusek, 1987, for review) show that infected animals exist far outside the recognized areas of endemic human disease and hantaviruses are being recognized as causing fevers of unknown origin in an increasing number of countries where they were not thought to exist. A brief review of the emergence of newly recognized hantaviral disease follows, as it is relevant to problems and processes in disease recognition in the United States.

Nephropathia epidemica (NE), a relatively mild form of HFRS, with a case-mortality rate of less than 1%, is found in Scandinavia, the western Soviet Union, and other European countries (Lähdevirta,1971, 1982: Sommer et al., 1988). While nephropathia epidemica was first described in Scandinavia in the 1930's, it is only now emerging as a relatively common cause of acute renal failure elsewhere in France (Kessler et al., 1986; Rollin et al., 1987), Italy (Savadori et al., 1989), Belgium (van Ypersele de Strihou et al., 1983) and other European countries (Koolen et al., 1989; van Ypersele de Strihou et al., 1989; Walker et al., 1984). As was the case with Hantaan virus, the virus that causes nephropathia epidemica, Puumala virus, was only recently discovered and isolated.

Within the last decade, a extremely severe type of HFRS was recognized in the mountainous regions of Greece (Antoniades et al., 1987a, 1987b; LeDuc et al., 1986), Albania, parts of Yugoslavia (Avsic-Zupanc et al., 1990; Gligic et al., 1989a), and Bulgaria. This disease is far more severe than nephropathia epidemica seen elsewhere in Europe and more closely resembles the Asian form of HFRS. Although relatively few cases have been recorded, the mortality rate appears to be even greater than that seen in Asia; preliminary reports suggest that as many as 15 to 30% of patients hospitalized with this disease may succumb to their infection. The causative agent, originally named Porogia virus, has more recently been shown to be closely related to Hantaan virus (J. W. LeDuc, personal communication). It is also

maintained by a small rodent, the yellow-necked mouse, Apodemus flavicollis (Gligic et al., 1989b). Unlike HFRS in Asia and other parts of Europe, cases in the Balkans peak during the warmer months of the year, with most cases occurring around August.

In 1982 workers detected evidence of hantaviral infections among Norway rats (Rattus norvegicus) in cities of Korea (Lee et al., 1982). This finding was the most significant event in the recent history of HFRS research, from the global public health perspective, as it raised the potential for global dissemination of a novel zoonosis by means of international shipping. Rat-borne hantaviruses were subsequently detected in the United States, and two isolates were made (LeDuc et al., 1984; Tsai et al., 1985). This was followed, in 1985, by the characterization of another Hantavirus in the U.S. (Prospect Hill virus; Lee P-W et al., 1985) in meadow voles (Microtus pennsylvanicus) first detected in Maryland. Most recently, an additional virus of the genus was described (Leakey virus) from house mice (Mus musculus), in Texas (Baek et al., 1988).

Studies conducted in the intervening years since the initial identification of hantaviruses in the United States, had shown that HFRS due to rat-borne viruses in Asia caused an illness that was less severe in its hemorrhagic and renal manifestations than that caused by Hantaan virus. The disease associated with rat-borne viruses also showed a higher degree of hepatic involvement (Chan et al., 1987; Lee 1989). These findings indicated that indigenous hantaviral infections in the United States may be sufficiently mild in the majority of cases or sufficiently different from classical HFRS that they may have been misdiagnosed or even overlooked.

Studies examining hantaviral infections first in rodents, and subsequently in humans were initiated in Baltimore in 1983. Following several years of study, Childs et al. (1987a) characterized a Hantavirus and provided a detailed examination of the epizootiology of the virus in R. norvegicus (Childs et al., 1985; Childs et al., 1987b). Extensive investigations were initiated to study this Seoul-like virus in inner-city Baltimore rats. Infected rats were found widely distributed throughout the city (Childs et al., 1985, 1987a), but were especially abundant in the lower-income neighborhoods, where accumulated trash, litter, and garbage provided ideal conditions for rat infestations (Childs et al., 1987b). Infections had been stably enzootic within the rats of Baltimore for at least six years (ten years now, J. E. Childs and G. E. Glass, unpublished data), based on long term trapping of specific alleys. In addition, Norway rats do not move great distances in urban environments, so that widespread dissemination of a virus, without an arthropod vector, is likely to take many years. Thus, Seoul-like viruses were not recent introductions to the United States, but had been here for some time and had become widely disseminated. Rat populations of several areas were followed longitudinally over two years, and we were able to demonstrate that virus was transmitted among rats

throughout the year, and about 11% of the population became infected per month (Childs et al., 1987b). As older cohorts of rats were examined, their antibody prevalence rates increased until virtually all of the oldest rats had become infected. At that time we concluded that there was potential for transmission of Seoul-like viruses to humans in Baltimore wherever the species came into close contact.

Indigenous human infection with a rat-borne hantavirus was first proven in Baltimore (Childs et al., 1988). We have since shown that infection in the general inner city population of Baltimore is on the order of 2-3/1000 (Childs et al., 1991 and this report), but is dramatically increased in patient populations with clinical laboratory findings of proteinuria (>250 mg/24 hours; see this report). We have been able to expand these observations through the enlistment of dialysis patients throughout Baltimore and Detroit, MI. We have recently demonstrated a significant statistical association between the occurrence of neutralizing antibody to Baltimore rat virus and the presence of chronic renal disease and hypertension (Glass et al., 1990 and this report). These are the first data to demonstrate any association between serological positivity to a domestic hantavirus and disease, and point to a potentially major complication of hantaviral infection, the subsequent development of chronic renal disease.

The possibility of long-term renal dysfunction following a hantaviral infection has not been well studied in any location. One of the few studies examined Korean Conflict veterans who had suffered hantaviral infections, and a group of matched controls in 1956, about 3 to 5 years after most cases would have been infected (Rubini et al., 1960). This study found a significant increase in the rate of genito-urinary hospital admissions among the HFRS cases, that increased with the severity of their original disease. Other findings included hyposthenuria, persistent mild albuminuria, and hypertension. Similarly, Lähdevirta examined patients with nephropathia epidemica, 1-6.5 years after disease and found evidence of depressed renal tubular function, and hypertension in some of these patients (Lähdevirta 1971; Lähdevirta et al., 1978). Hypertension was especially common in his followup population with nearly 75% of the individuals hypertensive. These indications of chronic renal dysfunction and hypertension are consistent with the hypothesis that their condition could evolve over time to conditions similar to those seen among the Baltimore residents.

Studies being conducted in Baltimore are also addressing the critical area of rapid diagnosis of hantaviral diseases. Even with severe cases of HFRS a diagnosis based solely on clinical manifestations may be accurate in only about 50% of cases (LeDuc and Lee, 1989a). Definitive diagnosis by seroconversion requires acute and convalescent sera in the case of IgG assays (LeDuc and Lee, 1989a), although a positive IgM test has promise for early diagnosis (Chen et al., 1987). In most circumstances viral isolation is not the method of choice in aiding diagnosis, as

hantaviruses can be difficult to isolate from patient specimens, (LeDuc and Lee, 1989b) and then require passage in cell culture or animal inoculation.

An additional problem arises when the goal of study is to define the probable source of viral infection and the reservoir involved in transmission. Most epidemiological studies have relied on comparative serological results using convalescent serum in a number of different assays (Childs et al., 1988; Groen et al., 1991). One widely accepted test uses relative neutralizing antibody titers to a number of different viruses, to implicate the specific virus involved (Dalrymple, 1989). This assay requires maintenance of reference serotypes of hantavirus and Biohazard level 3 containment. Also, it is not sensitive enough to differentiate between strains of infecting virus within serotypes.

Therefore, the availability of a polymerase chain reaction assay to detect viral RNA in patient specimens and to provide DNA fragments for restriction analyses, could revolutionize the rapid diagnosis of HFRS and the epidemiological of transmission. PCR has been previously adapted for amplification of the M segment of the tripartite hantavirus genome (Xiao et al., 1991). In this report we describe a PCR assay utilizing consensus primers capable of amplifying S segment RNA obtained from hantaviruses of each serotype. Restriction enzyme patterns that distinguish among each serotype and, in the case of Seoul-related viruses, among several strains are also described.

With these observations and background we initiated the current contact on the Epidemiology of Hantaviral Infections in The United States with the following six specific aims:

1) Monitor human populations in Baltimore, MD for evidence of acute HFRS.
2) Determine the extent of infection with hantaviruses in selected patient populations in Baltimore, MD and Detroit, MI .
3) Examine the association between hantaviral infection and three chronic human diseases; chronic renal failure, hypertension, and congestive heart failure.
4) Determine risk factors associated with human infection, and to characterize demographic patterns of infection.
5) Examine stored human tissues for evidence of previously unidentified cases of infected individuals, and correlate with clinical disease.
6) Adapt polymerase chain reaction techniques to study infection patterns in rodent and human tissues.

Owing to interesting preliminary results promising significant contributions to the rapid diagnosis of hantaviral disease globally, we departed somewhat from specific aim 5. Although this aim will be addressed as techniques are perfected, we would offer the following specific aim for this midterm report:

7) Investigate the potential use for consensus S segment PCR primers in the detection of all four serotypes of

Hantavirus; and the potential for limited restriction enzyme analyses of small (280 bp) fragments to be used in the identification of specific virus types.

Potential Military Significance

There are seven major areas of potential military significance to this contract:

1) The absence of reported HFRS in an area where infection in reservoir species is high may be due to the presence of nonpathogenic strains, or the spectrum of disease may be poorly understood. In the former case such strains would be useful candidates for either vaccine development, or for detailed molecular studies of pathogenesis of HFRS by comparison with known pathogenic strains. Additionally, if nonpathogenic strains do exist it would suggest that current surveillence techniques of reservoir species should be augmented to evaluate the risks to troops entering areas where hantaviruses of unknown virulence are found. Alternatively, if the spectrum of disease is not fully recognized, then troops could currently be becoming infected and their disease may be unrecognized. This has serious implications if sequelae result from infection.

2) HFRS due to rat-borne hantaviruses is poorly characterized clinically. Given that commensal rats are world-wide in distribution, these diseases are important, numerically. Additionally, commensal rats are potentially always a risk at military bases throughout the globe, as well as any place troops are stationed for extended periods of time.

3) Hantaviruses are transmitted by aerosol, as such, they represent an offensive BW risk to troops, as well as the known risks to troops from natural reservoirs of the viruses in the field.

4) HFRS is known to represent a serious acute illness, but preliminary evidence (including data gathered after the Korean conflict) indicates that long-term sequelae which require expensive, life-long treatment also may result. This possibility needs to be evaluated as it may require extensive health-care benefits for affected military personnel.

5) Selected populations surveyed in this study will permit us to evaluate the additional risk that individuals engaged in military service may engender relative to civilian populations.

6) Rapid diagnostic methods using recently developed molecular biological thechniques may be used to identify previously recognized, as well as new hantaviruses in areas of operations. The rapid diagnostic methods also permit treatment of infected troops with currently available medical methods within a sufficiently short time frame that therapies are efficacious.

7) Understanding viral and antigen distributions within tissues may aid in understanding how disease is produced, and help identify new therapies for troops.

BODY-SUBJECTS, MATERIALS AND METHODS

Subjects

Johns Hopkins Hospital (JHH)

All in-patients and out-patients using the JHH from 15 January 1986 to 30 May 1991 receiving 24 hr. proteinuria tests and having blood drawn were considered for inclusion in the study if they excreted >250 mg of protein/24 hrs. (More than 70% of patients with quantitative urine protein tests also had blood drawn.) This criterion was selected because significant proteinuria is a consistent clinical finding in all forms of HFRS regardless of the infecting virus. In addition, 2 patients/week with proteinuria <150 mg/24 hrs from the same population were included as controls. The first two individuals from a randomly chosen collecting day with this criterion were selected. Patients were excluded if currently undergoing chemotherapy for cancer, or treatment for human immunodeficiency virus (HIV). A history of previous cancer treatment or HIV infection was not, however, grounds for exclusion.

In addition, beginning in June 1990, all in-patients posted in the renal units at JHH were reviewed to ensure that specimens were being obtained during our routine screening of clinical laboratory records. This procedure has identified additional candidates for HFRS screening.

Johns Hopkins Hospital Emergency Room (JHH-ER)

Through a collaborative agreement with Drs. T. Quinn and G. Kelen, we obtained aliquots of 3400 patient sera from the JHH-ER during 1988. The samples were drawn based on CDC sampling guidelines for HIV screening, and provided a more adequate representation of the inner-city Baltimore population over age 30 than could be acquired from our sexually transmitted disease clinic (STD) population (see Childs et al., 1991). The samples are essentially from consecutive visits, however, CDC provided sampling protocols that slightly weighted the effort towards the very young (<5 years). This population, with the STD population, are used to provide a background estimate of hantaviral antibody prevalence with which to compare our populations with presumed higher risk of being seropositive (JHH proteinurics and dialysis patients).

Dialysis Patients

Baltimore Hospitals

Patients using dialysis equipment at Francis Scott Key, Good Samaritan, and Bon Secours Hospitals were enrolled in a study to define their prevalence of antibodies to hantaviruses. Initial contacts were made in 1988 and continue through the present. All specimens were collected after obtaining informed consent as specified by the Joint Commission of Clinical Investigations, and the Human Volunteers Committee of the Johns Hopkins University Schools of Medicine and Hygiene and Public Health, respectively.

Information regarding patients was obtained from dialysis unit records and includes; patient hospital number (for retrieving clinical records if needed), age, race, sex, ZIP Code of residence, date of first dialysis treatment and diagnosis and IDC number when available.

Detroit Hospitals

Patients using any of the six dialysis units in Detroit under the auspices of the Henry Ford Hospital were enrolled in a study to define the prevalence of hantaviral antibody in this populations. This population was approached as Henry Ford Hospital performs all cnronic renal dialysis for end-stage renal disease patients in the Detroit area. Initial contacts were made in 1988, and collections were begun in 1989. All specimens were collected after obtaining informed consent from the patients. Information was gathered on the age, sex, race, ZIP code of residence, ICD code, duration of dialysis, serum creatinine and urea nitrogen values.

Veteran's Administration Medical Center

Patients from the hypertension and the genitourinary clinics at the Baltimore Veteran's Administration Medical Center were enrolled in a study to determine hantaviral antibody prevalence in a population of veterans many of whom resided in Baltimore. The purpose was to determine if this population showed any increased risk of hantaviral infection compared to other inner city populations, that might be attributed to military service, and to determine the source of the infecting virus to determine if infection might be related to nondomestic sources of infection.

Serological Tests

Hantaviral Assays

Four serological tests were utilized for the detection of antibodies to hantaviruses; indirect immunofluorescent assay (IFA), plaque reduction neutralization (PRN), enzyme-linked immunosorbent assay (ELISA) and western blotting. The serological assays and the viral stocks commonly utilized (Seoul virus (strain Baltimore rat), Hantaan virus (strain 76-118), Prospect Hill virus (PHV-1) have been described previously (LeDuc et al., 1984; Childs et al., 1987a, b, 1988; Glass et al., 1990).

The ELISA technique was used to screen all sera and the other tests were used to confirm specificity (WB, PRN) and to serologically incriminate the rodent source of hantaviral infection by differential responses to a battery of known viruses (PRN; see Childs et al., 1988). Sera were considered suspect positive if their optical densities were greater than the mean plus three standard deviations of a group of three known negative human sera included on each ELISA plate. Suspect positive sera were further examined by WB analyses using prototype Hantaan virus (HTNV) and BRV as antigens. Sera producing a single band

at approximately 50 kd (corresponding to nucleocapsid antigen) were considered positive. To determine the specific virus causing infection, each positive serum, and a representative group of negative sera, were further examined by PRNT using prototype HTN, BRV and PHV. The PRN yields at least 4-16 fold differences in antibody titers between heterologous and homologous hantaviruses allowing identification of persons who may have been infected with HTN while traveling overseas, or were infected by a strain of Hantavirus from indigenous small mammals. Two other serological assays not previously utilized by our laboratory are described below.

Other Serological Tests

Antigen extracted from Vero cells infected with Armstrong strain LCMV was used in an ELISA similar to that for SEOV (Ksiazek et al., 1989). A sample of 23 sera found LCMV positive by ELISA and 10 negative sera were further tested by PRNT using heat inactivated, complement reconstituted serum at dilutions beginning at 1:8. The highest serum dilution at which 80% of the virus challenge (45-50 plaque forming units) was neutralized was considered the endpoint.

Sera tested for antibodies to leptospires were screened for IgG by ELISA using mwogolo, a serovar of the Icterohaemorrhagiae serogroup as antigen. Antigen was produced by a modification of the outer membrane protein procedure described previously (Terpstra et al., 1980), and has been shown to be genus specific (R. Ross Graham, unpublished data). Sera reactive at 1:200 dilution were considered positive. A subsample of 37 sera positive for leptospiral IgG by ELISA were examined by the microscopic agglutination test (MAT) by standard methods (Cole et al., 1973) and for IgM by ELISA. Samples were tested by MAT against the following serogroups (serovars); Australis (australis), Autumnalis (autumnalis), Ballum (ballum), Bataviae (bataviae), Canicola (canicola), Grippotyphosa (grippotyphosa), Sejroe (hardjo), Icterohaemorrhagiae (icterohaemorrhagiae, copenhageni), Pomona (pomona), Pyrogenes (pyrogenes), Shermani (shermani), and Tarassovi (tarassovi). A titer of 1:50 was considered positive for all serovars with the exception of shermani where a criterion of 1:800 was used due to the potential autoagglutination of the antigen.

Tested sera were collected from a sexually transmitted disease clinic so we were concerned with potential cross reactions to Treponema pallidum. To evaluate this possibility, all ELISA positive sera and 163 negative sera were tested for syphilis antibodies by a standard rapid plasma reagin (RPR) assay.

Polymerase Chain reaction (PCR) Methods

RNA Extraction

Two methods were employed to extract RNA from wild caught rat tissues for subsequent reverse transcription (RT) and then

DNA amplification by PCR. The first method required minimal handling but it was determined that the quality and quantity of RNA obtained was sub-standard for the methods we wished to employ. The method is still described below to provide details of our negative results.

Method 1-A 2-3 mm^3 piece of rat lung, spleen, kidney or bladder was minced with sterile scalpel blades within a laminar flow hood. The mince was diluted in PBS made with diethylpyrocarbonate (DEPC) treated water and pelleted at 500 X g for 5 min. and washed. Pellets were resuspended in 400 μl of ice-cold IHB/0.5% NP-40 containing 0.01% DEPC and vortexed. Post-nuclear supernatant fluids were placed in a screw top microcentrifuge tube in a humidified 37°C water bath (20-30 min.) followed by a 70°C bath (10-20 min.) and a return to 37°C (20 min.). Precipitates were pelleted and supernatants transferred to a second tube. Five-ten μl of this supernatant was used for RT/PCR reactions, as detailed below.

Method 2-Method 2 followed previously described techniques (Chomczynski and Sacchi, 1987), and was a single-step RNA extraction using the acid guanidinium-phenol-chloroform (AGPC) method. Briefly, 100 mg of tissue was homogenized with 1.0 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. The homogenized tissue was transferred to a 4-ml polypropylene tube and, sequentially, 0.1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were 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 1 ml of isopropanol and placed at -20°C for at least 1 hr. Sedimentation of precipitated RNA was again 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 μl of DEPC treated 1.0% Laureth-12 at 65°C for 10 min. Two μl of this material was then used in a RNA PCR protocol as recommended by Cetus (Perkin Elmer Cetus, GeneAmp RNA PCR Kit).

Hantavirus Strains

Twenty strains of hantaviruses were used in the effort to identify universal primers for detection and rapid diagnosis of hantaviruses and HFRS (Specific Aim 7). Viruses, their country of origin and the animal species from which each strain was isolated is shown in Table 1 (Appendix 2). Two strains were isolated from humans, seven from a single species of rat (Rattus norvegicus), six from three species of mice (Apodemus agrarius, Mus musculus, Apodemus flavicollis), three from two species of voles (Clethrionomys glareolus and Microtus pennsylvanicus) and one each from a shrew (Suncus murinus) and bandicoot (Bandicota

indica). The initial isolations of Girard Point virus, Houston and Baltimore rat viruses were performed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). The other hantaviruses were provided to USAMRIID by the WHO Collaborating Center for Virus Reference and Research (HFRS) in Seoul, Korea.

Preparation of Viral RNA-Total cytoplasmic RNA was prepared from Vero E6 cells that were infected with each strain of virus (Chirgwin et al, 1979; Glisin et al., 1973).

PCR Conditions

(a) First-strand synthesis of CDNA-Annealing reactions were performed in 10 μl volumes of DEPC-treated water containing 0.01 μM of the HTN-S4 primer (Fig. 1, Appendix 1) and 1 μl of cytoplasmic RNA from infected cells. The reaction mixture was incubated at 95°C for 3 min., then at 42° 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, 1X reverse transcriptase reaction buffer, 1 Mm of each of the deoxynucleotide triphosphates (dNTPs), 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 30 min., then at 95°C for 3 min. to eliminate reverse transcriptase activity.

(b) Selection of primers and probes-The nucleotide sequences of the S-segments of Hantaan 76-118 (Schmaljohn et al., 1986), SR-11 (Arikawa et al.,1990) and Prospect Hill virus (Parrington and Kang, 1990) were examined for regions of homology and oligonucleotide sequences for primers and probes selected, as shown in Fig. 1 (Appendix 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 identical homology so that this single set of "consensus" primers could be used to amplify viruses in the Hantavirus genus. During synthesis, inosine was inserted in positions with nucleotide mismatches (Fig. 1, Appendix 1). A 20 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. The length of the regions of these three viruses that were targeted for amplification was 281 nucleotide pairs (np).

(c) 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 HPLC-purified oligonucleotide primer (HTN-S4 and HTN-S5), 2.5 units AmpliTaq polymerase (Perkin Elmer Cetus, Norwalk CT) and 1X 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 thermal cycling. Amplification of target DNA was accomplished using a thermal cycle of 30 sec at 95°C for denaturing the DNA, 30 sec at 42°C

for annealing of primers and 30 sec at 72°C for sequence extension. Each sample was subjected to 30 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk CT).

(d) Analysis of reaction products-

(i) Agarose gel electrophoresis-A 10 μl 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.

(ii) Hybridization-Amplified hantavirus sequences were identified by Southern transfer hybridization from agarose gels (Southern, 1975). Following transfer, the nylon membrane (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 3X SSPE (20X SSPE; 3.6 M NaCl, 200 Mm NaH_2PO_4 [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. Five ng/ml of the HTN-S5 oligonucleotide probe (Fig. 1, Appendix 1), end-labeled with ^{32}P-ATP and T4 polynucleotide kinase (United States Biochemical Corp., Cleveland OH) to a specific activity of 2-5 X 10^8 cpm/μg, was added and incubated for one hr. at 42°C. The filter was then washed for five min. at room temperature in 3X SSPE, three times for five min. in 1X SSPE containing 0.1% SDS, and once for ten min. at 55°C in 5X SSPE with 0.1% SDS. Autoradiograms were prepared by exposure of filters to Kodak X-omat XAR-5 film with intensifying screens for 2 to 24 hr at -70°C.

The filters were then washed at 95°C to remove the oligoprobe and the hybridization procedure was repeated with a ^{32}P-labeled PCR-amplified hantavirus product. The probe was prepared by 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 exceptions: the final concentration of each dNTP was decreased to 25 μM, 6.25 picomoles each of ^{32}P-labeled dATP, dGTP, and dCTP (800 Ci/mMol) and 1 U of AmpliTaq were used in the reaction mixture. 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. For hybridization, the labeled 76-118 probe (sufficient volume to obtain 1-5 X 10^6 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 described for the oligoprobe and incubated for 1 hr. at 43°C. The filters were washed one 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 exposure periods of 1 to 6 hrs.

(iii) Restriction endonucleases-The following

endonucleases were used to analyze restriction length fragment polymorphism in the PCR-amplified hantavirus products: *Alu*I, *Bam*HI, *Dde*I, *Eco*RI, *Hae*III, *Hha*I, *Mbo*I, *Mbo*II, *Rsa*I, *Taq*I. Reaction products (1-5 μl) were digested for one hr. with 10 U of enzyme and in a total reaction volume of 15 μl. Appropriate buffer and temperature conditions were used for each enzyme to obtain optimal activity.

Agarose gel electrophoresis of the 15 μl digestion reaction was performed as described above. Estimates of the size of the restriction fragments were determined by comparison with molecular weight standards (*Msp*I digest of pUC18) using simple linear regression techniques. The logarithm of the size for each standard was plotted against the distance migrated in the gel. Migration distances of the hantavirus restriction fragments were measured and the size estimated by extrapolation from the regression curve.

Epidemiological Methods

Epidemiological Survey for Acute Disease

Subject Population

The surveyed patient population was drawn from the Blood Chemistry Department at the Johns Hopkins Hospital in Baltimore, MD from 1986 to 1990. All patients with proteinuria exceeding 250 mg/24 h were included in the survey if serum samples were available, excluding patients undergoing chemotherapy for cancer[8].

Case Definition

Diagnosis of hantaviral infection was determined serologically because of the diverse array of clinical presentations. Charts of patients were reviewed for evidence of illness consistent with HFRS (Lee, 1989), at the time of seroconversion. Serodiagnosis is a standard procedure even in endemic areas, as misdiagnosis based on clinical presentation approaches 50% (Yanagihara, 1990; Lee, 1989).

Acute infection was determined by at least a four-fold rise in neutralizing antibody titer. In situations where only a single serum sample was available, acute infection was defined as the presence of neutralizing antibody, and a significant (>1:800) IgM titer as IgM antibodies typically persist for only 1-2 months.

Association Between Hantaviral Infection and Chronic Disease

Matched Study

Seropositive patients by ELISA and PRNT were matched with patients from the remaining seronegative population. Seronegative patients were randomly selected and matched to

seropositives for age (within 3 years), and sex. Five seronegatives were selected for each seropositive, with the exception of two positive females aged 87 and 88 for which only 8 negative patients could be identified. Medical charts were examined without prior knowledge of serological status by one of us (Alan J. Watson) to determine clinical diagnoses. Variables recorded for each individual included race, age, occupation, home address and length of residency, travel history (if available), history of military service (if available), reason for admission, original admission ward, and medical history, including chronic disease history. Persons suffering from more than one chronic condition were included in each applicable category.

Diagnoses

Individuals with a history of systolic pressure greater than 150 mm, and/or diastolic pressure greater than 90 mm on more than three visits, were considered hypertensive. Those with impaired glucose tolerance, elevated blood sugar, especially if associated with retinopathy, or receiving appropriate medications were considered to have diabetes mellitus. The category of cerebrovascular accidents included individuals with strokes or ischemic attacks. Chronic renal disease was identified based on a history of serum creatinine exceeding 1.4 mg/dl for more than 3 months.

Within the category of chronic renal disease, several potential causes were further identified. Diagnosis was made on the basis of previous medical histories, physical examinations, and previously obtained laboratory tests. Where available, biopsy information was obtained to provide a definitive diagnosis. In the absence of biopsy data, specific attention was paid to the presence of a family history of hypertension, the temporal relationship between the onset of hypertension and discovery of kidney disease, a history of diabetes mellitus, or any evidence suggesting glomerular process e.g., nephrotic range proteinuria.

Individuals with chronic renal disease and at least a 10-15 year history of diabetes mellitus and/or associated retinopathy were considered to have diabetic nephropathy. Those with chronic renal disease in the setting of longstanding hypertension were diagnosed as having hypertensive renal disease. Individuals with a history of nephrotoxic drug ingestion and/or injection, followed by chronic renal disease, were categorized as drug induced. Obstructive renal disease was diagnosed for those with urological, radiological, or clinical evidence of obstruction, and autoimmune renal disease was diagnosed for individuals with positive laboratory findings for autoantibodies. In the absence of sufficient documentation, individuals that met the criterion of chronic renal disease were diagnosed as having disease of unknown origin.

Data Analyses

Analyses were performed with either SAS (1985) or BMDP

(Dixon 1988) statistical software systems. Descriptive analyses were performed for all variables and initial comparisons were made by contingency table or analysis of variance (ANOVA), as appropriate.

For risk factor analyses, variables were dichotomized to ease interpretation of results and to allow calculation of odds ratios. Crude odds ratios were calculated for the association between all study variables and rodent exposure, contact and leptospiral serological status (positive, titer $\geq$ 1:200 vs. negative, titer < 1:200). Stratification and logistic regression were employed to assess interactions between variables and to control for potential confounding variables. A set of variables thought to be important a priori were included in the initial logistic regression models, then all other variables were assessed for their contribution to this initial model and partial F-tests were calculated. After the selection of a logistic regression model incorporating the group of a priori variables and others found to be important risk factors or confounding variables, all possible interactions between these variables were assessed and retained in the final models if statistically significant ($p < 0.05$) or if felt to be biologically important.

Both individual and composite rodent exposure and contact variables were evaluated in the logistic regression models. History of foreign travel and individual travel destinations (modelled as five travel location dummy variables; Hosmer and Lemeshow, 1989) were examined, as were 12 occupational categories (office, construction, mechanical, food services, miscellaneous services, transportation, animal handlers, manufacturing, arts, outdoor work, professional, or unemployed). Crude odds ratios and adjusted odds ratios from the final logistic regression model, with 95% confidence intervals, are reported.

For analyses exploring the association of hantaviral antibody and acute and chronic disease, values are given as mean $\pm$ 1 standard deviation, except as noted. All frequency variables were tested as simple Chi-square tests for homogeneity, and odds ratios (OR) with 90% confidence interval (CI) were derived. Two-tailed tests were used in all comparisons. Statistical examination of primary diagnoses for chronic renal disease considered four categories; none, diabetes mellitus, hypertension, and other. Differences in cumulative distributions were examined using Smirnov two sample tests. Logistic regression was used to assess interactions between variables and to control for confounding variables.INSERT

In Situ Hybridization Methods

To document the tissue distribution of hantaviral proteins and nucleic acid we performed several experimental infections. Three day old Harlan strain mice were inoculated i.c. with 10^5 PFU's of either BRV (R - 58), Hantaan (76 - 118), or Rift Valley Fever Virus (ZH501) in 0.2 ml of media, or sham inoculated with media. At 17 - 19 days mice were euthanatized by CO_2

asphyxiation, and their tissues fixed in 10% buffered formalin. After 14 days, tissues were paraffin imbedded and sectioned at 4 um.

Sectioned tissues were probed for S-segment HTNV genome, using ^{32}-P labeled RNA made from c-DNA (Schmaljohn et al., 1987), by in situ hybridization (Gupta et al., 1987a, b), or were tested for antigen using an Avidin-Biotin Complex (ABC) test (Hall, unpubl. data). Details are given below. Tissues from RVF infected, and sham inoculated mice were used as controls to measure the degree of background labeling.

RNA probes to the S-segment of HTNV were prepared from cDNA of the entire S segment cloned into pGEM-1 by Dr. C. Schmaljohn, of USAMRIID. The cDNA was cloned into the Pst I site in the Multiple Cloning Region (Schmaljohn et al., 1987). Viral sense probe (SB) was made from the preparation by Sal I digestion. Following digestion, DNA was precipitated in 3 M Sodium Acetate (1/10 volume) and 100% Ethanol (2.5 volumes) overnight at -20°C. The preparation was centrifuged at 10 K for 30 minutes at 4°C, the supernatant was decanted and the pellet dried in a vacuum for 10 minutes. The pellet was resuspended in 70% Ethanol (-70°C), centrifuged for 10 minutes, dried, and resuspended in TE.

Radioactively labeled probe was made by combining 1 μg of DNA template with 5X buffer, RNAsin, and 10mM stocks of ATP, CTP, GTP, 35-S labeled UTP, and polymerase. The cocktail was mixed at room temperature, briefly spun in a microfuge, then incubated at 40°C for two hours. A 1:50 dilution of the cocktail was made in DEPC water to determine the amount of RNA incorporated and the specific activity of the probe. Cocktails with specific activities on the order of 10^9were then treated with DNAse, incubated at 37°C for 20 minutes followed by the addition of 0.1 M EDTA in DEPC water. The probes were then frozen at -80°C, overnight.

The next day, the probes were thawed on ice and a Sephadex column prepared. The probe cocktail was added to the column and radioactively labeled probe collected as the first peak of radioactive material passing through the column. Total and precipitable counts were determined, to assess the purity, and amount of RNA incorporated. Probes were frozen at -80°C until ready by hybridization studies.

For in situ hybridizations, probes were thawed at room temperature and the sample evaporated in a speed vacuum to a final concentration of 2 ng/ul. Tissue samples, previously mounted on organisilane treated slides, were deparaffinized in xylenes, and rehydrated in successive steps in 100%, 95%, 70% ethanols, and 2X SSC. Slides were treated with 25 ug/ml Proteinase K in a buffer of 10mM Tris and 2mM CaC12, for 30 minutes at 37°C. Slides were acetylated with 0.1 M TEA-HCL with 0.25% acetate anhydride for 10 minutes, then placed in 2X SSC, 70%, 95% ethanols, and air dried. A cocktail of formamide dextran sulfate and probe were made so that the final concentration of the probe was 0.5 ng/ul, and 20 μl of cocktail

was added to each slide. Cover slips were placed over each tissue section and sealed with rubber cement. The slides were then placed on a sponge soaked with 1M NaCl, in an airtight container, and incubated at 46°C overnight.

The following day, the cover slips were removed and the slides washed in 0.5 M SSC containing 10mM DTT. The slides were washed twice in 0.5 M SSC and then in 0.5 M NaCl in 10 mM Tris. The slides were then transferred to a bath of 0.5 M NaCl in 10 nM Tris containing 40 µg/ml of RNAse A, at 37°C for 40 minutes. The slides were washed four times in 0.1 M SSC containing 10 mM DTT, at 60°C, for 10 minutes per wash, then dehydrated in 70% and 95% ethanols containing 0.3 M Ammonium Acetate. The slides were dipped in photographic emulsion, placed in a holding box, at 4°C until they were ready to be developed (3-21 days). Slides were developed using standard procedures, counter-stained in hematoxylin and eosin, and mounted with permount. Tissues were examined under high power light microscopy for the presence of characteristic silver grains associated with labeled RNA probe.

Attempts then were made to hybridize the Hantaan probe with tissues from naturally infected rats, and BRV infected and uninfected humans. Fifteen wild rats were collected, eight infected and seven uninfected. Kidney and liver tissues from two infected and two uninfected Baltimore residents also were obtained from JHH. These tissues were treated as described above for experimentally infected mouse tissues.

BODY-RESULTS

Specific Aim 1) Monitor human populations in Baltimore, MD for evidence of acute HFRS.

a) Seroepidemiology-JHH-Proteinurics

A total of 1795 sera from 1341 patients at JHH were screened for hantaviral antibodies from 15 January 1986 to 30 April 1990. Twenty-two individuals were confirmed positive (1.54%); all possessed highest titers to Baltimore rat virus (BRV), the local Seoul-like hantavirus. Eighteen patients had unchanging IgG and neutralization titers and no IgM titer, indicating past infections. These are discussed below (Specific Aim 2).

b) Case Reports

Four patients from the JHH-Proteinuric population met the definition for acute hantaviral infection. This is an incidence rate of approximately 2.2%/patient-year. All four patients had histories compatible with mild HFRS associated with their seroconversions. Specimens from the patients were negative against all other infectious agents examined. The frequency of presenting symptoms (Table 2, Appendix 2) and clinical findings (Table 3, Appendix 2) are outlined below.

Case 1. A 62 year old black female was admitted to the Johns

Hopkins Hospital with a three day history of fever (>38.0°C), nausea, vomiting, a productive cough, diarrhea, dyspnea, and vertigo. Chest examination showed bilateral pleural effusions. Abdominal examination showed hepatomegaly. Initial laboratory evaluation showed a serum urea nitrogen of 27mg/dl, with a serum creatinine of 2.4 mg/dl. Serum electrolytes were normal, as were tests of urine function. Her hospital course was characterized by epigastric pain, chills, myalgias, diarrhea, vomiting and nausea. The patient developed microscopic hematuria and proteinuria of 1700 mg/24 h, and urine specific gravity decreased to 1.005. On day 13 the patient became hypotensive and anuric. The white cell count was 18,200/mm^3. The prothrombin time was 16 seconds, and the activated partial thromboplastin time was 35 seconds. The urea nitrogen rose to 79 mg/dl, and the creatinine was 3.0 mg/dl. Lactic dehydrogenase was 275 IU/L (normal=0-222), and amylase reached 123 IU/L (normal=15-120). Anuria persisted for a second day. The patient developed mild hyponatremia, and hyperkalemia. Anuria was followed by several days of polyuria with urine output of > 3 L/day. Prothrombin and APPT times peaked with the onset of polyuria at 17.0, and 35.9 seconds, respectively. At the onset of polyuria, AST increased to 104 IU/L, while ALT peaked the following day at 82 IU/L. Alkaline phosphatase was 140 IU/L prior to the onset of polyuria, before decreasing towards normal. With the onset of polyuria, serum urea nitrogen, and creatinine decreased, but remained elevated compared to values during admission.

She was discharged 4 weeks after admission with normal liver function panels, but a persistent, mild, elevation of serum nitrogen, and creatinine. Subsequently, the patient developed end-stage renal disease, and required renal hemodialysis.

An serum sample collected 7 days after the onset of illness was IgG positive by ELISA at 1:1000, and had neutralizing antibody titer of 1:20 to BRV, but failed to neutralize HTNV or PHV. Convalescent sera showed an IgG ELISA titer of 1:1200, and a four fold rise (1:80) in neutralizing antibodies to BRV. Sera collected two years after infection remained positive at 1:1000 by Ig-G ELISA and neutralized BRV at 1:80.

Case 2. A 58 year old black male presented with dyspnea, and anasarca, presumably due to congestive heart failure. Routine evaluation three months prior to admission indicated normal renal function at that time. Four days after admission the patient was noted to have new onset proteinuria, which was quantitated at 954 mg/24 h. The following day he developed nausea, vomiting, and diarrhea. Epigastric pain and hepatomegaly developed on day 8. The patient was noted to have hematuria and a low grade fever. His erythrocyte sedimentation rate was 27 mm/h, hematuria was noted and his urine specific gravity was 1.009. On day 11, he developed hypotension, which persisted a second day.

On day 13 the patient became oliguric with a urine output of 125 ml/24 h. Prothrombin time was increased to 20.9 seconds 10 days after the onset of illness, and APPT increased to 37.8

seconds. Recovery of renal function began after 5 days. The patient was discharged 3 weeks after admission. By this time the patient was subjectively better but retained elevated BUN, creatinine and alkaline phosphatase. The patient was lost to followup at this time.

A serum sample collected the day of the onset of illness was tested for hantaviral antibodies. It was negative for IgG by ELISA and PRNT. A sample collected 1 week later had an IgG titer of 1:12,800 and a neutralizing titer of 1:20 to BRV. There were no neutralizing antibody titers to HTNV or PHV.

Case 3. A 71 year old black female reported to the Johns Hopkins Hospital emergency room with a >1 week history of illness characterized by anorexia, chills, and hemoptysis. She also complained of epigastric pain and melena. On examination she had pleural effusions. The patient was admitted to the hospital where three days later she developed clinical features of deep vein thrombosis. At that time the urea nitrogen and creatinine were noted to be elevated. Liver transaminase levels were normal but alkaline phosphatase was elevated (163 IU/L). Proteinuria was quantitated at 550 mg/24 h; pyuria, and a few granular casts but no hematuria were noted. She showed no evidence of leukocytosis.

A blood sample obtained 18 days after the onset of illness had an IgG titer of 1:1000 by ELISA and a neutralizing titer of 1:20 to BRV. Convalescent sera showed a four-fold rise in neutralizing titer to BRV, and an IgG titer of 1:1200. Followup examination showed normal liver enzyme panels, but continued elevated serum urea nitrogen, and creatinine up to 13 months after the illness.

Case 4. A 79 year old black female was admitted to Johns Hopkins Hospital complaining of a >1 week history of an illness with sudden onset characterized by; productive cough, nausea, vomiting, diarrhea, and chills. Urinalysis indicated proteinuria of 4100 mg/24 h. The urea nitrogen was 91 g/dl, and creatinine was 3.4 g/dl. The prothrombin and APPT times were elevated. Physical examination showed pleural effusion, epigastric tenderness, and hepatomegaly. The patient was treated for acute renal failure by diuretic therapy with little effect, recovered spontaneously, and subsequently was released. Two weeks later the patient was readmitted for a cerebrovascular accident.

A single serum sample was obtained for serological testing 3 days after admission. She had an IgG titer of 1:4000 by ELISA and IgM titer of 1:800 by ELISA, and a neutralizing titer of 1:640 to BRV. She also had a neutralizing titer of 1:20 to HTNV, but did not have neutralizing antibodies to PHV.

Specific Aim 2) Determine the extent of infection with hantaviruses in selected patient populations in Baltimore, MD and Detroit, MI.

a) Seroepidemiology-JHH-ER

A total of 3400 sera were tested from patients visiting the JHH-ER and 4 (.1%) were positive by PRNT to BRV after initial screening by ELISA. This antibody prevalence was in excellent accord with our previous study population from the Carolyn Street sexually transmitted disease clinic (STD). A comparison of the stratified analyses of these two study populations (Table 4, Appendix 2) provides an indicator of background levels of hantaviral infection in the inner city of Baltimore. The combined data from the STD and JHH-ER populations (n=6060 individuals) are subsequently used to compare infection rates in other targeted populations.

b) Comparative Serology

A comparison of the age-stratified antibody prevalences in the JHH-Proteinuric population with that in the combined JHH-ER and STD populations revealed an overall significantly greater risk of seropositivity in the population with some deficit in renal function (OR=4.90; 95% CI(2.37,10.16)). Comparisons based on 2-decade intervals showed a linear trend of increasing risk of seropositivity in the proteinuric group (Table 5, Appendix 2), however, statistical significance was precluded by small numbers of seropositives.

c) Dialysis Populations in Baltimore

Data were obtained from 352 patients in the renal hemodialysis units in Baltimore. The population was predominantly black (79.9%), with a median age of 55 years, and nearly evenly divided by sex (52.2% female). There was no difference in the sex ratio between races (OR=1.21; 0.68-2.14). Most (55.4%) of the patients resided in centrally located zip codes in Baltimore City, 36.9% resided in peripherally located zip codes, while only 7.7% resided outside of the city limits. Thus, the population focuses, as intended, on Baltimore residents, with nearly equal representation of the sexes.

Primary diagnoses for the cause of endstage renal disease were available for 317 (91.1%) patients. Diagnoses were obtained from the International Classification of Diseases codes that were recorded in patient charts. Among this population the leading causes of end stage renal disease were hypertension (40.7%) and diabetes mellitus (29.0%). Other significant causes of renal disease were: glomerulonephritis due to various causes (7.6%), obstructive uropathies (3.2%), polycystic disease (2.6%), and IV drug abuse (2.2%).

Serological data have been completed on 189 patients from Baltimore. Hantaviral exposure was confirmed in six individuals (3.2%), three males and three females. Based on PRNT results all exposures were to a Seoul-like hantavirus. None the patients had histories of overseas travel, indicating that domestic exposure was the source of infection. All six patients had hypertensive renal disease as the primary diagnosis for their end-stage renal disease. Thus, there is a strong statistical association between hantaviral infection and hypertensive renal disease in this

population (p=0.0032, Fisher's exact test). This supports the observed association between hypertensive renal disease and hantaviral infection observed in the JHH Proteinuric population. Among the hypertensive population, 8.6% of the patients were seropositive for exposure to rat-borne hantaviruses.

The six patients resided throughout the city; four in the centrally located zip codes, and two in peripheral areas. None resided outside the city limits. There was a tendency for residents of centrally located regions in the city to have a higher likelihood of exposure than those in peripheral areas (4.1% vs 2.4%) (OR=1.72, 0.24-19.43), but the difference was not statistically significant.

d) Dialysis Populations in Detroit

A total of 402 patients were sampled from the Detroit renal hemodialysis population. This population was similar to Baltimore city in its age, sex and race composition. The population had a median age of 60 years old, and was predominantly black (70.9%). However, there tended to be a greater proportion of males in this population than observed in Baltimore (56.9% vs 47.8%) although the difference was not statistically significant (OR=1.44; 0.92-2.28).

Fifteen patients (3.73%) were positive when screened by IgG ELISA. None of the patients were confirmed positive by PRNT to either Baltimore rat or Hantaan viruses. Sera from these patients were tested by IgG ELISA at USAMRIID, and confirmed positive. Thus, these patients either represent false positive ELISA tests or possible hantaviral infections with a noncross-reacting hantavirus. Currently, we are aware that patients with autoimmune diseases, especially SLE, may produce false positive results in ELISA tests. However, none of the seropositive patients are reported to have autoimmune disease. Thus, we are attempting to determine if another hantavirus is responsible for the serological patterns we have observed in this population. We have contacted the City of Detroit Rodent Control Board to receive permission to serologically sample rats within the city for evidence of hantaviral infection. If infection is found, isolation attempts, using standard protocols will be used to isolate the virus, and characterize it relative to other domestic strains of virus.

e) Populations in Veteran's Administration Medical Center

We obtained 822 samples from patients in the Veteran's Administration Medical Center in Baltimore. One hundred and ninety-seven patients were being treated for hypertension, and 625 for urogenital diseases. Ten patients in the genitourinary clinic also were treated for hypertension. All patients were male, 77.3% were black. The median age was 65 years old.

Crude seroprevalence in the hypertension clinic was 2.67%. All exposures were to a rat-borne hantavirus. Seroprevalence was highest among 50-60 year old patients (4.17%), decreasing in younger and older patients. All seropositive patients were black, but the racial difference was not statistically

significant, as only one white patient was expected to be positive. All seropositive patients lived within Baltimore city, especially in the south central and southwest areas. Only one patient reported overseas duty in Korea, or other previously recognized endemic zones.

Crude seroprevalence to hantaviruses in the genitourinary clinic was 1.30%. All exposures were to a rat borne hantavirus. Exposure to hantaviruses tended to occur more frequently among hypertensive patients than among the genitourinary population (OR=2.03; 0.52-7.12), although the difference was not statistically significant. In part, this may be due to the small sample size in the hypertension clinic population, and secondly, long-term genitourinary complications may be a sequela of hantaviral infection (Rubini et al. 1960).

Specific Aim 3) Examine the association between hantaviral infection and three chronic human diseases; chronic renal failure, hypertension, and congestive heart failure.

a) Antibody Prevalence and Chronic Disease

Among the JHH-Proteinuric population, 72% of patients excreting $\geq$ 250 mg protein /24 hr also had blood collected. Sera were not available from 446 patients with total urine protein >250 mg/24 hrs. A total of 1,669 sera were obtained from 1,148 individuals who met the criteria for inclusion (x = 1.34 samples/person). Females predominated in the population (1.6:1), and averaged 41.4 $\pm$ 20.9 years of age. Men were significantly older (51.0 $\pm$ 19.2 years) than women (t = 8.25, 1146 df; $p<0.005$). The median proteinuria for men (720 mg/24 hours, 25-75% Quartiles 332.5 - 1614 mg/24 hours) was significantly higher than for women (532 mg/24 hours, 210-1425 mg/24 hours).

Twenty samples (x = 1.33 bleeds/patient) from fifteen patients (1.31%) were seropositive for hantaviral antibodies by ELISA and confirmed by Western blot. All seropositive persons had markedly higher ($\geq$ 10 fold) titers by PRNT to BRV than to HTN, and none neutralized PHV, implicating BRV in their infections. Sera from seronegative individuals (n = 93) were negative by Western blot, and failed to neutralize any of the hantaviruses indicating none of these individuals had been previously infected. Individuals with proteinuria (>150 mg/24 hrs) were 2.7 times (0.5-14.7; OR $\pm$ 90% CI) more likely to be seropositive than individuals with normal protein levels. However, this difference was not statistically significant.

Most (10/15) seropositives were admitted for complications from chronic diseases. Of the seropositives without chronic disease, one was admitted for severe preeclampsia, one for a psychiatric disorder, and three for complications from their inabilities to care for themselves. None of the seropositives reported histories of foreign travel. The median time of residence in Baltimore for 10 patients was 43 years. The duration of residence was not obtained for two Baltimore

residents. Three individuals lived outside of Baltimore (one from Annapolis, Maryland, and two from New York, New York).

The seropositive individuals consisted of 10 women and 5 men, of whom 13 were African-American and 2 were Caucasian. The sex ratio among seropositive individuals was similar to the population as a whole (χ^2 = 0.16, 1 df, n.s.). There was no significant difference in the ages of exposed men and women (Smirnov test (T_1 = 0.5, p = 0.20). Their ages ranged from 23 to 90 with a median age of 65 years.

Demographics of Seropositives and Seronegatives

The home addresses for 73 age and sex matched controls (seronegatives) was similar to that of the seropositives. Fifty-five resided in Baltimore, while 18 lived outside the city. Mapping of residences indicated that seropositives and control patients were drawn primarily from neighborhoods in Eastern Baltimore, (Fig. 2, Appendix 1), where seropositive rats had been captured (3). The proportion of Baltimore residents among seropositives and seronegatives, (χ^2 = 0.15, 1 df; n.s.), and the length of their residencies (median 43.0 vs 38.5 years) did not differ significantly, nor did their occupational histories (Table 6, Appendix 2). No seropositives and only one seronegative held a white collar position, and 57.4% of seronegatives and 42.9% of seropositives were either unemployed or held unskilled/day labor jobs. African-Americans represented 86.7% of the cases and 64.4% of the controls, a difference that was not statistically significant (χ^2 = 2.93, 2 df, 0.1<p<0.5).

b) Clinical Study

Examination of patients' charts did not identify any consistent laboratory finding that differentiated cases from controls. Although seropositive patients tended to have higher values for systolic blood pressure, serum creatinine, and proteinuria levels, they were not significantly different (Table 6, Appendix 2; p>0.10). Diastolic blood pressure was higher among seronegatives; (p<0.10), but did not reflect a lack of treatment in this group, as all hypertensive patients were medicated.

In contrast, seropositives represented a defined subgroup of patients in this population with regard to clinical histories of chronic disease. They had higher rates (80.0% vs 43.8%) of chronic renal disease than seronegatives (OR= 5.1, 1.6 - 13.3), and there was a marked difference between seropositives and seronegatives in the presumed etiology of renal disease (Table 7, Appendix 2). Seropositives had significantly higher rates of hypertensive renal disease (70% vs 9.4% of those with assignable diagnoses), while diabetic nephropathy was the most common diagnosis in the control group (50% vs 20% among cases). Other sources of chronic renal disease occurred at lower frequencies (6.2 - 12.5% and did not differ between groups). The differences in primary diagnoses among cases and controls were highly significant (χ^2 = 26.75, 3 df, p<0.005), and were primarily due

to the overall higher prevalence of chronic renal disease among seropositive patients, and the differences in the frequency of hypertensive renal disease between the two groups (Table 7, Appendix 2). Fourteen seropositive individuals (93.3%) had hypertension. This prevalence was significantly higher (OR= 7.7, 1.2 - 23.5) than among the controls (64.4%) (Table 7, Appendix 2). Seropositives also were 4.9 times (CI = 1.5-15.8) more likely to have suffered cerebrovascular accidents compared to controls. There was no difference in the prevalence of diabetes mellitus between the two groups (OR= 0.7, 0.3 - 1.9). Biopsies were available only for a minority (<20%) of patients, as the procedures were not generally performed on individuals with chronic renal failure. However, in all individuals, diagnoses were made by attending physicians, and none of our diagnoses differed from theirs.

Given the high rate of hypertensive disease among inner city African- Americans (Rostand, Brown Kirk, et al. 1989), their somewhat higher representation among the seropositive population was considered a possible source of confounding. However, these effects persisted after stratifying by race. Among African-Americans, prevalences of hypertension (χ^2 = 3.05, 1 df, p<0.10), chronic renal disease associated with hypertensive renal disease (χ^2 = 19.05, 3 df, p<.005) and cerebrovascular accidents (χ^2 = 3.15, 1 df, p<0.10) remained significantly higher among seropositive individuals. The small sample size for seropositive Caucasians prevented a meaningful analysis.

Chronic heart failure also was examined among seropositive and seronegative patients. There was a tendency for seropositive patients to have higher rates of heart failure than seronegative patients (OR=2.33; 0.62-4.03). However, the difference was not statistically significant.

Results from logistic regression supported the results of the univariate analyses. Hypertensive renal disease (OR=8.35), and hypertension (OR=4.13) remained as significant independent variables in the model predicting serological status, while residence inside Baltimore city vs outside the state of Maryland approached significance (OR=2.12), with city residence associated with exposure. No other variable helped predict serological status.

Specific Aim 4) Determine risk factors associated with human infection, and to characterize demographic patterns of infection.

Owing to the low overall prevalence of hantaviral neutralizing antibody in Baltimore and Detroit populations, we were limited in our epidemiological approaches to identify risk factors for hantaviral antibody, beyond those described under specific aims 1 and 2. We decided upon two complementary approaches, which, although not directly addressing the aim, allow for: a) Determination of demographic and other characteristics associated with exposure and physical contact

with the rodent reservoir (Rattus norvegicus) of hantaviruses in urban areas; b) Document and compare the exposure history and antibody prevalence of our sample population for two other pathogens associated with rodents (lymphocytic choriomeningitis virus associated with house mice (Mus musculus) and leptospirosis associated with Norway rats and domestic pets) to allow the relative estimation of the risk of hantaviral infection compared to other rodent-borne pathogens.

a) Reservoir Exposure and Contact

Demographic Characteristics of Study Population

Interviews, with at least partial completion of questionnaires, were conducted with 1363 persons at the STD Clinic. The racial characteristics of the respondents were 94.3% black, 4.9% white and 0.8% classed as other (hispanic, Native American and oriental). Males comprised the majority of the sample with male/female ratios of 2.3 for blacks, 2.4 for whites and 1.8 for other races. The median age for all respondents was 23.5 years, with females, in each racial group, being consistently and significantly younger than males. As only 11 respondents were identified in groups other than white or black, and these individuals were excluded from detailed analyses.

Levels of education, for individuals over 18 years of age, were comparable across all groups and indicated a median education level of between 11 and 12 grade. Reported income was consistently higher for males than females, and the overall median was $8-10,000. Unemployment was similarly high in all groups with 40.2% of blacks, 41.0% of whites and 40.0% of other races, over the age of 18, reporting no current occupation. Overall, these characteristics indicated that this population was drawn from a low socio-economic stratum of the City.

The majority of the respondents were born in Baltimore, and over 99% were currently living in the City. ZIP Code mapping indicated that the majority of STD respondents lived within the multi- unit residential sections of the inner city. The median residency time in both current and previous dwellings was 3-4 years (range, 2 weeks-45 years)

Reports of Sightings of Rodents

Reports of rats and mice within residences, on streets and in alleys at the current home address, or at the workplace are summarized for black and white respondents in Figure 3 (Appendix 1). Overall, mice were reported within residences significantly more frequently than rats (48.8% and 5.9% of respondents, respectively; $\chi^2 = 602.9$, $p < 0.0001$), although rats were more commonly sighted on streets or in alleys around residences (63.8% and 32.1%, respectively; $\chi^2 = 269.3$, $p < 0.0001$). Mice were also more frequently reported than rats at the work place (23.4% and 16.0% respectively; $\chi^2 = 19.7$; $p < 0.001$).

The patterns or trends in sightings of rodents were

generally similar across race (Fig. 3, Appendix 1). Blacks and whites reported rat and mouse sightings within their residences at similar frequency, but blacks reported rats on streets and in alleys more frequently than whites (65.3% compared to 39.4%; χ^2 = 18.38, p <0.001). Conversely, more whites reported mice at their workplace than did blacks (28.6% and 22.7%, respectively; χ^2 = 13.57, p = 0.001).

In general, male and female respondents reported rodent sightings at similar frequency. The only exception was that black females reported mice more frequently (52.6% vs. 47.2% for males) and at heavier infestation levels (9.8% reported frequent sightings compared to 4.8% for males; χ^2_2 = 11.84, p = 0.003) than did males. In all other univariate comparisons black or white males and females reported infestations at similar frequencies.

In accounts of past rodent infestations at previous residential and work locations, a similar pattern emerged to that seen for current residences. Overall, the percentages of respondents who reported exposure to rats at some time, either in current dwellings or in prior residences, was 14.8% of blacks and 19.8% of whites, and for mice 61.3% of blacks, and 61.2% of whites.

Rat exposure was primarily reported as sightings of animals on streets or in alleys, with relatively few reports of this species in homes or at the workplace. Overall, 16.0% of individuals reported some exposure to rats at work, and jobs involving pest control/sanitation and construction/home repair had significantly (P<0.025 in each case) higher risks of exposure (crude odds ratio =4.55, [1.5,14.7; 95% CI) and 2.15 [1.2,4.0]), respectively. Day laborers/maintenance workers and warehouse/factory workers also reported increased rates of rat sightings, but not significantly more than expected from the overall sample. Significantly decreased exposure to rats was indicated for office (clerks, secretaries, bank tellers, receptionists) or store workers (crude odds ratio=0.22, [0.10,0.54], P<0.025).

The overall rate of reporting of mouse exposure at the workplace was 23.4%. Only pest control/sanitation workers had significantly higher (crude odds ratio=4.04, [1.3,12.5]) rates of exposure to mice, otherwise the exposure was fairly evenly distributed among occupations.

Contact with Rodents

Contacts with rodents, were similar across racial groupings (Fig. 4, Appendix 1). The major distinction between reports of rodent contact was a consistently higher percentage of white respondents reported picking up rats (10.6 % vs. 4.5 % for blacks; χ^2 = 5.18; P=0.023) and mice (27.3 % vs. 9.5%, χ^2 = 21.18, P<0.0001).

Respondents indicated significantly higher contact rates with mice compared to rats, with regard to trapping (49.1 % for mouse and 19.8 % for rats), droppings (30.8 % and 13.0%), and

picking up a rodent (10.4 % and 4.8%; P<0.0001 for all comparisons). However, there was no difference in the frequency with which bites were reported for rats or mice (0.9 % and 1.0 % respectively).

There were some significant differences in patterns of rodent contact between the sexes. Black males reported more frequent contact with rats through trapping (20.8 % compared to 13.0 % for females) and picking up rats (5.5 % and 2.1%), and more frequent contact with mice through trapping (52.6 % and. 41.2%) and picking up mice (11.8 and 4.4%; P<0.01 for all four comparisons). White males reported significantly more contact with rat droppings (25.5 % versus 0.0 % for females; $\chi^2 = 5.93$, P = 0.01), and there was a strong tendency for both black and white males to pick up rats and mice more frequently than females (P<0.1 in both comparisons).

Contacts with rats or mice were predominantly through interactions with wild animals, although in 3 cases of contact with rats via droppings/picking up animals and in 10 cases of mouse contact via droppings/picking up animals, the rodents in question were either pet or laboratory rodents. In 1 of 9 reports, mouse bite was also associated with a laboratory animal.

Rat bite was reported by 8 (mean age when bitten = 11.1 years [range 2 - 29]) and mouse bite by 9 individuals (mean age when bitten = 14.4 years [range = 1 - 26]). In 3 cases of rodent bite the individuals reported being bitten at age 2, and may not have had personal memories of the bite. One individual reported being bitten by a rat while in Vietnam rather than in Baltimore. One individual reporting mouse bite claimed to have been bitten by a field mouse, rather than a house mouse.

Geographic Distribution of Rodent Exposure

The geographic distribution of reportings of rats on streets and alleys or within residences indicated greater infestations in central, inner-city locations, than from peripheral locations (Fig. 5, Appendix 1). Frequencies of reported rat sightings on streets and alleys were 2-4 fold higher in inner-city locations than in more peripheral locations. In addition, in peripheral ZIP Code areas most individuals reported never having seen rats within residences, while inner city residents reported a low, but consistent number of sightings.

A similar pattern to that of rat reports was seen for house mice (Fig. 6, Appendix 1). However, the differences in frequency of reports of mice within residences were less distinct between peripheral and inner-city locations.

Multivariate Analyses

Logistic regression indicated a different suite of variables contributed to reports of mouse and rat sightings (exposure) compared to mouse and rat contact (physical contact with a rodent or its excreta; Table 8, Appendix 2). In most instances multivariate analyses confirmed patterns present in univariate

comparisons.

Mouse and rat exposures were significantly associated with respondents with central as compared to peripheral location of residence and with current ownership of pet dogs (Table 8, Appendix 2). Increasing residence time was significantly associated with reports of mouse exposure and was marginal ($P<0.1$) for reports of rat exposure. Lower income was independently associated with rat exposures (adjusted odds ratio=1.6 [1.1,2.1], $P<0.01$) but was not significantly associated with mouse exposure or contact with either rodent. Reporting of rodent exposures were generally independent of age and sex; in a single analysis race was significantly associated with risk of rat exposure (adjusted odds ratio=2.0, [1.1,3.5], $P<0.05$).

Risk of actual contact with rodents was independently associated with older age, male gender, dog ownership, and residence within central Baltimore (Table 8, Appendix 2). Risk of rodent contact was independent of race, income, residence time in house, and cat ownership.

b) Exposure of Study Population to Rodent-Borne Pathogens

Serological Tests

Fifty-four of 1149 (4.7%) persons had antibody titers of >1:100 to LCMV by ELISA. The prevalence was similar across races and there were no differences attributable to sex (Table 9, Appendix 2). Antibody prevalence increased with age, with mean ages of seropositives of 28.2 ± 8.8 and 23.3 ± 6.4 years for males and females, respectively. Seropositive individuals were found throughout the City. The reciprocal geometric mean titer (GMT) for ELISAs to LCMV was 600 (range, 100-12,800), and 14 of 23 positives, and none of the negatives, neutralized LCMV at serum dilutions > 1:8 (concordance of 72.7%). The GMT of neutralization positive sera was 28 (range, 8-512).

Three of the 1180 (0.25%) sera tested for antibodies to SEOV were positive by ELISA (Table 9, Appendix 2; GMT = 1170; range, 200-4000). All of the positives had neutralizing antibody titers (GMT = 100; range, 20-320) to the Baltimore rat isolate of SEOV and each was positive by western blot. Two of the three sera with neutralizing antibody to SEOV neutralized prototype Hantaan virus, strain 76-118, at reciprocal titers of 10 and 20 (8- and 32-fold lower than their neutralizing titers to the Baltimore rat virus). None of the sera tested had neutralizing activity against Prospect Hill Virus.

The SEOV seropositive individuals were all black males with a mean age of 24.3 ± 1.4 (range, 23-27). Each was born in Baltimore and currently lived there, and none had traveled to a foreign country or served in the military.

There was no significant statistical differences between seropositive or seronegative individuals for either virus with regard to frequency of reported rodent exposure and cat or dog ownership. Hamsters were not implicated in LCMV exposure as seronegative individuals indicated history of ownership more

often than seropositives (3.8% and 1.9%, respectively).

The overall prevalence of ELISA IgG titers $\geq$ 1:200 for leptospirosis was 16 % (185/1150), but only a single (1/37) IgM titer $\geq$ 1:200 was found. The geometric mean titer of positive sera was 566, with 40 (21.6 % of positives) individuals with reciprocal titers of 200, 59 (31.9 %) with 400, 55 (29.7 %) with 800, 18 (9.7 %) with 1600, 7 (3.8 %) with 3200, and 5 (2.7 %) with the highest observed titer of 6400.

Of the subsample of 37 sera positive by ELISA, 31 (83.8 %) had MAT titers of $\geq$1:50 to one or more pathogenic serovars. The median number of positive reactions was to 3 serovars, with one serum agglutinating 8. The most common serotype eliciting a response was Batavia (20/37) followed by Icterohaemorrhagiae (16/37; including serovars icterohaemorrhagiae and copenhagi), while no response was observed to Shermani. Geometric mean titers to all serovars were low (range 50-70), and indicated few, if any, recent infections.

There was no indication of significant cross reactivity with T. pallidum as 6/163 (3.7 %) leptospire seronegative and 13/185 (7.0 %) seropositive individuals were positive by RPR (χ^2=1.88, P=0.17). Although not statistically significant, these data suggested that almost twice as many leptospire seropositive samples were syphilis seropositive than leptospire seronegative samples. This association was likely to be overestimated, however, as the leptospire seropositive individuals were significantly older than leptospire seronegative subjects.

Descriptive Epidemiology

Crude prevalences of antibody to leptospires were examined for all variables. Individual variables included in composite variables, such as the unique rodent contact and exposure histories and the 12 employment categories, also were examined, but none were associated with leptospiral seropositivity. There was a strong association with age, such that prevalence of leptospiral antibody peaked in age groups over the age of 20. Frequency derived division of age into quartiles indicated that this population could be dichotomized into individuals $\leq$19 (the lowest quartile of age, 25.9 % of the total population; 12.8 % seropositive) and >19 (20.7 % seropositive). Males had a higher seroprevalence than females, although males were also significantly older than females regardless of race (for blacks, mean age for males=25.5, females=22.9, $p<0.001$; for other races males=31.2, females=22.7, $p<0.001$, ANOVA). None of the other demographic variables of race, education, employment or income were associated with leptospiral seropositivity. Animal contact, exposure and ownership variables were infrequently associated with presence of leptospiral antibody. Only current ownership of cats was significantly associated with lower antibody prevalence. None of the composite rat and mouse exposure or contact variables were associated with antibody, and multivariable analyses incorporated individual rather than composite variables.

Analytic Epidemiology

Demographic variables: Logistic regression indicated that several study variables were significantly associated with increased or decreased risk for leptospiral antibody (Table 10, Appendix 2). Age and sex remained as independent risk factors, even after adjustment for confounding variables, with individuals over age 19 possessing approximately 3-fold higher odds of leptospiral antibody. Both black race and low income were also independent risk factors in the main effects model.

Travel variables: History of travel to Canada was positively associated and travel to Mexico, the West Indies or South America was negatively associated with leptospiral antibody.

Animal variables: Self-reported contact with rat excrement was negatively associated with seropositivity. Two pet ownership variables were independent risk factors for leptospiral antibody, but in opposite directions. History of bird ownership (which included farm birds such as chickens as well as pet birds) was associated with leptospiral antibody. Current cat ownership was negatively associated with antibody, and was the only study variable to show significant interactions with two other risk factors, age and race (Table 11, Appendix 2). Current cat ownership eliminated the risk of leptospiral seropositivity associated with older age ($\geq$19) and race (black vs. other). Not owning a cat resulted in a 3-4 fold elevated odds of leptospiral antibody for these groups (Table 11, Appendix 2). However, cat ownership did not eliminate the risk associated with male gender. Similar associations between these variables were observed when cat ownership was categorized as ever owning a cat (past or current ownership) vs. never owning.

Specific Aim 5) Examine stored human tissues for evidence of previously unidentified cases of infected individuals, and correlate with clinical disease.

In situ hybridization of experimentally infected suckling house mice tissues was successful. Pathology examination of infected mice with H&E staining revealed perivascular mononuclear cuffing with gliosis and cellular leptomeninges with blast cells in the brain. In addition, reactive endothelial cells were present. Perivascular mononuclear infiltrates with blast cells also were present in the lung. The liver showed periportal mononuclear infiltrates, and the spleen had lymphoid hyperplasia. In lymph nodes the architecture was effaced and blast cells were present. Other tissues appeared normal.

Hybridization with ^{32}P labeled S-segment Hantaan virus genome revealed no signal in Rift Valley Fever infected, and sham inoculated mice. The probe showed strong signal in the hippocampus and cerebellum of Hantaan and Baltimore Rat virus infected mice, indicating good cross reaction among the different hantaviruses. Virus was located in the neurons of the cerebral cortex within the external granular, external pyramidal, and

internal pyramidal layers. Astrocytes near the lateral vertricle and cells within the choroid plexus also were infected. In the cerebellum, Purkinje cells, and neurons of the granular layer were infected. Probe also was found in thalamus, and pituitary. Lung cells also showed signal but poor preservation made cell identification difficult. Strong signal was detected in myofibers of the heart. Probe also was observed in pancreatic islet cells,, periacinar cells of the salivary glands, and in lymph nodes.

A labeled probe for Rift Valley Fever virus also was developed, and was used successfully to label tissues from experimentally infected house mice. Rift Valley Fever virus was localized to the hippocampus, and hepatocytes.

Attempts to hybridize probe to tissues from naturally infected rats, and humans were, generally, unsatisfactory. Hybridization to tissues from the 15 rats and two humans revealed no substantial signal. Data were subsequently obtained from Dr. C. Schmaljohn, at USAMRIID, that indicated mismatch in the sequences between Baltimore and Hantaan viruses was approximately 30%, suggesting that stringency conditions were too high to obtain a good signal. Subsequent attempts to alter stringency conditions were not successful in that background levels increased substantially at cooler temperatures, obscuring any signal. Attention, therefore, was focused on obtaining a useful Baltimore rat virus probe for naturally infected tissues. PCR (see below) was used to incorporate ^{35}S labeled nucleotides in the 281 np BRV S segment product. These results have just recently been successful, and will be used on naturally infected animals and humans to determine the extent of infection and tissue tropisms.

Specific Aim 6) Adapt polymerase chain reaction techniques to study infection patterns in rodent and human tissues.

Forty seven rats were trapped from Baltimore alleys and 8 were initially selected for RNA extraction by method 1. Three of these animals were IFA negative and 5 had IFA reciprocal titers ranging from 2048-4096. Rats with IFA titers to BRV were preferentially sampled as in the past, seropositive rats have yielded isolates of Seoul virus by co-cultivation and tissue passage through laboratory rats (Childs et al., 1988a). None of these 8 animals were positive by PCR as indicated by a specific band of appropriate size or Southern hybridization followed with ^{32}P-labeled probes. The original oligomer probe used, HTN-S5, was subsequently shown not to hybridize with most strains of Seoul virus (Fig.7, panel B, Appendix 1), including BRV. We have since developed two additional probes which hybridize with all rat viruses (Fig. 7, Panel C, Appendix 1). Filters initially probed with HTN-S% were boiled and re-hybridized with the Hantaan 76-118 probe with negative results. These same 8 rat tissues will be re-extracted by the AGPC protocol and retested.

Using the AGPC protocol we have tested 5 rats. Three are IFA negative and two are positive with reciprocal titers of 4096 and 16,000. The rat with the 16,000 titer revealed bands of approximately 280 bp with RNA extracts from all three tissues tested (kidney, lung and spleen). The second IFA positive rat was negative for the same tissues. The IFA negative rats were negative by PCR.

Specific Aim 7) Investigate the potential use for consensus S segment PCR primers in the detection of all four serotypes of Hantavirus; and the potential for limited restriction enzyme analyses of small (280 bp) fragments to be used in the identification of specific virus type.

Consensus Primer Amplification of 20 Hantaviruses

Nucleic acid sequences from all 20 hantavirus strains tested 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 viral RNA was the template from which products were produced. A single amplified product was seen on agarose gels for all strains except Thailand 749 which had one prominent and two minor bands (Fig. 7, Appendix 1). The size of the amplified fragments were consistent with the 281 np product predicted by sequence data for Hantaan 76-118 (Schmaljohn et al., 1986), SR-11 (Arikawa et al.,1990), and Prospect Hill virus (Parrington and Kang, 1990) with one exception. Amplification of Thottopalayam produced a larger fragment of approximately 320 np. The S-segment of this virus has previously been shown to be larger than that of other hantaviruses. The amounts of product generated in the amplification reaction were comparable for all but two viruses. Amplification of Jinhae 502 and Puumala (Fig. 7, lanes 4 and 18, respectively; Appendix 1) resulted in less product. This suggests that homology between the primers and target sequences of these viruses is less conserved.

Amplification of hantavirus sequences was confirmed by hybridization of the reaction products with ^{32}P-labeled probes. The consensus oligonucleotide probe HTN-S5 however, did not detect amplified products from all virus strains indicating the presence of sequence diversity in the regions flanked by the primers. Despite the appearance of roughly equivalent amounts of amplified product on the gels, strong signals were seen with ten viruses, weak to moderate signals with seven viruses and no signals with three viruses. The strongest hybridization with the oligoprobe was with the mouse strains (Fig. 7, Panel B, lanes 1-8; Appendix 1). In this group, Jinhae 502 produced the weakest signal but this was probably due to a smaller amount of DNA on the filter. Hybridization with rat strains varied considerably; from no signal for Baltimore rat virus (Fig. 7, Panel B, lane 11; Appendix 1), weak signals for SR-11, Girard Point, Tchopitoulas and Seoul (lanes 10, 12, 13, and 15, respectively), a moderate

signal for Singapore (lane 9) and a strong signals for Houston (lane 14). Thailand 749, the bandicoot strain, also produced a strong signal (Fig. 7, Panel B, lane 16; Appendix 1). Hybridization with the multiple bands of this virus that were seen on agarose gel indicated that the bands represented specific products. The oligoprobe did not hybridize with the Eurasian vole strains, Puumala and USSR 18-20 (lanes 18 and 19, respectively) but produced a strong signal with Wisconsin (lane 17), the vole virus from the United States. Moderate hybridization occurred with Thottopalayam (lane 20), the shrew strain from India.

A second probe was prepared in an effort to detect the amplified sequences from all of the hantavirus strains. To produce this probe, 32P-labeled dNTPs were incorporated in the amplification product of Hantaan 76-118 as it was being synthesized by the polymerase chain reaction. It seemed reasonable that a longer probe would increase the probability of finding regions of homology in the amplified products. The same filter that was hybridized with HTN-S5 was tested with this probe after the oligoprobe was removed by boiling the filter. The Hantaan 76-118 probe detected all amplified hantavirus fragments (Fig. 7, Panel C, Appendix 1). The strength of the signal was strong for all strains from mice, humans and rats, but less intense with viruses from the other animals species. Moderate signals were observed with Thailand 749, Wisconsin, Puumala, and USSR 18-20 (Fig. 7, Panal C, lanes 16, 17, 18, and 19, respectively; Appendix 1). Thottopalayam had a weak, but detectable signal (lane 20).

Restriction Enzyme Analyses

To further investigate the differences in the sequences of the amplified genomes of these viruses, the PCR products were analyzed by a panel of restriction endonucleases. Sequence data in the targeted regions of Hantaan 76-118, SR-11, and Prospect Hill virus was examined for restriction sites that might be useful in differentiating the amplified hantavirus sequences. Enzymes that have recognition sites spanning four nucleotides comprised most of the panel. The small 281 np amplified products were more likely to contain cleavage sites for these enzymes than they would for enzymes with longer recognition sequences. The restriction length fragment polymorphism (RLFP) patterns for each enzyme were determined and the viruses were then grouped on the basis of the patterns.

The restriction patterns generated by treatment of all 20 strains with each of the 10 endonucleases were grouped and are shown in Table 12 (Appendix 2). The patterns for one enzyme, *AluI*, is shown for illustrative purposes in Figure 8 (Appendix 1). Seven groups of patterns were observed among the 19 viruses with amplified products of 281 np in length (Table 12, Appendix 2 and Fig. 8; Appendix 1). Patterns were based on bands that were visible on 4% agarose gels, thus the sum of the sizes of all fragments did not always account for 281 np since small fragments

would not have been detected. A comparison of the RLFP patterns of viruses for which sequence data was available, e.g., 76-118 and SR-11, indicated that the estimates were within 5 np. For example, the predicted fragments of *Alu*I digestion of SR-11 were 153, 115, 11 and 2 np. The observed fragment sizes were 157 and 117 np (Fig. 8 lane 2; Appendix 1). Similar analyses with other enzymes, for the most part, produced comparable results. The only exception was with *Hae*III; sequence data indicated that Hantaan 76-118 has a *Hae*III site, but it was not cleaved. Based on the overall agreement between expected and observed results, we concluded that analysis of PCR-amplified by endonucleases was a reasonable approach for examining the relatedness of these hantaviruses and for identifying specific viruses with unique patterns.

The patterns of all restriction enzymes for each virus were compared to those of other viruses. These profiles are shown in Table 13, Appendix 2. The profiles separate the viruses into three groups; mouse viruses, rat viruses and a group of diverse profiles for the remaining viruses. These groups could be clearly differentiated with a single enzyme, *Alu1*. *Dde*I and *Mbo*II also separated the viruses but results were less consistent. Mouse virus sequences were highly conserved as evidenced by identical profiles in 5 of the 6 viruses. The only exception was Jinhae 502 which produced a unique profile among all the viruses and differed from the other mouse viruses with 6 of 10 enzymes. Greek and CG 3883, the viruses isolated from humans, had mouse virus profiles indicating the infections were acquired from mice.

Sequences among the rat viruses were also conserved, but differences in *Dde*I, *Mbo*II, *Rsa*I and *Taq*I patterns resulted in unique profiles for several viruses. Singapore was an outlier among the rat virus group because it had a profile identical to he mouse viruses. Although Houston had a profile similar to other rat viruses, it was distinctly different in that it was cleaved by both *Mbo*II and *Rsa*I, whereas the other rat viruses were not. This was consistent with the hybridization data in that Houston was the only rat virus to produce a strong signal with the HTN-S5 oligoprobe. SR-11 had a unique *Dde*I compared to the other viruses, Girard Point and Tchopitoulas had identical profiles, and the profile for Seoul and Baltimore rat virus were identical.

Each virus from the other animal species had a unique profile. Surprisingly, Puumala and USSR 18-20, both isolated from Clethrionomys glareolus, had different patterns for 6 of the 10 enzymes. Wisconsin, the only other vole virus tested, differed considerably from the Eurasian vole viruses. The profile for Wisconsin was however, similar to that of the expected profile for Prospect Hill virus. But these viruses, both isolated from Microtus pennsylvanicus in the United States, were not identical. Wisconsin was cleaved by *Hha*I, whereas the Prospect Hill virus sequence data indicates that there is no site for this enzyme in the region amplified by the primers. Although

the multiple bands that were produced by amplification of Thailand 749 complicated restriction endonuclease analysis, some sites between this virus and Puumala appeared to be conserved. The RLFP restriction patterns for Thottopalayam were unique for all enzymes since the amplified product from this virus was larger (320 np).

CONCLUSIONS AND DISCUSSION

Antibody Positive Individuals and Association with Acute and Chronic Disease

Hemorrhagic fever with renal syndrome (HFRS) is an acute, febrile illness which presents with a wide array of symptoms (Bruno et al. 1990). The diverse array of presentations is such that, in the absence of serologic testing, the diagnosis cannot be established in more than 50% of cases.

The pathogenesis of hantaviral disease is unclear, although the basic lesion is endothelial cell damage or dysfunction (Yanagihara and Gajdusek, 1987), and viral antigen has been detected in vascular endothelia of experimentally infected animals (Kurata, et al. 1983) and fatal human cases (Yanagihara and Gajdusek, 1987). Despite its name, HFRS presents with hemorrhagic manifestations in only 20 - 30% of cases. Rather, patients with moderate to severe illness experience an acute onset of fever, severe retroorbital headache, blurred vision, photophobia, nausea, and abdominal, back, and flank pains. Laboratory findings during the febrile and oliguric stages include proteinuria, thrombocytopenia, leukocytosis with a left shift, elevated blood urea nitrogen and serum creatinine, and a slight transaminase elevation. Differential diagnoses of classic HFRS might include: leptospirosis, allergic interstitial nephritis, acute glomerulonephritis, rapidly progressive glomerulonephritis, and other causes of acute renal failure. However, at least 30% of clinical cases are mild, and may be misdiagnosed as influenza (Lee, 1988).

The potential occurrence of HFRS in the United States was suspected shortly after it was determined that rodents of the genus Rattus were chronic carriers of a hantavirus(Yanagihara, 1990; Tsai et al. 1985; Lee et al. 1982). Surveys of selected human populations demonstrated that infection occurs in the United States, however, they did not attempt to identify acute disease. The current study provides evidence that infection with hantavirus in the United States is associated with an illness similar to HFRS described elsewhere for rat-borne hantaviruses (Salvadori et al. 1989; Chan et al. 1987). As such, these are the first indications that hantaviral infection in the United States produces human disease.

Rat-borne hantaviruses produce what is considered a mild form of HFRS, in that hemorrhagic manifestations are rare, and, minor. Moreover, the duration, and severity of hypotension, and

the duration of renal failure are less (Chan et al. 1987; Lee, 1989). However, the derangements in liver enzymes occurs to a greater extent in patients exposed to rat-borne hantaviruses. The first two presumptive cases from Baltimore show a typical presentation of HFRS, with acute onset, nausea, vomiting, chills, fever, vertigo, abdominal pain, proteinuria, hepatomegaly, and acute renal failure. The febrile phase was followed by a 1-2 days of hypotension, with a short duration of acute renal failure. Recovery of renal function was heralded by polyuria. Generally, the only evidence of hemorrhagic manifestations were the occurrence of melena, and/or heme positive stools. Also generally absent was the characteristic retroorbital headache associated with other HFRS, although this is less commonly reported with rat-borne hantaviruses (Lee, 1989).

The substantial delay in arrival for care of the second two patients suggests that individuals who become ill following infection may experience milder symptoms and delay seeking medical care. Failure to recognize HFRS in the United States may be in part due to the failure to seek treatment. This may be especially true, as individuals with a high likelihood of non-occupational exposure to rats in the United States are usually urban poor with less access to medical care. An additional factor may be that most cases of HFRS are treated symptomatically, and most patients recover with supportive care (LeDuc et al. 1986). Thus, in the absence of serological testing most cases go unrecognized.

Of particular concern, however, is the potential development of sequelae associated with infection. The renal sequelae in two patients, and the cerebrovascular accident in the third is of concern because of the association between past hantaviral infection and hypertension, hypertensive renal disease, and cerebrovascular accidents (Glass, et al. 1991). Generally, recovery from HFRS is thought to be complete. However, this study is consistent with earlier observations by Lahdevirta (1971) and Rubini (1960) who noted that up to 75% of patients in followup from HFRS were hypertensive, and nearly 30% had decreased renal function. If these results are confirmed they would suggest that at least part of the substantial rates of hypertension, and its consequences in the inner cities of the United States, may have at its basis an infectious origin. Given the world-wide distribution of Rattus in urban centers, attention should focus on evaluating this risk.

Although previous studies demonstrated human infection in the United States (Childs et al. 1988; Tsai et al. 1985; Yanagihara et al. 1984), most could not identify the probable source of the infecting virus and none identified associated illnesses (Childs et al. 1988). Results presented here are the first to indicate that hantaviral infection in the United States, acquired by contact with domestic rodents or their excretion, may be associated with chronic human disease.

Most of our proteinuric population was drawn from inner city, lower socioeconomic status areas. Although most

seropositive individuals resided in Baltimore, cases from New York and Annapolis indicates exposure is geographically widespread. There was no significant difference in exposure of the two sexes. This differs from other studies (Yanagihara and Gajdusek, 1987), in which males have higher infection rates and exposure is job-related. This suggests exposure to BRV may occur in non-occupational settings, such as in and around home. Childs and colleagues (1987) have documented high rates of infection and abundant rat populations in most of these areas.

Clearly, the data concerning chronic diseases are associational, and necessary precautions must be taken to consider alternate, confounding variables and hypotheses (Lilienfeld and Lilienfeld, 1980). Matching of seropositives with seronegatives gave excellent agreement where it could be assessed. There were no significant differences in ages, sex ratios, geographic distribution of residencies, lengths of residence in Baltimore, occupational types, or uses of the hospital system (based on blood samples/person) for seropositive and seronegative individuals that would suggest other obvious confounding factors. In addition, stratification by race did not alter the association of seropositivity with chronic disease.

One potential explanation for the observed association is that individuals with pre-existing hypertensive disease have lifestyles that expose them to virus at increased frequency. In this situation chronic disease would predate exposure to a hantavirus. Our primary indicator of lifestyle, occupation, does not support this hypothesis. There were no differences in the types of work performed by seropositives and controls and seropositives showed no greater tendency to be incapacitated than controls. Among controls, 18/36 individuals under the age of 65 were retired, unemployed, or listed no occupation, while 3/7 cases were in this category. Similarly, the absence of any difference in the number of samples obtained from seropositive and seronegative patients suggests that seropositives were no more likely to have received hospital treatment than seronegative patients.

Another explanation is that seropositive patients may have had more complete histories obtained by their physicians. However, this seems unlikely, as all but one of the patients with chronic renal disease had been diagnosed by their physicians and histories emphasized evidence of hypertension and diabetes. A third possibility is that hypertensive individuals may be more susceptible to some renal pathology caused by hantaviruses. If so, hypertension may predispose individuals exposed to hantaviruses to develop renal disease.

Alternatively, hantaviral infection may precede and in some manner contribute to the onset of hypertension and hypertensive renal disease. Several uncontrolled studies of HFRS patients suggest that hypertension and chronic renal diseases may be a consequence of hantaviral infection in an unknown proportion of patients. Rubini and colleagues (1960) noted that 2 - 5 years after apparent recovery from HFRS 7/13 patients had acquired

hyposthenuria, and 2/13 had developed hypertensive vascular disease. In addition, they reported one case of chronic glomerulonephritis and two cases of pyelonephritis among 31 cases after presumptive recovery. Lahdevirta's (1971) study of NE patients also reported abnormal findings for blood pressure, sedimentation, and creatinine clearance, rates among most of the 20 patients examined 1 - 6 years after infection. Cizman and colleagues' recent report lends credence to these associations. Examination of patients two years following another outbreak in Yugoslavia revealed that 18% had impaired tubular function, 9% were hypertensive and 9% had chronic renal insufficiency (Cizman, et al. 1988). Our apparently higher rates of chronic diseases associated with infection may reflect study design. Whereas earlier studies followed patients who had HFRS, this study examined patients with high levels of proteinuria for prior exposure to hantaviruses. Population surveys need to be conducted to obtain comparable data.

The relationship between hantaviral infection and subsequent development of chronic renal disease in studies of HFRS patients bears continued investigation. Evaluation of our various populations indicates that exposure to hantaviruses increases as attention moves from the general hospital population (JHH-ER), to those with renal difficulties from various causes (JHH-Proteinuric), to more narrowly defined risk groups, especially those with hypertension (VA Medical Center), and hypertensive renal disease (Baltimore dialysis populations). Thus, in the hypertensive renal disease population, nearly 10% of the patients are exposed. In addition, the development of chronic renal disease in two of the acutely infected patients suggests that infection is responsible for development of renal disease. However, infection may act on individuals with marginal renal function, possibly due to hypertension, and aggravate their medical condition. Continuing efforts will focus on establishing this relationship.

Risk of Hantaviral and Other Rodent-Borne Infections

The prevalence of antibodies to LCMV and SEOV were in rank order of reported sightings and contacts with mice and rats, respectively. However, the 19-fold greater prevalence of LCMV (4.70%) compared to SEOV antibody (0.25%) was greater than expected from the frequency of reports of different rodent contact. Overall, the prevalence of seropositive individuals to both viruses was low considering the high frequency of self-reported rodent contact.

The potential for human contact with rats infected with SEOV in Baltimore is considerable, as prevalence of this infection in this City is high even by global standards (LeDuc et al., 1986), and infected rats have been found throughout the city (Childs et al., 1985, 1987b). From 1980-1986, 853 rats were tested for antibody to this hantavirus and 42% were seropositive (Childs et al., 1985, 1987a,b). In the STD population, as in other studies

from Baltimore (Glass et al., 1990), PRNT analyses indicated all infections were caused by a rat-associated hantavirus, and the absence of foreign travel suggested that infections were locally acquired. The relatively infrequent occurrence of rats within residences, compared to their common presence on streets, may substantially reduce the risk of human-rodent contact and infection with SEOV in Baltimore. Similar epizootiological studies have not been conducted on the prevalence of LCMV infections in Baltimore house mice.

The leptospiral study revealed a high overall prevalence (16 %) of antibody that peaked in the population over age 19. The prevalence of this potentially rodent-borne pathogen was >3 times that for LCMV and 64 times that for SEOV, indicating the relative scarcity of hantaviral infection among residents of the city. The low rate of infection may be due to the combination of relatively low direct contact rate between humans and infected animals, and the inefficiency of aerosol transmission to humans in this environment. This would suggest that the low rates of acute HFRS in the U.S. may be due to low rates of contact with infectious agents. It also supports the view that the relatively high rates of infection in hospitalized patients indicates that infection is associated with unrecognized disease, and has significant sequelae.

Beyond the commonly observed associations between antibody with increasing age and male sex, there were several interesting study variables linking increased or decreased risk with animal contact or ownership (in the case of birds and cats, respectively).

The clearest and most unusual association between leptospiral antibody and animal contact was the relationship between cat ownership and lowered risk of antibody. The finding that current cat ownership (or history of previous ownership) reduces the risk of leptospiral antibody in age and race groups at excess risk (those >19 and black, respectively) is intriguing. Cats could reduce the risk of human exposure to rodent-borne pathogens if they kill or reduce human contact with the reservoir. The emphasis on rat-borne leptospires in the urban environment (Thiermann, 1977, 1981) makes this an attractive hypothesis. Although cats are not effective in killing adult rats (Childs 1986), they could exclude them from households. The relationship between cat ownership and race is also of interest, as surveys of cat-ownership in Baltimore demonstrated that blacks own significantly fewer cats than whites, although dog ownership is equal (Childs 1990). This difference in cat ownership could place inner-city black residents at increased risk for contact with rodents and the zoonoses they carry. These conclusions are consistent with our observations on hantaviral infections in inner city Baltimore.

PCR Analysis of Hantaviruses and Amplification of Viral RNA from Wild Rat Tissue

PCR has been widely applied for the diagnosis of infectious diseases produced by parasitic, bacterial and viral pathogens. A principal consideration in applying PCR for disease diagnosis is the selection of appropriate genetic targets. Multiple hantavirus strains, some occurring in the same geographical region, produce disease in humans. The spectrum of disease, HFRS, varies in severity. Although specific groups of hantaviruses are usually associated with various degrees of disease severity, the infecting virus can not be predicted on this basis alone. Therefore, as a first step in the development of a diagnostic test capable of detecting all hantaviruses, we selected highly conserved regions in the S genomic segment to prepare a single pair of "universal" primers. We used these primers to amplify genomic sequences from all 20 hantavirus strains that we tested. These included classic Hantaan-like strains isolated from humans and mice, rat strains, vole strains and strains from other animal species. Although some of these viruses have not been implicated in human disease, their amplification with these primers indicate the broad utility of this PCR method for detecting hantaviruses. Amplification of hantavirus sequences from rat tissues, demonstrates the potential for diagnosis of infections in humans.

For this test to have diagnostic utility, it must be sensitive, specific and have timely turnaround. With respect to sensitivity, PCR routinely detects 1-10 copies of target sequence. Although all 20 hantavirus strains were amplified, we did not determined the sensitivity of our assay. Base pair mismatches could potentially affect the sensitivity of detection of any particular virus. This is less likely to be a problem with viruses that are documented pathogens, since the primers were selected on the basis of sequence data for these viruses. With respect to specificity, available sequence data from viruses in the other genera of the *Bunyaviridae* were analyzed for homology to the hantavirus primer sequences and none was observed.

The rapidity with which PCR can be performed is a distinct advantage over other diagnostic techniques such as culture and antibody measurements of acute and convalescent serum. Results can be obtained by PCR on the same day or the day after specimen receipt. Further improvements in specimen processing techniques may further reduce turnaround time. In contrast, culture requires days to weeks of incubation and must be performed in high-level biosafety containment facilities. With serological tests, definitive identification of the infecting virus requires neutralization tests which are tedious and time consuming to perform.

Analysis of the amplified PCR products with restriction endonucleases, was useful for identifying specific viruses or groups of viruses. With the exception of Jinhae 502, the mouse viruses appeared to be identical, whereas some members of the rat virus group could be differentiated by the panel of restriction enzymes that were used. The greatest diversity was seen in the

amplified region of viruses from other animal species. This suggests that viruses from these animals will have unique patterns and may be difficult to group. This approach to diagnosis of human hantavirus infections by PCR and restriction enzyme analysis should provide an indication of the specific reservoir species from which the infection was acquired.

The use of labeled nucleic acid probes permits efficient and sensitive detection of amplified hantavirus sequences in the reaction products. Our attempts to use an 20 base oligonucleotide probe with homology to a conserved region in the amplified products were not successful for detection all viruses, particularly the rat viruses. A Hantaan 76-118 probe produced by PCR amplification detected all 20 hantaviruses, although the signal for the shrew virus Thottapalayam was weak. Southern hybridization format was required for this probe since it contained primer sequences. The use of oligonucleotide probes with homology to sequences in the amplified product that are flanked by the primer is preferable since it can be used in a efficient dot/slot blot format.

Knowledge of the nucleic sequences in the amplified region of the viruses that have not been sequenced previously will be of value in several respects. First, these data should permit the development of a group or virus-specific oligonucleotide probes. This seems feasible for the viruses from animals other than mice, but the RFLP data suggest that the sequences of the mouse viruses may be too highly conserved to differentiate the viruses with oligonucleotide probes. Second, the comparison of sequence data of the strains should provide insight into the genetic relatedness of these viruses that may lead to an understanding to how these viruses have evolved and become distributed.

We have successfully used reverse transcription followed by PCR to amplify BRV from tissues collected from wild rats. This successs has only been achieved within the last week so no figures are available and numbers are small. Our previous efforts in this direction were hindered by lack of a probe for detecting BRV (see Specific Aim 7 for details), poor controls (since overcome by the availibility of large quantities of RNA from Seoul isolate Houston, provided by Dr. R. Lofts, USAMRIID), and poor quality RNA (overcome by AGPC RNA extraction technique that still avoids cumbersome overnight ultracentrifugation). Although these data represent few rats, they are a methodological breakthrough for our research effort. To our knowledge these represent the first application of PCR followed by restriction analysis to identify a strain of Hantavirus from field collected material. In the future we will concentrate on expanding the rat sample, including tissues fixed by different means, and attempting the same process on human tissues at JHU and USAMRIID for the potential rapid diagnosis of HFRS.

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APPENDIX 1

Figures 1 through 8

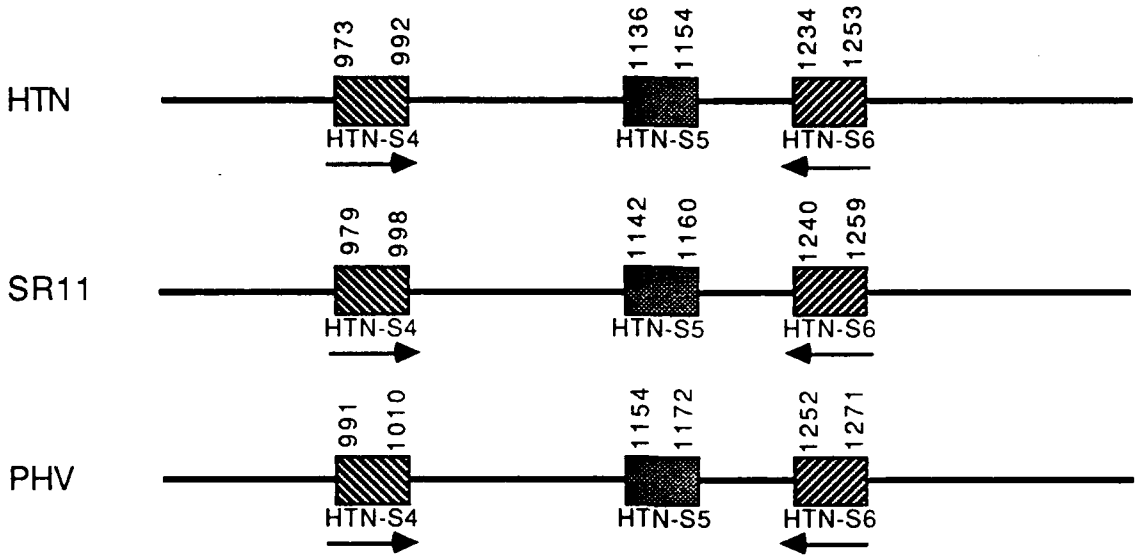
CONTRACT NO.: DAMD17-89-C-9093

TITLE: EPIDEMIOLOGY OF HANTAVIRUS INFECTIONS IN THE UNITED STATES

PRINCIPAL INVESTIGATOR: James E. Childs

PI ADDRESS: The Johns Hopkins University
School of Hygiene & Public Health
Dept. of Immunology & Infectious Diseases
615 N. Wolfe St., Room 4307-B
Baltimore, MD 21205

PREPARED FOR: US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21701-5012



HTN-S4	5'	GAIIGITGTCCACCAACATG	3'
HTN-S5	5'	GAAGIACACAATCIATGGG	3'
HTN-S6	5'	AGCTCIGGATCCATITCATC	3'

Figure 1. Consensus oligonucleotide primers (HTN-S4 and HTN-S6) used for amplification of hantaviruses by PCR. Also shown is the consensus probe HTN-S5. The targeted sequences are on the S segment of the hantavirus genome and were selected using sequence data for Hantaan 76-118, SR-11 and Prospect Hill virus. Numbers are in nucleotides.

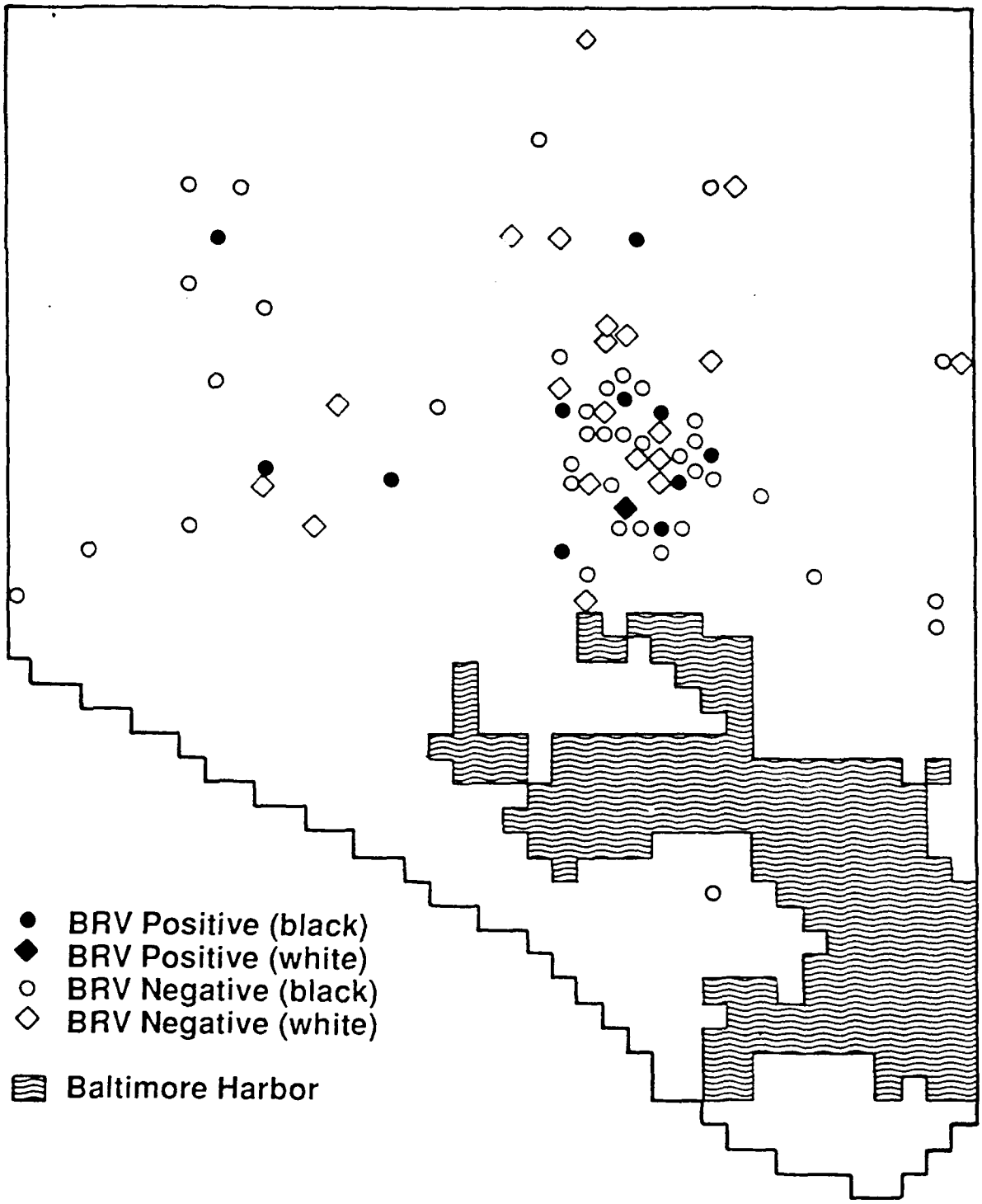


Figure 2. Geographic distributions of residences for seronegative and seropositive individuals in Baltimore, Maryland.

Seropotives are indicated by closed symbols and seronegatives by open symbols. African-Americans are indicated by circles and others by diamonds.

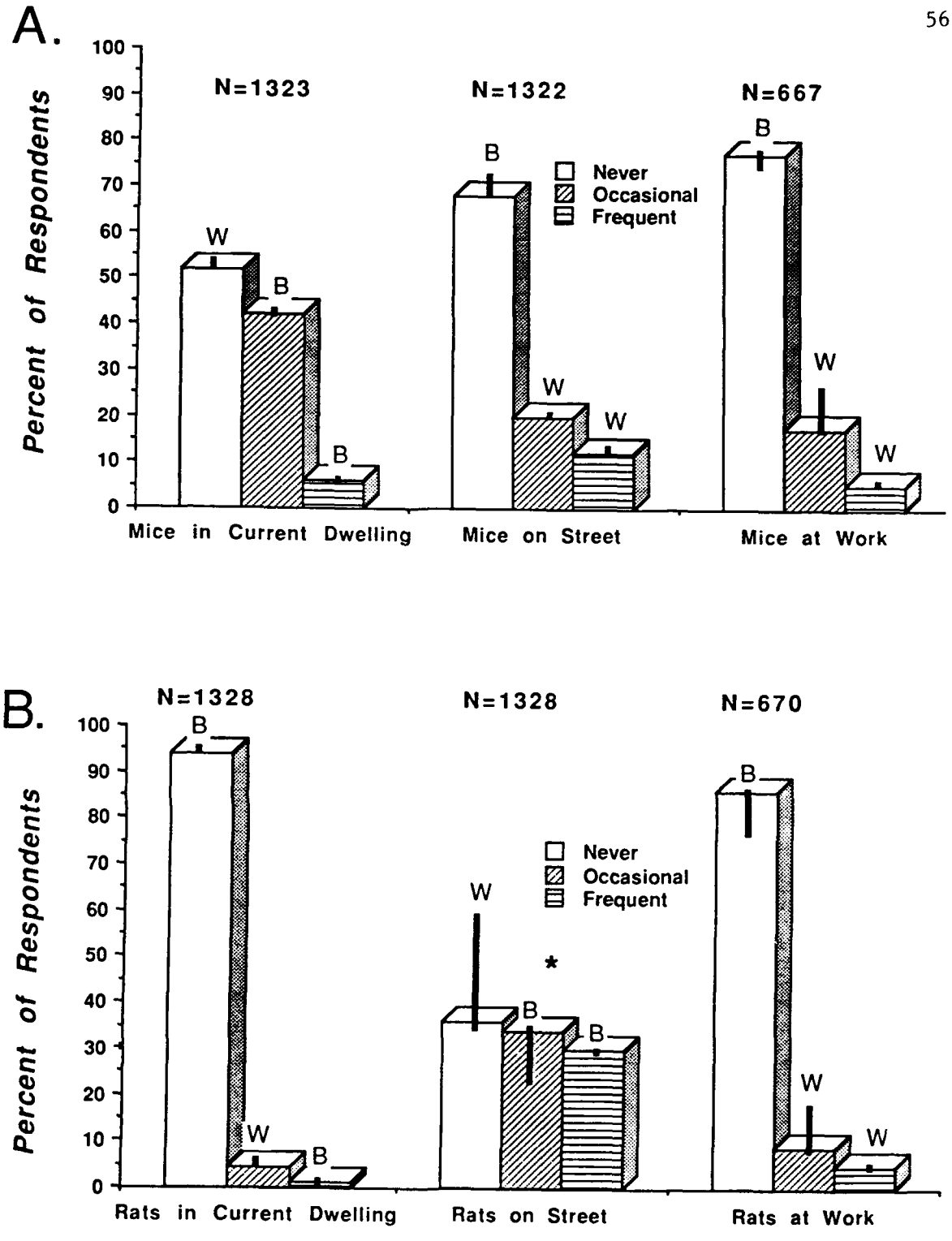
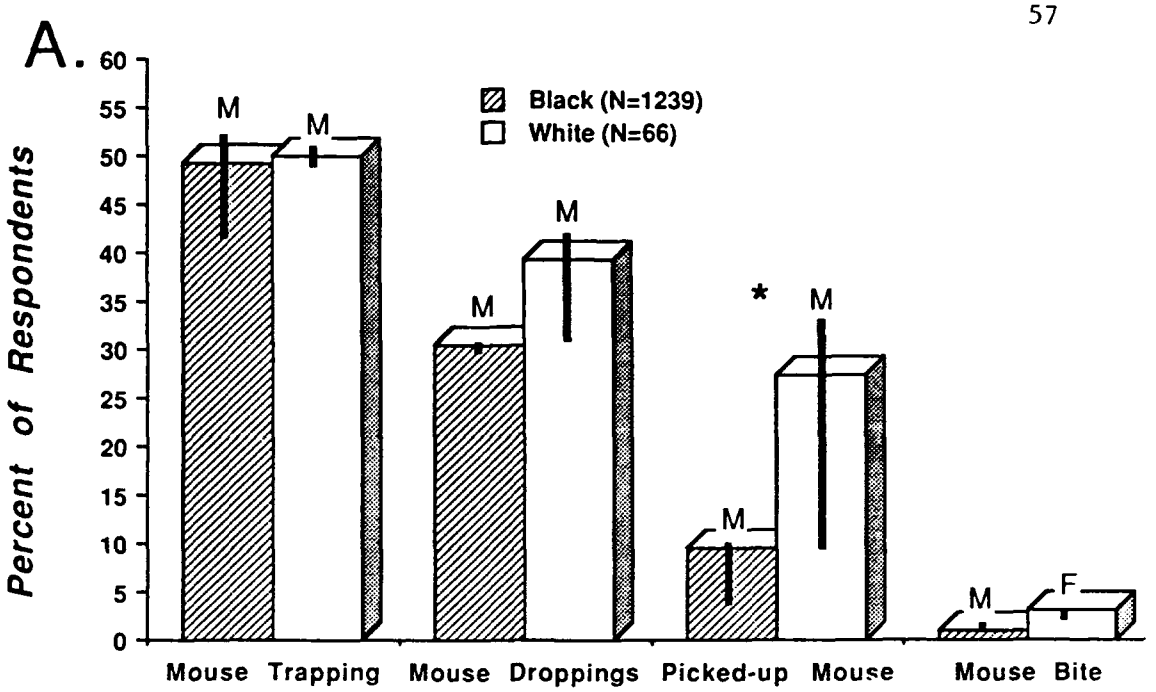


Figure 3. Reports of rodent sightings from Baltimore. A. house mice; B. Norway rats. Vertical bars indicate the range of reponses for black and white respondents. The asterisks indicate a significant difference ($P<0.01$) between black (B) and white (W) respondents, indicated at the top of each range bar.



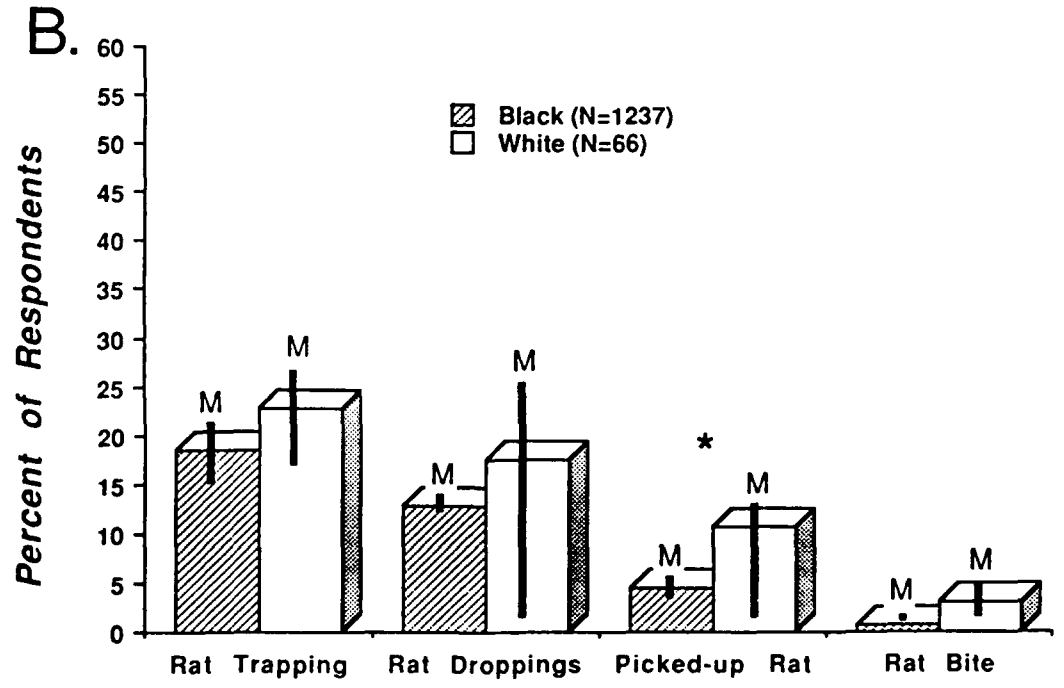


Figure 4. Reports of rodent contacts from baltimore. A. house mice; B. Norway rats. Vertical bars indicate the range of responses for male and female respondents. The asterisks indicate a significant difference ($P < 0.01$) between male (m) and female (F) respondents, indicated at the top of each range bar.

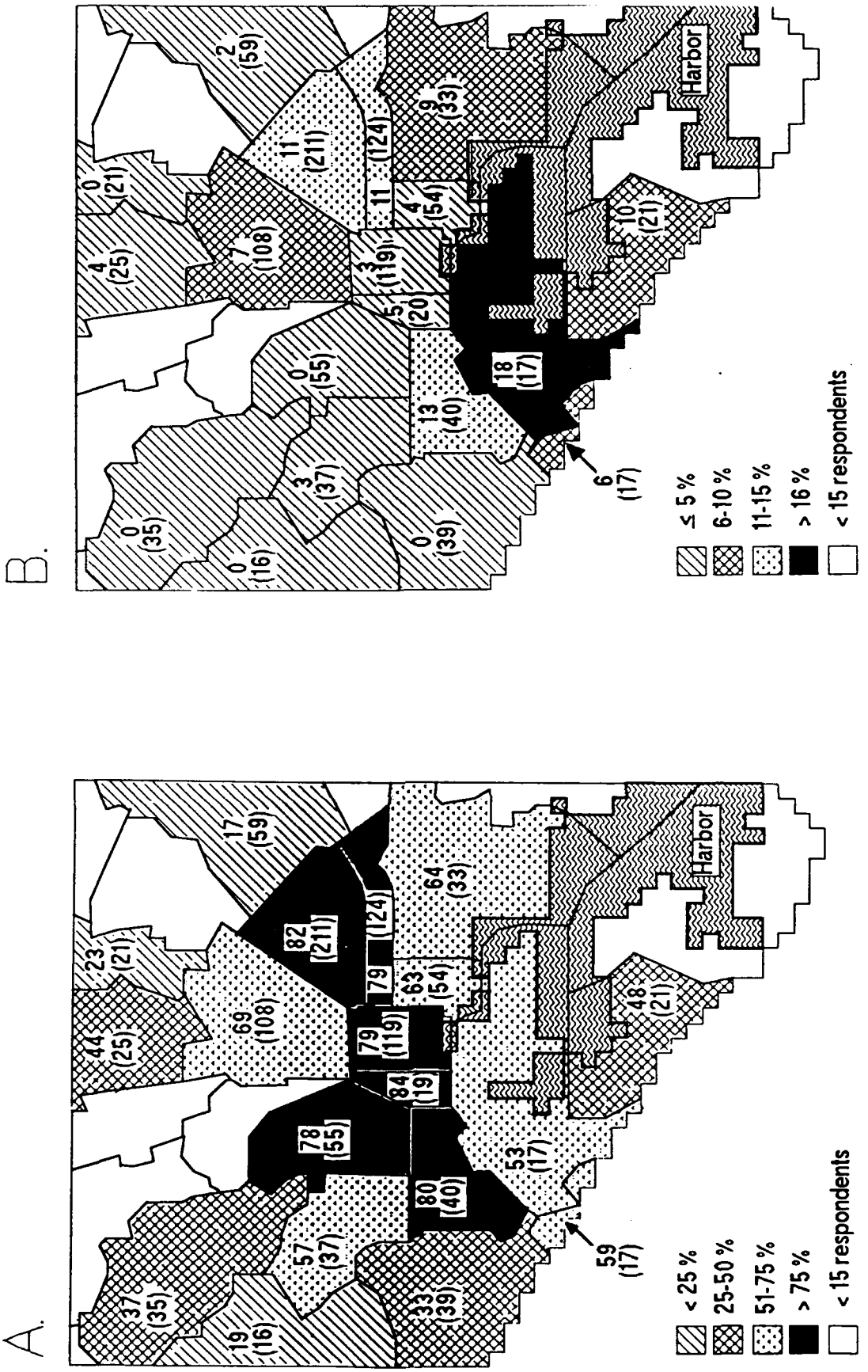


Figure 5. Distribution of reports of Norway rat sightings in Baltimore ZIP Code areas. A. on streets or in alleys; B. within residences. The bold lines delimit central from peripheral areas as defined in the text. The number in each ZIP Code area indicates percent of respondents indicating rats present in the specific location, and the number in () indicates the sample size for each area. Only ZIP Code areas with more than 15 respondents were plotted.

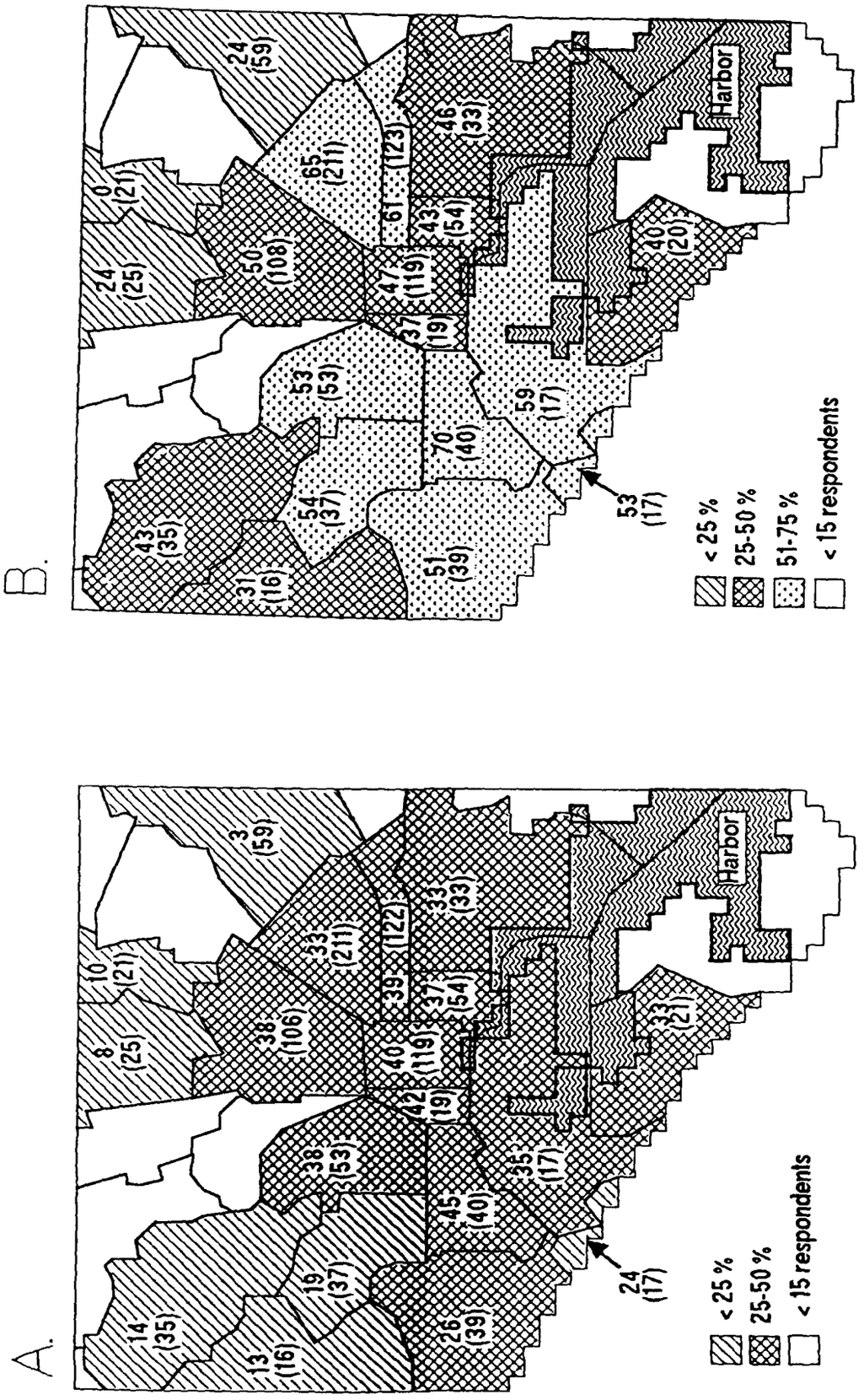


Figure 6. Distribution of reports of house mouse sightings in Baltimore ZIP Code areas. A. on streets or in alleys; B. within residences. The bold lines delimit central from peripheral areas as defined in the text. The number in each ZIP Code area indicates percent of respondents indicating mice present in the specific location, and the number in () indicates the sample size for each area. Only ZIP Code areas with more than 15 respondents were plotted.

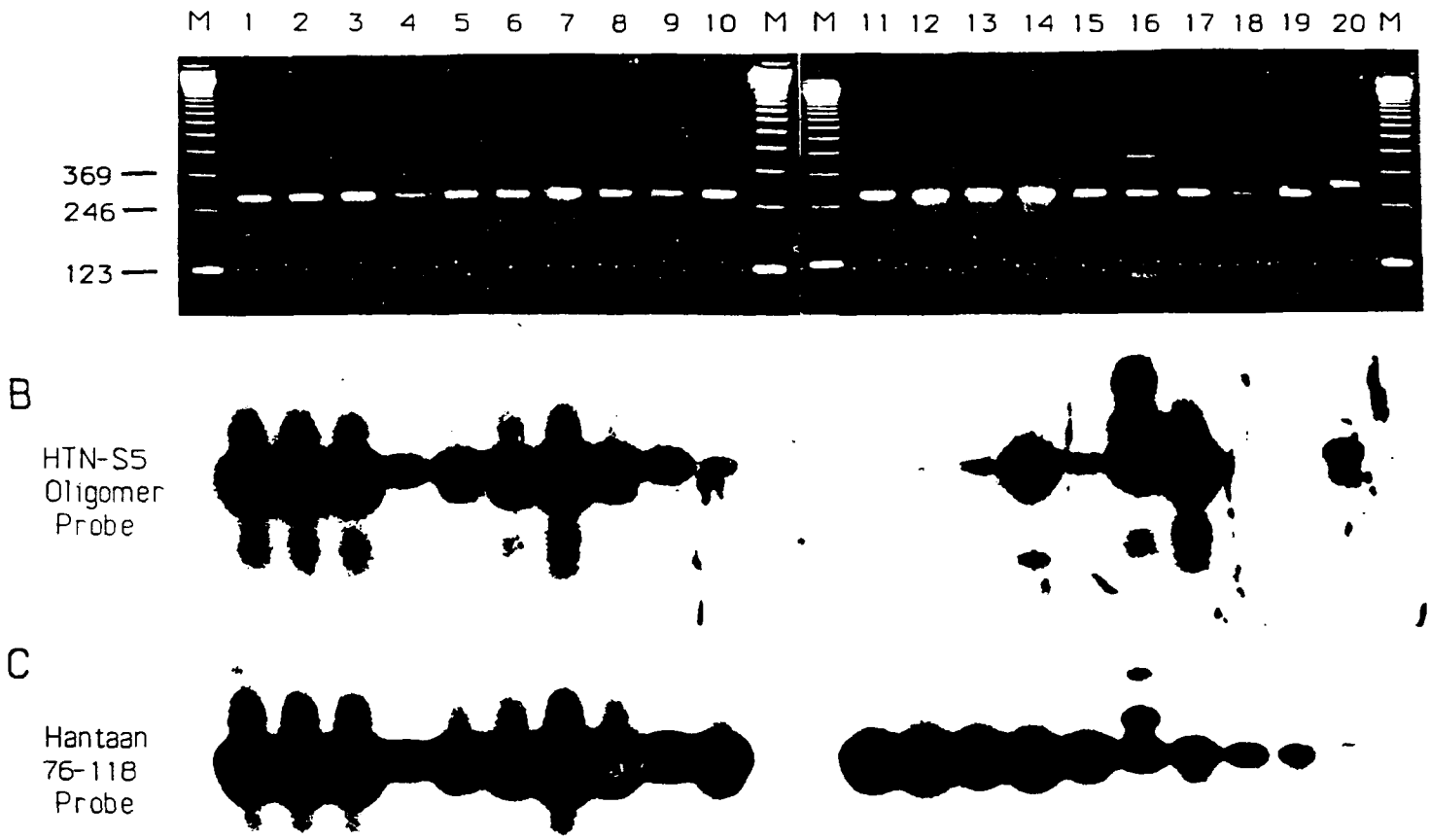


Figure 7. Analysis of PCR-amplified produced from 20 hantaviruses by gel electrophoresis (A) and southern transfer hybridization with oligonucleotide (B) and PCR-amplified (C) probes. Lane M contains size markers (123-base pair ladder; Bethesda Research Laboratories-Life Technologies). Lane 1 Hantaan 76-118; lane 2 CG 3883; lane 3 Jinhae 494; lane 4 Jinhae 502; lane 5 Greek (Porogia); lane 6 Leaky; lane 7 Maagi; lane 8 Yugoslavia; 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 1820; lane 20 Thottopalayam. 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 from 6 to 24 hrs.

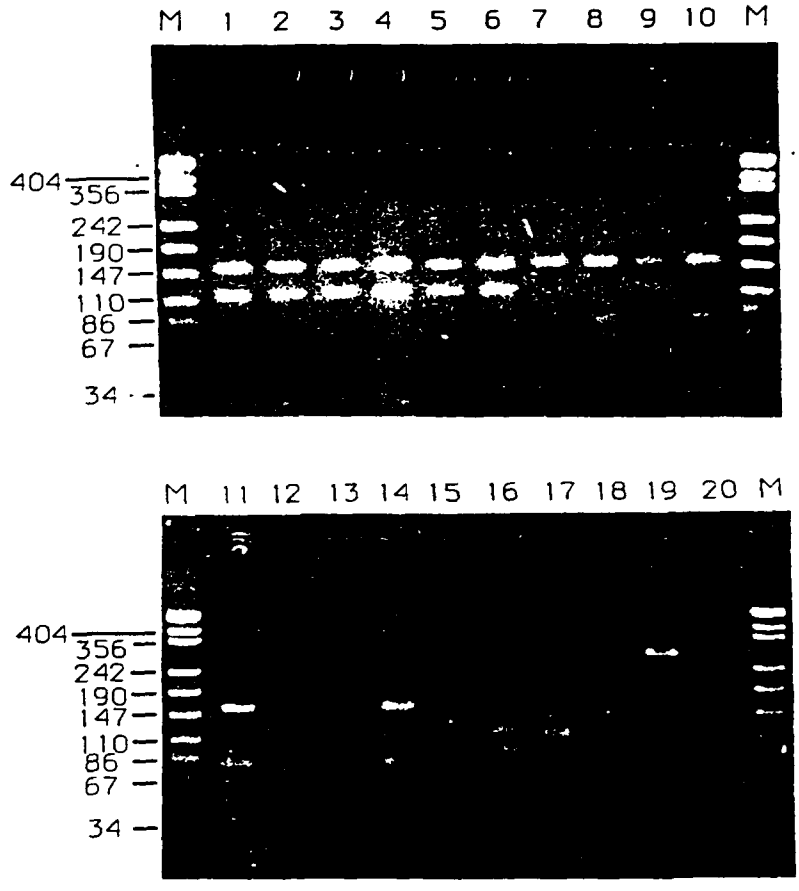


Figure 8. AluI digests of PCR-amplified products from 20 hantaviruses. Lane M contains size markers (MspI digest of pUC18). Lane 1 Seoul; lane 2 SR-11; lane 3 Baltimore Rat; lane 4 Girard Point; lane 5 Tchopitoulas; lane 6 Houston; lane 7 Singapore; lane 8 Hantaan 76-118; lane 9 Greek (Porogia); lane 10 Jinhae 494; lane 11 CG 3883; lane 12 Yugoslavia; lane 13 Leaky; lane 14 Maagi; lane 15 Thailand 749; lane 16 Wisconsin; lane 17 Jinhae 502; lane 18 Puumala; lane 19 USSR 18-20; lane 20 Thottopalayam.

APPENDIX 2

Tables 1 through 13

CONTRACT NO.:	DAMD17-89-C-9093
TITLE:	EPIDEMIOLOGY OF HANTAVIRUS INFECTIONS IN THE UNITED STATES
PRINCIPAL INVESTIGATOR:	James E. Childs
PI ADDRESS:	The Johns Hopkins University School of Hygiene & Public Health Dept. of Immunology & Infectious Diseases 615 N. Wolfe St., Room 4307-B Baltimore, MD 21205
PREPARED FOR:	US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND FORT DETRICK FREDERICK, MARYLAND 21701-5012

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
Leaky	Mus musculus	United States
Maagi	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
Thottapalyam	Suncus murinus	India
USSR 18-20	Clethrionomys glareolus	USSR
Wisconsin	Microtus pennsylvanicus	United States
Yugoslavia (Fojnica)	Apodemus flavicollis	Yugoslavia

TABLE 2-Clinical presentation of 4 presumptive HFRS patients.

CLINICAL SYMPTOMS	
Symptoms	Fraction
Fever	4/4
Generalized weakness	4/4
Melena/heme positive	4/4
Epigastric pain	4/4
Productive cough	3/3
Chills	3/3
Diarrhea	3/4
Nausea	3/4
Vomiting	3/4
Vertigo	2/4
Headache	1/3

Denominator are the number of individuals in which either positive or negative findings were recorded .

TABLE 3-Clinical and laboratory findings of 4 presumptive HFRS patients

FINDING	POSITIVE/EXAMINED
Clinical	
Pleural effusion	4/4
Hypotensive (<90 mm systolic)	2/2
Hepatomegaly	3/4
Periorbital edema	1/4
Petechiae	0/4
Pharygeal injection	0/4
Urinalysis	
Proteinuria	4/4
Urine S. G. <1.024	4/4
Oliguria (<400 ml/24 h)	2/2
Polyuria (>2000 ml/24 h)	2/2
Microhematuria	3/4
Casts	3/4
Serum enzymes	
Elevated Creat (>1.4g/dl)	4/4
Elevated Alkaline phos (>95IU/L)	4/4
Elevated BUN (>25g/dl)	3/4
Elevated AST (>35IU/L)	3/4
Elevated ALT (>30IU/L)	2/4
Elevated Amylase (>120IU/L)	2/3
Hematology	
Increased prothombin time	3/3
Increased APPT	3/3
WBC >11,000/mm^3	1/4
Thrombocytopenia	1/4

Denominator are the number of individuals in which either positive or negative findings were recorded

TABLE 4-Prevalence of neutralizing antibody to Baltimore rat (Seoul) virus in patients visiting an emergency room (ER) and sexually transmitted disease clinic (STD) in Baltimore, 1985-1989.

		ER		STD	
Characteristic		N	% positive	N	% positive
Race					
	Black	2474	.36	2563	.16
	White	897	.22	83	-
	Other	29	-	14	-
Sex					
	Male	1820	.36	1878	.21
	Female	1578	.22	782	-
Age					
	<20	1067	.19	772	-
	21-40	1663	.24	1780	.22
	41-60	399	.75	100	-
	>61	271	.74	13	-

TABLE 5-Comparison of neutralizing antibody prevalences to Baltimore rat virus between Johns Hopkins Hospital patients with proteinuria and persons visiting an emergency room and sexually transmitted disease clinic, 1985-1991.

	JHH (proteinuria >250)	ER and STD		
Age	positive/negative	positive/negative	Odds Ratio	95% CI
≤ 20	0/150	2/1839	-	-
21 - 40	2/547	8/3443	1.57	0.16 - 7.91
41 - 60	6/373	3/499	2.68	0.57 - 16.62
≥ 61	11/498	2/284	3.14	0.68 - 29.29
TOTAL	19/1568	15/6065	4.90	2.37 - 10.16

TABLE 6 -Epidemiological characteristics of seropositive and seronegative individuals examined for chronic disease. There were no significant differences between groups in any of these variables.

VARIABLE	SEROPOSITIVE	SERONEGATIVE
Race		
African-American	13	47
Caucasian	2	24
Other	0	2
Age		
Male ($\bar{X}$ ± sd)	58.2 ± 12.5	58.0 ± 12.0
Female	66.7 ± 22.0	67.3 ± 21.2
Occupation		
Unemployed	1	7
Unskilled	5	28
Blue Collar	2	13
Service Oriented	3	12
White Collar	0	1
Retired	6[a]	35[a]
Unknown	3	5
Residence		
Baltimore	12	55
Non-Baltimore	3	18
Clinical		
Blood pressure (mm/Hg)	164/76 ± 32/10	151/89 ± 34/18
Serum creatinine (mg/dl)	2.69 ± 2.28	2.06 ± 1.96
Proteinuria (mg/24 hrs)	2140 ± 3000	1660 ± 2440

[a]includes individuals in other job categories

TABLE 7-Prevalences of clinically diagnosed chronic disease among individuals seropositive or seronegative to Baltimore rat virus.

DISEASE	SEROPOSITIVE	SERONEGATIVE	ODDS RATIO	90% CONFIDENCE INTERVALS
Chronic Renal Disease	12	32	5.1	(1.6-13.3)
Hypertension	7[a]	3	20.4	(5.1-60.5)
Diabetes mellitus	2[b]	16	0.5	(0.2- 2.2)
Drug-induced	1[b]	6	0.8	(0.2- 3.9)
Obstructive	0	3	---	
Autoimmune	0	4	---	
Unknown	1[c]	0	---	
None	3	41	---	
Hypertension	14/15	47/73	7.7	(1.2-23.5)
Cerebrovascular accident	4/15	5/73	4.9	(1.5-15.8)
Diabetes mellitus	6/15	35/73	0.7	(0.3- 1.9)

[a] Case deleted with chronic hypertension and chronic renal disease but requiring of further testing
[b] IV drug user with diabetes mellitus included in drug-induced category
[c] Case with insufficient clinical data

TABLE 8-Multivariate relative-risk regression analysis of reported exposures and contacts to mice and rats in Baltimore. Adjusted odds ratios (above) and 95 percent confidence intervals (below) are shown, except when not significantly different from 1.0. Sample sizes for the exposure and contact analyses ranged from 1206-1208 respondents.

	Outcome			
	Exposure		Contact	
Characteristics	Mouse	Rat	Mouse	Rat
Sex (male vs.female)	1.0 -	1.0 -	1.5‴ 1.1,1.9	1.9‴ 1.4,2.6
Age (>28 vs. ≤28)	1.1 -	1.0 -	1.7‴ 1.3,2.1	1.7‴ 1.3,2.2
ZIP Code (central vs. peripheral)	3.5‴ 2.7,4.5	5.8‴ 4.4,7.6	1.3* 1.0,1.7	1.4* 1.0,1.8
Dog owned (≥1 vs. none)	2.1‴ 1.5,2.8	1.8‴ 1.3,2.5	1.8‴ 1.4,2.4	1.6‴ 1.2,2.2

* P<0.05
‒ P<0.01
‴ P<0.001

TABLE 9-Demographic characteristics and prevalence of infections with LCMV and SEOV in Baltimore residents.

	Antibody			
	LCMV	n	SEOV	n
Prevalence	4.70	1149	0.25	1180
Race				
African-American	4.73	1079	0.27	1110
White	3.28	61	0	61
Other	11.11	9	0	9
Sex				
Male	4.76	820	0.35	845
Female	4.56	329	0	335
Age				
≤ 20	3.25	369	0	379
21-30	4.62	563	0.52	580
31-40	7.30	178	0	183
> 40	7.90	38	0	38

TABLE 10-Crude and adjusted Odds Ratios (95% Confidence Intervals) for presence of leptospiral antibody in patient sera collected from a STD clinic. Sera were tested for other rodent-borne pathogens.

Study Variable	Odds Ratio (95% CI)	
	Crude	Adjusted
Age (>19 vs. ≤19)	2.3 (1.5, 3.5)	2.9 (1.8, 4.6)
Sex (male vs. female)	1.5 (1.0, 1.9)	1.5 (1.0, 2.2)
Race (Black vs. other)	1.8 (0.8, 3.9)	2.0 (0.9, 4.6)
Income (≤$8000 vs.>$8000)	1.2 (0.8, 1.7)	1.6 (1.1, 2.3)
Foreign Travel (ever vs. never)		
Canada	1.9 (0.8, 4.2)	2.4 (1.0, 5.9)
Mexico/West Indes/ South America	0.4 (0.2, 1.2)	0.4 (0.1, 1.1)
Contact with Rat Excrement (some vs. none)	0.7 (0.5, 1.1)	0.5 (0.3, 0.9)
Current Cat Ownership (none vs. ≥1)	2.1 (1.1, 3.8)	1.9 (1.0, 3.5)
Bird Ownership (ever vs. never)	1.4 (0.8, 2.5)	1.7 (1.0, 3.0)

TABLE 11-Adjusted odds ratios for presence of leptospiral antibodies in selected groups after stratifying on cat ownership. Note reduction of Odds ratios in cat owning groups.

	Current Cat Ownership	
	None	≥1
Age		
>19 vs. ≤19	3.4 (2.1, 5.7)	0.8 (0.3, 2.8)
Race		
Black vs. other	3.4 (1.2, 9.6)	0.4 (0.1, 1.6)

TABLE 12-Restriction Endonuclease Patterns of Hantavirus S-Segment Sequences Amplified by PCR Using Consensus Primers

Enzyme:	*AluI*
Group	
1	Not cleaved
2	157,117
3	159,83,52
4	115,85,75
5	148,92
6	119,96,67
7	130,95,68
8	166,146

Enzyme:	*BamHI*
Group	
1	Not cleaved
2	189,91
3	308

Enzyme:	*DdeI*
Group	
1	Not cleaved
2	122(2),48
3	122,164
4	98,62,48
5	187,102
6	183,157

Enzyme:	*EcoRI*
Group	
1	Not cleaved
2	182,90
3	182,97

Enzyme:	*HaeIII*
Group	
1	Not cleaved
2	227,53

Enzyme:	*HhaI*
Group	
1	Not cleaved
2	213,81

Enzyme:	*MboI*
Group	
1	Not cleaved
2	211,83
3	211,59
4	194,92
5	213,75
6	253,74

Enzyme:	*MboII*
Group	
1	Not cleaved
2	235,51
3	157,63(2)
4	139,100

Enzyme:	*RsaI*
Group	
1	Not cleaved
2	175,115
3	227,73
4	168,74,63

Enzyme:	*TaqI*
Group	
1	Not cleaved
2	154,134
3	173,119

TABLE 13-Restriction enzyme analysis of PCR-amplified products for 20 hantaviruses. Patterns are defined in TABLE 12.

	AluI	*BamHI*	*DdeI*	*EcoRI*	*HaeIII*	*HhaI*	*MboI*	*MboII*	*RsaI*	*TaqI*
Girard Point	2	1	2	1	1	1	1	1	1	1
Tchopitoulas	2	1	2	1	1	1	1	1	1	1
SR-11	2	1	3	1	1	1	1	1	1	1
Baltimore Rat	2	1	2	1	1	1	1	1	1	2
Seoul	2	1	2	1	1	1	1	1	1	2
Houston	2	1	2	1	1	1	1	2	2	1
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
Leaky	3	1	4	1	1	1	1	3	1	1
Maagi	3	1	4	1	1	1	1	3	1	1
Jinhae 502	4	1	C[a]	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
Thottopalayam	8	3	6	1	1	1	6	C	1	1

[a] C - Cleaved, incomplete pattern analysis due to weak band intensities.