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I am submitting herewith a dissertation written by Shawn Lee Lewis entitled "A Newly Identified Hantavirus: The Development of Immunologic Diagnostic Assays and Phylogenetic Analysis for Detection and Characterization." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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A NEWLY IDENTIFIED HANTAVIRUS: THE DEVELOPMENT OF IMMUNOLOGIC DIAGNOSTIC ASSAYS AND PHYLOGENETIC ANALYSIS FOR DETECTION AND CHARACTERIZATION

A Dissertation

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Abstract

Hantaviruses are the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. As of July 2005, 396 HCPS cases in 30 U.S. states with a 36% mortality rate have been confirmed since reporting began in 1993.

The primary rodent host of numerous U.S. hantaviruses is *Peromyscus maniculatus* (deer mouse) although other strains have been found in association with a distinct rodent host and geographical region. Reservoir hosts are asymptomatically, persistently infected and shed virus particles intermittently in urine, saliva and feces. Thus, the primary transmission route for hantaviruses from infected small mammals to humans is inhalation of aerosolized excreta.

Between September 13, 2000 and December 1, 2000; November 28 and 29, 2001; May 27, 2002 through July 24, 2002; and May 1, 2004 through May 8, 2004 trapping was conducted in the Great Smoky Mountains National Park (GSMNP). A total of 310 rodents and 35 insectivores (a total of 345 animals) were captured. Blood samples were obtained from 305 animals and subsequently tested for anti-hantavirus antibodies with enzyme linked immunosorbent assays (ELISAs) and immunofluorescent assay (IFA).

We performed RT-PCR, PCR and sequencing analysis encoding the immunodominant region of the nucleocapsid (N) protein contained within the S gene. A 59 amino acid peptide was synthesized based on the deduced amino acid sequence of the 59 residue epitope region of the N protein. This 59-mer was applied as a serodiagnostic antigen in an ELISA for the detection of anti-hantavirus antibody in rodent and insectivore sera. The sensitivity and specificity of this ELISA were comparable to those

of an IFA using virus infected cells. The only seropositive animals detected were deer mice and white-footed mice (*Peromyscus leucopus*). The overall estimated seroprevalence in GSMNP was 8.9% (27/305).

In our study, we also developed a one-step real-time detection-PCR (RTD-PCR) assay based on Superscript III reverse-transcriptase-Platinum *Taq* polymerase enzyme mixture. PCR amplicons were detected in real time with the use of a 5'hybridization probe. Results were compared to RT-PCR/nested PCR amplification products.

We detected our target genome sequence in one *Sorex fumeus* (smoky shrew), one *Clethrionomys gapperi* (Southern red-backed vole) and 16 mice of *Peromyscus spp.* by RTD-PCR. The overall estimated viral prevalence in GSMNP was 5.2% (18/345). Sequence analysis of the amplicon detected in the smoky shrew was identical to that previously taken from a deer mouse. This is the first reported case of a New World hantavirus being detected in a shrew and the first evidence of a hantavirus detected in a *Clethrionomys spp.*

Lastly, we sequenced G_1 and G_2 segments of the M gene and performed phylogenetic analysis with these segments and the N segment. We have designated this hantavirus strain Newfound Gap virus (NGV). The G_1 and G_2 segments demonstrated homology to New York virus, a pathogenic strain maintained in white-footed mice while the N segment demonstrated greater homology to Monongahela and Sin Nombre viruses, also pathogenic strains maintained in deer mice.

The incongruous homologies of the NGV S and M segments to other closely related hantaviruses suggest that genetic reassortment resulting in a hybrid virus may

have occurred. NGV possesses unique characteristics and is closely related to pathogenic strains that have resulted in HCPS case fatalities in this region.

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Glossary of Abbreviations

ARDS: Acute Respiratory Distress Syndrome

BCC: Black Creek Canal virus strain

DOB: Dobrava-Belgrade virus strain

EHF: Epidemic Hemorrhagic Fever

ENSO: El Nino Southern Oscillation

GSMNP: Great Smoky Mountains National Park

HCPS: Hantavirus Cardiopulmonary Syndrome

HFRS: Hemorrhagic Fever with Renal Syndrome

HPS: Hemorrhagic Pulmonary Syndrome or Hantavirus Pulmonary Syndrome

HTN: Hantaan virus strain

IF: Immunofluorescent

KHF: Korean Hemorrhagic Fever

NE: Nephropathia Epidemica

PHV: Prospect Hill virus strain

PUU: Pumuula virus strain

SEO: Seoul virus strain

SNV: Sin Nombre virus strain

SR-11: virus strain known to cause HFRS in Asia

Part 1

INTRODUCTION

1. LITERATURE REVIEW

1.1 History of Hemorrhagic Fever with Renal Syndrome in Eurasia

An assortment of hemorrhagic fevers have been recognized and reported across Eurasia since the early 1900's. Clinically similar cases of a milder form of hemorrhagic fever, Nephropathia Epidemica (NE) were reported as "War Nephritis" as early as the American Civil War (1861-1865). During this time, approximately 14,000 cases were described among the Union army battalions occupying what were most likely Pennsylvania, Maryland and Delaware (Lee 1982).

Accounts describing Hemorrhagic Fever with Renal Syndrome (HFRS) have been found in a Chinese medicine text, *Whang Jae Nae Kyung*, which was written circa 960 A.D. (Lee 1982 and Yanagihara and Gajdusek 1988).

HFRS or Hemorrhagic Nephroso-nephritis as it was known in Russia, has been recognized since 1944 although several thousand cases involving severe, moderately severe and mild forms of the disease have been described since 1913 (Smorodintsev et al. 1959, Lee 1982 and Schmaljohn 1988). The severe and moderately severe forms of HFRS in Russia are now known to be caused by the *Hantaan* (HTN) and *Dobrava-Belgrade spp.* (DOB) of viruses. Their principal reservoir hosts are *Apodemus agrarius* (striped field mouse) and *Apodemus flavicollis* (yellow-neck mouse), respectively. Human outbreaks of HTN and DOB range from southwestern Russia to the Urals; however, infected animals have been captured in far eastern Russia as well (Johnson 1986 and Schmaljohn and Hjelle 1997).

The incubation period for HTN and DOB infections can be 4-42 days, 14-21 days on average. Clinical symptoms are fever, vomiting, prostration, shock and renal

involvement. The clinical course may or may not include proteinuria, hemorrhagic manifestations and renal failure (Johnson 1986). The mortality rate is approximately 5-10% (Lähdevirta et al. 1971).

The mild form of HFRS, which has been reported since 1939 throughout Russia and Scandinavia, came to be known as Nephropathia Epidemica (NE) in 1945 (Lähdevirta 1971). Several thousand cases of "Trench Nephritis" or "War Nephritis", clinically similar to NE, had been described among British troops in Flanders (a region in northwest Europe) during World War I (1914-1918) (Lee 1982).

In the case of NE, the principal reservoir, *Clethrionomys glareolus* (wild bank vole) was identified in 1980 through antigen detection but the etiologic agent; *Pumuula* (PUU) virus was not identified until ca. 1984 (Brummer-Korvenkontio et al. 1980 and Lähdevirta et al. 1984). The incubation period for NE is about 14-35 days. The clinical symptoms are acute febrile illness, abdominal pain and proteinuria. Patients rarely develop hemorrhagic manifestations (Johnson 1986). The mortality rate for NE is between 0-5% (Lähdevirta et al. 1984).

Epidemic Hemorrhagic Fever (EHF) is the form of HFRS that has been recognized in Asia and Eastern Europe. In China, about 20,000 cases had been reported annually from 1931 to ~1982 (Brummer-Korvenkontio et al. 1980 and Lee 1982). EHF cases were described among Japanese troops in Manchuria in 1932 and again during World War II (1939-1945) in Japan but were not identified as EHF until 1964 (Lee et al. 1979 and Lähdevirta et al. 1984). EHF had been reported in Eastern Europe in 1962 (Gajdusek 1962). A close antigenic relationship between EHF and HTN was demonstrated by Lee et al. (1978) and again by Gan et al. (1983). Lee also definitively

identified that the principal reservoir for EHF was *Apodemus agrarius* (Black-striped field mouse) (Gan et al. 1983 and Lee et al. 1979). The symptomology of EHF follows the same clinical courses as the three forms of HFRS described earlier (Lee et al. 1979).

From March to July of 1981, outbreaks of a mild hemorrhagic fever occurred in the Henan and Shaanxi provinces of northern China. This disease had a very short course (7-14 days), a very low mortality rate (<1%), and was characterized by acute febrile illness, proteinuria and slight hemorrhagic manifestations (Johnson 1986).

Thus far, HFRS cases had a sylvatic or rural association, primarily affecting agricultural workers, soldiers engaged in military operations and those involved with economic development (Johnson 1986). In the Chinese provincial cases, epidemiological evidence revealed that the patients had been in contact, sometimes frequently, with *Rattus norvegicus* (house rats) also known as Norway rats, in an urban setting. Samples of lung tissues from *R. norvegicus* collected from patients' homes were found to be antigenically similar to HTN but clinical manifestations in patients were similar to NE found in Scandinavia and Russia (Gan et al. 1983). An earlier study in Japan reported that all cases of EHF during 1960-1972 had been among urban dwellers of Osaka City where there were *R. norvegicus* but no *Apodemus spp.* (Lee et al. 1979).

One of the most important epidemiological events to occur in the history of HFRS was surrounding the Korean Conflict (1950-1953). Approximately 3,200 cases of HFRS were reported among United Nations forces from 1951-1954 in Korea, with a mortality rate of 10-15%, and first attracted the attention of western physicians (Schmaljohn 1988 and Schmaljohn and Hjelle 1997). The disease became known as Korean Hemorrhagic Fever (KHF) and the prototype viral agent was subsequently coined "*Hantaan*" virus,

named for the Hantaan River in South Korea that flows through a KHF endemic area (Schmaljohn 1988).

A viral etiology had been suspected since the early 1940's when Russian investigators were able to successfully reproduce disease in human volunteers injected with sera and urine from HFRS patients (Lee 1982 and Schmaljohn and Hjelle 1997). A rodent association with HFRS had been suspected for decades, but it was not until an HFRS outbreak in a Russian laboratory conducting research on tick-borne encephalitis in 1961 that a viral transmission from rodents was established. Two weeks after wild-caught rodents were brought into the laboratory, 113 workers, many of whom did not have any contact with the rodents or the animal rooms, were diagnosed with HFRS. This established a rodent correlation with the disease and strongly suggested that the virus could be aerosolized (Lee 1982 and Schmaljohn 1988).

Lee et al. (1978) identified the etiologic and reservoir host of KHF. An antigen, isolated from the lungs of wild-caught *A. agrarius*, produced a specific immunofluorescent (IF) reaction with sera from convalescing KHF patients (Lee et al. 1978, Lee 1982). With IF, combined with electron microscopy, other investigators were able to subsequently identify antigenically related, pathogenic viruses such as DOB, PUU, and Seoul (SEO) (Johnson 1986).

Given the primarily, sylvatic association with HFRS, and that the symptoms may be clinically mild, this disease is probably underreported. It is estimated that there are 150,000-200,000 cases of HFRS that require hospitalization annually in Asia and Europe. More than half of these cases are reported in China, with Russia and Korea reporting

hundreds to thousands of cases annually. As of approximately 1997, the mortality rate has declined to 0.1%-10% depending upon the viral strain (Schmaljohn and Hjelle 1997).

1.2 Hantaan Agents

In 1975, the taxonomic family *Bunyaviridae* was established by the International Committee on Taxonomy of Viruses to encompass many morphologically and morphogenically similar arthropod-borne viruses. The *Bunyamwera* virus was the prototype for the *Bunyavirus* genus, the only genus within *Bunyaviridae* at the time. Several arboviruses were morphologically similar to *Bunyamwera* but antigenically unrelated to other viral species within the *Bunyavirus* genus. In 1982, 3 genera were added to the *Bunyaviridae* family: *Phlebovirus*, Sandfly fever prototype; *Nairovirus*, Sheep or Crimean/Congo hemorrhagic fever virus prototype; and *Uukuvirus*, Uukuniemi virus prototype (Martin et al. 1985 and Fauquet 2000).

The viruses assigned to the *Bunyaviridae* family thus far were 80-120 nanometers (nm) in diameter with a primarily spherical shape, 5-10 nm surface projections anchored in a lipid bilayered envelope, and a tripartite RNA negative sense genome (Schmaljohn et al. 1983 and Martin et al. 1985 and Fauquet 2000).

McCormick et al. (1982) purified the 76-118 Hantaan viral strain originally isolated by Lee et al. (1978) and later grown in tissue culture by French et al. (1981) in order to morphogenically characterize the virus. Viral strain 76-118 was inoculated into E-6 cells, a cloned line of Vero cells (a serially propagated heteroploid cell line used extensively for viral replication and plaque assays). Investigators found that the purified virus particles were morphologically similar to the viruses within the *Uukuvirus* genus

and that the particles averaged 92.5 nm in diameter (McCormick et al. 1982 and Schmaljohn et al. 1983).

Hung et al. (1983) attempted to further characterize Hantaan virus. HTN inoculated cells were harvested and fixed for indirect immuno-electron- microscopy. Investigators found both spherical and oval shaped particles with a larger average diameter than previously described, 122 nm and a variation of 110-160 nm (Hung et al. 1983). The virions possessed a membrane envelope comprised of granulofilamentous viroplasm arranged in a grid-like pattern and ~6 nm surface projections (Hung et al. 1983, Schmaljohn et al. 1983 and Martin et al.1985).

Biochemical data were required to more completely characterize HTN. Schmaljohn (1988) demonstrated that HTN had the same sedimentation characteristics as Rift Valley fever in the *Phlebovirus* genus and consistent with *Bunyaviridae.* Disruption of the HTN sedimentation in nonionic detergent resulted in 2 distinct components that are characteristic of an enveloped virus with nucleocapsid and membranous components. The sedimented nucleocapsids were resolved into small, medium, and large components with similarity to the 3 nucleocapsids of LaCrosse virus in the *Bunyavirus* genus (Morita et al. 1985). HTN possessed characteristics very similar to the other genera of *Bunyaviridae*, a Large (L), Medium (M), and Small (S) segmented, single-stranded RNA genome enclosed in a lipid envelope with 2 virus-specified glycoproteins (Morita et al.1985, Schmaljohn 1988 and Elliott 1991).

Attempts were made to antigenically relate the viruses throughout Eurasia and the Americas. Immunofluorescent (IF) antibody tests have demonstrated cross reactivity between the etiologic agents that cause KHF, EHF, NE, and HTN (Lee et al. 1981).

Antibody titres against the viruses that cause EHF and KHF, SR-11, and Hantaan are high regardless of the severity of the disease and the rodent reservoir (Lee et al.1979, Lee et al. 1981, Gan et al. 1983 and Kitamura et al. 1983). Investigators were beginning to realize that the viral isolates from different rodent hosts and geographical areas were not identical but closely related. IF assays demonstrated that Hantaan viral isolates from the *Rattus* genus would rarely produce viral antigen in experimentally inoculated *Apodemus spp.* This indicated that there was no cross infectivity of the viral isolates and suggested a unique viral strain in a respective host and that viral circulation amongst the *Rattus* hosts, at least, must be longstanding (Lee et al. 1981, Lee et al. 1982 and Kitamura et al. 1983).

Serological classification of HFRS viruses began in 1985. Three serotypes were identified at that time based on antigenic cross reactivity and blocking antibody titrations. Hantaan was designated serotype 1; Puumala, serotype 2; and Prospect Hill, a virus isolated from the *Microtus pennsylvanicus* (Meadow vole) in the United Stated but not associated with human illness, was serotype 3 (Goldgaber et al.1985). Seoul and Dobrava/ Belgrade viruses have been designated fourth and fifth antigenically distinct groups respectively. As of 1987, Hantaan and related viruses had become a new genus of the family *Bunyaviridae* and recognized by the International Committee on the Taxonomy of Viruses as the genus *Hantavirus*. Thailand, Thottopalam and Muerto Canyon/Four Corners have also been added to the *Hantavirus* genus as have several more serologically and genetically distinct viruses. (Schmaljohn 1988 and Elliott et al.1991).

Hantaviruses possess 3 segments of single-stranded RNA that comprise the major structural proteins and make up 1-2% of the virion particle. The large (L) segment,

encoding a putative RNA dependent viral polymerase, has a molecular weight of $2.2x10^6$ Daltons and is 6500-8500 bases. The medium (M) segment encodes for G_1 and G_2 envelope glycoproteins and a non-structural protein (NSm), has a molecular weight of $1.2x10^6$ Daltons and is 4200-5700 bases. The antigenic differences that occur among hantaviruses are most likely due to the less conserved and external amino half of the G_1 protein. The G_1 and G_2 coding sequences are contained within one continuous Open Reading Frame (ORF). This ORF is then transcribed as a single mRNA. The small (S) segment encodes for a nucleocapsid (N) core antigen and a non-structural protein (NSs), has a molecular weight of $0.6x10^6$ Daltons and is 1800-2300 bases (Morita et al. 1985, Yoo and Kang 1987, Schmaljohn 1988, Elliott et al. 1991 and Rawlings et al. 1996). These 3 antisense RNA strands of Hantaviruses are highly conserved at the 3'- terminal nucleotide sequence (3' AUCAUCAUCUG) and are different than the other *Bunyaviridae* genera (Yanagihara and Gajdusek 1988).

Each hantaviral strain is associated with a specific rodent host and geographical region. There is a stronger association between the hantaviral phylogenetic relationship and rodent/insectivore host phylogenetic relationship than geographical distribution and thus, Hantaviruses may have coevolved with their specific hosts. There is strong evidence to support cospeciation among viral strains and rodent hosts as well as host switching or spillover. For example, HTN viruses are associated with *Apodemus sp.*; however an HTN-like strain was isolated from *Emberisa elegans* (Yellow-throated bunting). PUU viruses are associated with *Clethrionmys sp*. but some viral isolates from Russian *Clethrionmys spp*. were more closely related antigenically to HTN than to PUU. It has also been found that while two viral strains are monophyletic, their respective host

species are much more divergent and distally related (Dzagurova et al. 1995, Ravkov et al. 1995, Hörling et al. 1996, Song et al. 1996, Monroe et al. 1999, Vapalahti et al. 1999 and Rhodes et al. 2000).

1.3 Pathogenesis of Human Disease

Hantaviruses throughout the Eurasian landmass are now referred to as "old world hantaviruses" and cause hemorrhagic fever with renal syndrome (HFRS). Hantaviruses found in North, Central and South America are referred to as "new world hantaviruses" and cause hantavirus pulmonary syndrome (HPS) without the severe renal involvement characteristic of HFRS, however not all new world hantaviral strains identified thus far have been associated with human illness (Schmaljohn and Hjelle 1997 and Mills et al. 1999*a*).

HFRS Clinical Features.-- HFRS may exhibit mild, moderate or severe disease symptomology depending upon the viral variant. The clinical course of classic Hantaan viral infection causing severe HFRS has been described by numerous investigators and is summarized in the following (Lee 1982, Lee and Johnson 1982, Ellis et al. 1995 and Schmaljohn and Hjelle 1997).

HFRS occurs in five, often overlapping, clinical stages or phases; however these stages are arbitrarily categorized and not all stages of the disease are present or apparent in all cases (Gajdusek 1962, Lee 1982, Schmaljohn 1988 and Schmaljohn and Hjelle 1997). The incubation period is usually 2-3 weeks but may vary from 4 days to 6 weeks (Lee 1982 and Yanagihara and Gajdusek 1988).

The first or febrile phase, lasting 3-8 days, has an abrupt onset of fever (102°F-104°F) accompanied by chills and intense frontal or retro orbital headache with

occasional photophobia and ocular pain due to chemosis. This is followed by vomiting, and anorexia with lumbar and abdominal pain as a result of plasma extravasation causing retroperitoneal or peritoneal edema. A petechial rash appears on the face, palate, pharynx, axillary folds, thorax and legs; erythema appears on the face and chest. There is a marked increase of albumin in the urine, urine specific gravity begins to decrease and proteinuria continues to increase. There is a decrease in blood platelets and an increase in hematocrit due to plasma extravasation (Myhrman 1951, Smadel 1953, Gajdusek 1962, Lee 1982, Yanagihara and Gajdusek 1988 and Schmaljohn and Hjelle 1997).

The second or hypotensive phase lasts a few hours to 3 days and is marked by polyuria, continued febrile illness and onset of hypotension due to increased capillary permeability. Tachycardia due to increased blood potassium is present. Hematocrit levels are at their highest during the clinical course. Hematuria, hepatomegaly and splenomegaly may be present in some patients. Blood platelets continue to decrease and blood nonprotein nitrogen levels increase, delirium and confusion is observed. Acute hypotensive shock occurs resulting in a 30% mortality during this phase (Myhrman 1951, Gajdusek 1962, Lähdevirta 1971, Lee 1982, Yanagihara and Gajdusek 1988, Ellis et al. 1995 and Schmaljohn and Hjelle 1997).

HFRS then progresses to the third or oliguric phase in which nearly half of all severe cases will result in death. The oliguric phase lasts from 3-13 days and at the beginning, blood pressure may normalize but in most cases, patients will become hypertensive due to hypervolemia. Erythema and petechia subside and hematocrit levels may return to normal. As the disease progresses, the clinical symptoms worsen; electrolyte imbalance occurs (hyperkalemia, hyponatremia and hypocalcemia), there is

epistaxis with hemoptysis and purpura; cerebral, conjuctival and gastrointestinal hemorrhage. Urinary output is decreased and there is a marked increase in blood urea nitrogen and azotemia is evident (Smadel 1953, Gajdusek 1962, Lee 1982, Schmaljohn 1988,Yanagihara and Gajdusek 1988, Ellis et al. 1995 and Schmaljohn and Hjelle 1997).

The fourth or diuretic phase lasts from days to weeks with an average of 10-28 days and during this time, clinical symptoms improve dramatically. Fluids or diuresis must be administered carefully to the dehydrated, electrolyte imbalanced patient. The small percentages of deaths that occur during this phase are due to fluid overload that cause hypertension and pulmonary edema. Urine output increases and there is significant renal improvement (Gajdusek 1962, Lee 1982, Schmaljohn 1988, Yanagihara and Gajdusek 1988 and Ellis et al. 1995).

During the fifth or convalescent phase, which may last several months, proteinuria and azotemia continue to subside but full renal function, particularly urine concentration and glomular filtration rate may take more than 6 months to recover (Smadel 1953, Gajdusek 1962, Lee 1982, Yanagihara and Gajdusek 1988 and Schmaljohn and Hjelle 1997).

HFRS Pathology.--The pathologic changes that occur with HFRS vary depending upon the severity and duration of the disease, also during which phase of the disease course the patient died in, particularly the histopathological changes in the kidneys that occur due to capillary damage (Smadel 1953 and Lukes 1954). The primary lesion observed with HFRS found during postmortem examination is endothelial cell damage (Yanagihara and Gajdusek 1988).

Patients that die of shock during the hypotensive phase, usually by the eighth day of clinical onset, are found to have large quantities of protein-rich gelatinous fluid in the abdominal cavity (Yanagihara and Gajdusek 1988). The kidneys are swollen and pale in color, the subcortical medullary vessels are congested but without tubular damage, and hemorrhagic necrosis is present (Gajdusek 1962). Petechiae and ecchymoses are commonly found in the epicardium; congestion with fresh erythrocytes causing severe hemorrhage in the right atrium is almost always present in patients dying during the hypertensive phase. Hemorrhagic necrosis and intense congestion of the anterior lobe of the pituitary is apparent. The adrenal glands have focal to diffuse hemorrhagic necrosis. The lungs are only slightly congested and are of normal size in most cases although mild pulmonary edema or bronchopneumonia may be observed. Congestion of the spleen and bone marrow, and thrombocytopenia are observed. There is massive enlargement of the lymph nodes and distention of small intestine submucosa lymphatics (Lukes 1954, Gajdusek 1962, van Ypersele de Strihou 1979 and Yanagihara and Gajdusek 1988).

The pathologic changes that occur during the oliguric and diuretic phases, after the ninth day of clinical onset, are much more severe with primarily renal involvement. The most distinctive characteristic of the renal lesion is intense congestion and hemorrhage at the corticomedullary junction with extensive tubular epithelial necrosis (Smadel 1953). Vascular congestion in the renal intertubular spaces increases and the tubular lumens become filled with desquamated cells, eosinophilic casts and hyaline material indicative of progressive tubular damage and medullary hemorrhage (Lähdevirta 1971). Severe necrosis of the pituitary is common in the diuretic group and in some cases, collapsed connective tissue stroma and absence of parenchymal cells may be

observed. There is a significant increase of pulmonary edema and/or abscess formation, both of which cause bronchopneumonia in many of the fatal cases during the diuretic phase (Lukes 1954, Gajdusek 1962 and Yanagihara and Gajdusek 1988).

HPS Clinical Features and Pathology.--The occurrence of hantavirus pulmonary syndrome (HPS) has thus far been limited to the Americas and there have been no viral HPS strains associated with renal involvement identified in North America (Bradshaw 1994, CDC 2000). The reported mortality rate of untreated HPS was 43%-66% and about 50% for treated cases, 5 times more fatal than the most severe forms of old world Hantaviruses (Eidson and Ettestad 1995 and Kitsutani et al. 1999).

Serosurveys of cricetid and microtine rodents conducted in the United States 11 years prior to the first, highly publicized, and diagnosed cases of HPS revealed antibody titres to a genetically distinct hantavirus. Prospect Hill virus (PHV) was isolated from *Microtus pennsylvanicus* in 1982 and antibody titres in *Peromyscus maniculatus* were higher against PHV than to HTN (Nerurkar et al. 1994). In 1984, several *Rattus norvegicus* were antibody positive for a hantavirus that was antigenically distinct from the prototype HTN and subsequently named Girard Point virus (GPV) after the granary in Philadelphia, PA where the rats were found (LeDuc et al. 1984).

Since there were no cases of archetypal hemorrhagic fever with renal syndrome diagnosed in the United States, it was unknown if these new hantaviruses were pathogenic to humans (LeDuc et al. 1984 and Nerurkar et al. 1994). It was discovered that in a 1985 serosurvey of patients with idiopathic febrile illness, along with healthy blood donors, that 13 subjects (out of 1699 serum samples) had antibody titres against hantavirus. None of the donors had traveled to geographical areas that were endemic

with HFRS nor had a history of HFRS illness. The absence of neutralizing antibody titres in 12 of the 13 donors with low titres of reactive antibodies may be suggestive of a cross reaction to an antigenically related Hantaan virus (Yanagihara et al. 1985).

In May of 1993, a highly publicized outbreak of HPS in the Four Corners area (New Mexico, Utah, Colorado and Arizona) of the United States was reported although hantavirus was not the suspected etiologic agent at the time. On May 14, 1993, the Indian Health Service reported to the New Mexico Department of Health that 2 young, previously healthy, adult Navajo Indians had died within 5 days of each other of acute respiratory failure. Several more adult Navajo Native Americans succumbed to the same illness in the Four Corners area. Those and the index cases were negative for tests to all suspected pathogens. The public health departments of Arizona, New Mexico and Utah were asked by the Centers for Disease Control and Prevention (CDC) to provide blood and tissue samples from suspected cases. Again, all test results were negative for known pathogens in the region except for positive reactions with Puumala virus. The CDC, Army and National Institutes of Health performed extensive immunologic and molecular tests, and on June 9, 1993 the CDC confirmed a previously unidentified genotype of hantavirus. As of November 5, 1993, there were 42 confirmed cases of hantavirus infection with a 62% mortality rate (Rand 1994, Weigler 1995). This novel hantavirus, originally called Muerto Canyon or Four Corners virus after the area HPS appeared was subsequently coined Sin Nombre virus (SNV). It is the prototype etiologic agent of HPS (Hjelle et al. 1994, Weigler 1995 and Kitsutani et al. 1999).

The most distinguishing feature of severe or fatal HPS is acute respiratory distress syndrome (ARDS) caused by noncardiogenic pulmonary edema (Eidson and Ettestad

1995, Kitsutani et al. 1999 and Leslie et al. 1999). HPS occurs in 3 arbitrarily assigned phases; prodromic, pulmonary or cardiopulmonary, and convalescent. The syndrome is characterized by a rapid clinical progression and high case fatality rate (Eidson and Ettestad 1995, Kitsutani et al. 1999 and CDC 2000),

The incubation period for HPS is on average 2-3 weeks but can be up to 45 days as was the case for the only HPS fatality in Rhode Island reported thus far (Kitsutani et al. 1999). The prodromic or febrile phase lasts 1-6 days and is a non-specific presentation. This phase begins with febrile illness with a fever between 101°F-104°F, generalized myalgia, prostration, nausea and diarrhea. Tachypnea and tachycardia may be present in the late prodromal phase but usually occur during the abrupt onset of the cardiopulmonary phase (Weigler 1995, Kitsutani et al. 1999 and CDC 2000). During the late prodromal phase, there is a marked increase in large atypical lymphocytes and thrombocytopenia. A characteristic of the differential white blood cell count that distinguishes HPS from other viral infections is an elevated left shift of neutrophils with circulating myelocytes (CDC 2000).

Six or 7 days after clinical onset, there is an abrupt progression into the cardiopulmonary phase marked by dyspnea, hypotension, hypoxemia and noncardiogenic pulmonary edema leading to respiratory distress, which can occur within 24 hours. Clinical pathologic findings at this time are a rise in hematocrit and a fall in serum albumin, which reflects a fluid shift from the circulatory system to the lungs. Radiographically, there is a progression of interstitial or alveolar infiltrates to severe bilateral pleural effusions. Immunohistochemically, there is a distribution of viral antigens within the capillary endothelium of various tissues particularly within the

pulmonary microvasculature, spleen and lymph nodes. The resulting lesion is the functional impairment of the vascular endothelium that may be attributed to the cellular effect of viral inclusions and/or virally induced, immune-mediated response within the pulmonary endothelium. The severe pulmonary edema, prolonged prothrombin time, proteinuria and high lactate dehydrogenase activity mark a poor prognosis. The convalescent phase is somewhat unremarkable except that it is quite rapid and polyuria is frequently present in most survivors (Hjelle et al. 1994, Rand 1994, Eidson and Ettestad 1995, Weigler 1995, Kitsutani et al. 1999 and CDC 2000).

The varied clinical courses observed with new world HPS infections might depend upon 2 factors. The first is the genetic diversity of the hantavirus, for instance, the previously described pathology is of infection due to SNV or closely related hantavirus. Bayou, Black Creek Canal and Andes virus infections have been associated with renal insufficiencies and elevated serum creatnine kinase. PHV infection has thus far not been associated with any apparent clinical course in humans. Clinical descriptions of some human cases have not progressed past the prodromal phase and are thought to represent unique hantaviral strains. The second determining factor for different clinical manifestations may be within the human patient; it has been hypothesized that some patients may have a weaker immune response to infection thus causing a less intense virally induced immune response. Integrins expressed on platelets and endothelial cells may serve as receptors for HPS viral cellular entry and variations in these receptor molecules may alter viral pathogenicity (Schmaljohn and Hjelle 1997, Kitsutani et al. 1999 and CDC 2000).

The answer to disease severity may be solved with the recent discovery of an animal model for hantavirus pathogenicity. Thus far, infected rodents have remained asymptomatic but now virologists at the U.S. Army Medical Research Institute of Infectious Diseases have reported that Syrian hamsters exhibit HPS symptoms when inoculated. Although the hamsters are susceptible only to Andes virus, theories about cellular entry and subsequent immune response to the virus can now be tested (Enserink 2001).

Hantavirus pulmonary syndrome (HPS) was previously the term used by investigators to describe clinical manifestations of patients infected with a new world hantavirus. However, since the majority of hantavirus case fatalities are a result of cardiac depression rather than hypoxia, many investigators prefer the term hantavirus cardiopulmonary syndrome (HCPS) (Schmidt et al. 2005 and Bharadwaj et al. 2000).

1.4 Rodent-Human Hantavirus Transmission

The primary route of human hantavirus infection is the inhalation of virus present in the urine, feces and saliva shed by infected murid rodents. After several proposed routes of infection, the aerosol route was established in 1961 after an outbreak in a Moscow laboratory that resulted in the infection of 113 of 186 employees and visitors. Several sylvatic rodents captured from endemic areas were brought to the laboratory during hantavirus field studies and many of the infected people had no contact with the rodents. Investigators had once believed that the other possible routes of infection, in addition to respiratory, were via rodent ectoparasites, mucous membrane penetration, and percutaneous since all of these potential confounders could occur with close contact with rodent carriers. The 1961 Moscow outbreak where several infected people had no

contact as well as a 1954 laboratory outbreak that occurred in another Russian laboratory studying ectoparasite-free rodents in which visitors that had no contact with rodents became infected convinced investigators that infection must occur via aerosolization (Lee 1982, Lee and Johnson 1982, Johnson 1986, Tsai 1987 and Hjelle and Glass 2000).

Since then, laboratory associated outbreaks have occurred throughout the world; imported Louvain (Lou/WSL/M) rats were responsible for an outbreak in a United Kingdom laboratory, as were Wistar rats in medical research laboratories in Japan and Belgium. Wild-caught rodents brought into laboratories have been associated with Korean and Chinese laboratory infections (Lee and Johnson 1982, Destmyter et al. 1983 and Lloyd et al. 1984).

The majority of hantavirus infections are associated with temporally sporadic outbreaks and sylvatic settings, occurring in localized foci, eliciting the concept of a "place" disease. Historically, the highest risk group for contracting a hantaviral infection had been those conducting military operations due to the disruption of the rodents' habitats (Gajdusek 1962, Johnson 1986 and Yanagihara and Gajdusek 1988). At-risk groups or individuals are persons that engage in any activities that bring them into contact with infected rodents. Agricultural activities such as threshing and working in grain silos along with forestry related activities bring humans in contact with potentially infected rodent excreta. Several rural cases are results of working and/or sleeping in closed structures such as cabins, barns and outbuildings that are or have been infested with rodents. Field investigators are also at a high risk (Yanagihara and Gajdusek 1988, Bradshaw 1994 and Ellis et al. 1995).

Urban hantavirus cases are more prevalent in Asia than in North America and outbreaks are of more epidemic proportions due to poorer housing conditions that would bring humans in contact with infected rodents in densely human populated dwellings. Infestations of house rats are responsible for outbreaks in dormitories, army barracks and apartment buildings (Lähdevirta 1971, Gan et al. 1983 and Schmaljohn and Hjelle 1997). There have been isolated urban cases in the United States such as the ones that occurred in New York and Rhode Island (Ellis et al. 1995).

Secondary routes of hantavirus infection occur as a result of rodent bites as was the case in Haute-Sovoie in rural France (Dournon et al.1984). This scenario is plausible since infected rodents excrete virus in their saliva and horizontal transfer of hantavirus between animals is often a result of fighting. While infection due to contact with contaminated fomites or food is possible, it is far less common than aerosol or even percutaneous transmission (Dournon et al. 1984, Ellis et al. 1995, Otteson et al. 1996 and Schmaljohn and Hjelle 1997). The possibility and perhaps occurrence of nosocomial infection has been reported (Enria et al. 1996). A Buenos Aires physician with no other risk factors such as visits to endemic areas, contracted HPS 27 days after direct contact with an HPS infected patient's blood. Early work by Russian investigators, from 1940-1941, produced HFRS in human volunteers by intramuscular and intravenous injections of serum and urine from naturally infected HFRS human patients (Smorodintsev et al.1959 and Gajdusek 1962). Japanese investigators, from 1941-1943 claimed to have produced HFRS infection with injected filtrates of *Apodemus agrarius* tissues along with ectoparasites collected from HFRS endemic areas (Gajdusek 1962). The possibility of nosocomial transmission of hantaviruses remains unclear although plausible since viral

isolates can be obtained from blood and urine of infected patients (Gajdusek 1962, Lee et al. 1978, Tsai 1987, Enria et al. 1996 and Schmaljohn and Hjelle 1997).

1.5 Hantavirus Epidemiology

Seasonal incidences of HFRS have been noted in Korea since 1951 and appear to be the highest during late spring and late fall. These seasonal peaks of incidence may be exclusively a result of an increase in rodent populations or confounded by activities that would bring humans into more contact with infected rodents and their excreta (Lee 1982, Lee and Johnson 1982 and Ellis et al. 1995).

Since the 1993 HPS outbreak in the Four Corners area, Investigators have tried to find a correlation between rodent populations and HPS cases (Mills et al. 1999*b* and Jay et al. 1997). Preceding the 1993 outbreak there was an increase in precipitation and a mild winter attributed to the El Nino Southern Oscillation (ENSO) of 1991-1992. This, in turn, resulted in an increase in abundant food sources such as insects and vegetation, primarily rich crops of pinyon nuts. Sevilleta National Wildlife Refuge in New Mexico reported a 20-fold increase in the rodent population over 1992 estimates. This scenario makes it likely that a rodent population explosion and subsequent increase of viral transmission via more rodent-rodent contact would also increase the likelihood of humans coming into contact with HPS infected rodents (Bradshaw 1994, Engelthaler et al. 1999 and Mills et al. 1999*a*). A similar pattern of increased precipitation followed by an HPS outbreak was observed in Paraguay from 1995-1996. A second ENSO event began in mid-1997 and prior to that time there were approximately 4 cases/year that had been reported throughout CO, AZ, NM and UT, the region of the original outbreak. HPS cases in the four states increased to 33 between January 1998-July 1999 from an

incidence of 6 cases that was predicted for that time frame (Engelthaler et al. 1999, Mills et al. 1999*a* and Hjelle and Glass 2000).

Attempts to construct a model that could predict epidemiological patterns of HPS in humans based upon rodent population dynamics alone have led to more questions than answers. The association between rodent populations and prevalence is complex (Lloyd et al. 1984, Childs et al. 1987*a,b*, Boone et al. 1998 and Engelthaler 1999).

Each viral strain known to cause HPS is associated with a single primary Sigmodontine host species. Although evidence suggests that the same hantavirus may be maintained by 2 closely related species, a secondary host species may represent a spillover from the primary reservoir. There is also cospeciation of virus-host, which will eventually lead to a unique viral strain maintained in that host. There have been no cyclical or seasonal fluctuations observed in Sigmodontine rodent populations but rather dramatic unpredictable increases or decreases in densities based on factors such as climatic changes, changes in biotic communities, inter- and intraspecific competition and predation (Schmaljohn and Hjelle 1997 and Mills et al. 1999*a*).

The *Peromyscus maniculatus* (deer mouse) is widely distributed throughout the contiguous United States and is the primary reservoir for SNV and SNV related viruses. Other Peromyscus species such as *P. boyii* (brush mouse) and *P. truei* (pinyon mouse) have a southwestern geographical focus and are also reservoir hosts for SNV and related viruses. Most of the host-habitat association work has been conducted in the Southwest United States because it remains the most HPS-endemic area known and has one of the richest assemblages of biotic and rodent communities in North America (Mills et al. 1997, Abbott et al. 1999 and Mills et al. 1999*a*).

A temporal pattern of fluctuating rodent densities and a linear correlation of hantavirus incidence is better documented in Korea, China and Scandinavia. The population density of the reservoir host of KHF in Korea and EHF in rural China, *Apodemus agrarius* experiences an increase in the late spring and more so in the fall; this corresponds to the increased incidences of human disease. In Sweden, the reservoir host of NE, *Clethrionomys glareolus* (an Arvicoline rodent) experiences a fairly regular population cycle every 3-4 years and this also corresponds to the incidence of NE in humans (Lee and Johnson 1982, Schmaljohn and Hjelle 1997 and Mills et al. 1999*a,b*).

The establishment of a temporal pattern of HPS cases in the United States is not so clear-cut. In studies of Sigmodontine rodents in the Southwest, population densities of *P. boyii* experience a slight spring-fall bimodal peak but HPS cases were unevenly distributed displaying a higher incidence in spring-summer. While this is an oversimplification, in actuality rodent population dynamics experience year-year and seasonal trends varied by biotic communities. In order to establish a temporal pattern of HPS cases, longevity of a study that also factored in environmental variables and increased statistical power would need to be conducted (Engelthaler et al. 1999, Kuenzi et al. 1999, Mills et al. 1999*a* and Boone et al. 2000).

Community types and altitudinal data are important variables for monitoring rodent densities. The lowest SNV prevalence in rodents is found at altitudinal and climatic extremes such as alpine tundra and salt desert scrub, $\geq$3,384m and $\leq$873m respectively. This corresponds to the majority of HPS cases found at mid altitudes (1,800m-2,500m) consisting of chaparral, pinyon-juniper and grassland. This sometimes coincides with increased densities of rodent reservoirs but is not consistently found from

study to study due to factors such as longevity of the study and biases introduced as results of survivability and seroconversion of rodents during mark-release-recapture monitoring (Mills et al. 1997, Abbott et al. 1999, Engelthaler et al. 1999, Parmenter 1999 and Boone et al. 2000).

There is a positive correlation between favorable climatic events initiating abundant mast and increased reproduction of *Peromyscus spp.* and visa versa. While this may lead to an increase in rodent densities and seroprevalence, data collected during the spring after a mild winter for instance, will be biased due to maternal antibodies conferred to the offspring. The same scenario could also lead to an increase in rodent densities but a decrease in seroprevalence if data were collected after juveniles have cleared maternal antibodies (the juvenile dilution effect). This may represent an increase in seroprevalence for the following year due to subsequent infection of older juveniles, even though population densities may decline. This has been observed as a result of unfavorable environmental conditions causing a decline in newborn mice. The subsequent population will consist of older mice that are commonly infected with hantavirus (Graham and Chomel 1997, Mills et al. 1997, Abbott et al. 1999, Kuenzi et al. 1999 and Mills et al. 1999*a*).

Another difficulty with predicting epidemiological patterns is the focality of HPS cases and viral infection in host reservoir populations. Long term monitoring in endemic areas has demonstrated that HPS outbreaks in the U.S. are temporally and spatially sporadic as are infections in host reservoir populations. Hantaviral infection appears as distinct "islands" associated with the preferred microhabitat of the reservoir host during periods of population stasis. These islands expand and contract temporally and during

periods of irruptions (extreme population explosions). These foci become difficult to identify for the purpose of assessing human risk. The same phenomenon has been observed in urban and suburban areas in the U.S (Korch et al. 1989, Mills et al. 1997, Boone et al. 1998, Abbott et al. 1999, Engelthaler 1999, Kuenzi et al. 1999, Mills et al. 1999*a* and Glass et al. 2000).

In order to develop a model that could predict the frequency or even timing of HPS outbreaks continual monitoring of endemic areas needs to be implemented. Long-term prospective studies need to include the following variables; temporal patterns of rodent host population dynamics and infection; altitudinal and climatic data, community types and reservoir host ecology all as a function of a geographical site's microenvironment, given the spatially incongruous nature of HPS (Graham and Chomel 1997, Mills et al. 1999*b*, Parmenter et al. 1999 and Boone et al. 2000).

As mentioned earlier, each hantaviral strain is associated with a primary reservoir host, most notably the Murid and Arvicoline families; *Peromyscus maniculatus* (deer mouse) and *Sigmodon hispidus* (Cotton rat, sub-family *Sigmodontinae*) and *Clethrionomys glareolus* (Bank vole) and *Microtus pennsylvanicus* (meadow vole, family *Arvicolidae*) (Yanagihara and Gajdusek 1988 and Nerurkar 1994) (Table 1). Other less frequently infected reservoir hosts include *Lemmus sibiricus*, *Arvicolidae* (Black-footed lemming), *Blarina brevicauda* (Northern short-tailed shrew), *Suncus murinus* (house shrew, *Soricidae*) and *Talpa europea* (European mole, *Talpidae*) and are responsible for the maintenance and transmission of their respective viruses (Tang et al. 1985, Yanagihara and Gajdusek 1988, Nerurkar 1994 and Vapalahti 1999).

Table 1. Distribution of hantaviruses in the continental U.S. by reservoir and disease syndrome.

Viral Strain	Primary Rodent Reservoir	U.S. Reservoir Distribution	Human Illness
Sin Nombre	*Peromyscus maniculatus* (grassland form) (Deer Mouse)	All of U.S. except the SE seaboard and southernmost CA	HPS
El Moro Canyon	*Reithrodontomys megalotis* (Western Harvest Mouse)	West and Central U.S. S to N Baja, CA	None documented
Monongahela	*Peromyscus maniculatus nubiterrae* (Deer Mouse)	Eastern U.S.	HPS
Blue River	*Peromyscus leucopus* (SW/NW haplotype) (White-footed Mouse)	Central U.S.	HPS
New York	*Peromyscus leucopus* (eastern haplotype) (White-footed Mouse)	Eastern U.S.	HPS
Bayou	*Oryzomys palustris* (Rice Rat)	SE Kansas to E Texas to S New Jersey and Florida	HPS
Black Creek Canal	*Sigmodon hispidus* (eastern haplotype) (Cotton Rat)	SE U.S. from S Nebraska to C Virginia to SE Arizona and Florida	HPS
Muleshoe	*Sigmodon hispidus* (western haplotype) (Cotton Rat)	Southern U.S.	HPS
Prospect Hill	*Microtus pennsylvanicus* (Meadow Vole) *montanus/ochrogaster* (Prairie Vole)	Throughout U.S.	None documented*
Isla Vista	*Microtus californicus* (California Vole)	California	None documented
Seoul	*Rattus norvegicus* (Black Rat)	Throughout U.S.	HFRS

*Human antibody reactive to Prospect Hill virus has been documented (Monroe et al. 1999)

(Childs et al. 1987*a,b*, Childs et al. 1994*a*, Mills et al. 1995, Rowe et al. 1995, Schmaljohn and Hjelle 1997, Bennett et al. 1999, Calisher et al. 1999, Ksiazek et al. 1997 and Monroe et al. 1999)

It is worth noting that no virus detection has been demonstrated in *B. brevicauda* and should not be considered a potential maintenance host (Yanagihara et al. 1987 and Lee et al. 1985).

Two species of bats, *Rhinolophus ferrum-equinum* (horse-shoe bat, *Rhinolophidae*) and *Eptesicus serotinus* (serotine bat, *Vespertilionidae*) were identified in 1994 as maintenance hosts for Hantaan or related viruses. Both species exhibited no differences in seropositivity in both summer and winter, which may be an explanation for year-round HFRS occurrences in non-endemic areas. Large amounts of antigen were detected in lung and kidney sections of both species. Genetic sequencing of the PCR products that were amplified with primer pairs for the S segment of Hantaan strain was not performed so exact homology to the prototyped virus is unknown. *E. serotinus* often inhabits human dwellings as do rats which makes it plausible for the bats to have contact with infectious rodent excreta and visa versa. More genetic analysis is required to determine the relatedness of the viral strains and if bats represent a spillover host or an example of cospeciation (Kim et al. 1994).

Other unusual non-rodent species that were seropositive to hantavirus during serosurveys are: *Mustela frenata* (long-tailed weasel), *Tamias spp.* (chipmunks), *Sylvilagus auduboni* (desert cottontail), domestic dogs, chickens, and pigs. Russian investigators claimed to have found hantaviral antigen in 13 species of birds in Eastern Russia. It is unlikely that these species are involved in virus maintenance and shedding, rather are end-stage hosts like humans (Yanagihara and Gajdusek 1988, Nerurkar 1994, Rand 1994 and Malecki et al. 1998).

Carnivores, particularly *Felis catus* may play a role in hantavirus epidemiology or be end-stage hosts themselves. Chinese investigators have reported that cats have been responsible for human HFRS cases in China and in a case-control study, cat ownership carried a significant relative risk (3.73; 95% CI=1.24-11.20), even when controlling for other variables. This evidence may be biased since households that have rodent infestations are more likely to have cats and/or cats may be bringing infected rodents into contact with humans as a result of hunting (Yanagihara and Gajdusek 1988, Eidson and Ettestad 1995 and Weigler 1995). Serologically positive cats have been found in residential areas of Baltimore, MD although hantavirus antigen was not detected (Yanagihara and Gajdusek 1988 and Weigler 1995). Serum samples from 200 domestic cats in Austria that were allowed outside had a seroprevalence of 5% and titred higher against PUU than HTN (Notwotny et al. 1994). A serosurvey in Great Britain of domestic cats resulted in a prevalence of 23% in some regions with a mean of 9.6%. Small foci of antigen were detected in the lungs of 2 (out of 100) cats that died of unknown illness and had histories of hunting; this is in contrast to rodent hosts where antigen is often found throughout the lungs (Notwotny 1994). Another study conducted in Great Britain found a 9.6% seroprevalence in domestic and feral cats. In addition, 23% of chronically ill cats were antibody positive and 8 (out of 19) were both FeLV and FIV negative (Bennett et al. 1990).

More extensive studies need to be performed such as genetic detection and characterization of viral isolates as well as serosurveys and accurate health data collected (in the cases of domestic cats). It remains to be determined if cats can shed infective virus and remain chronically infected, asymptomatic hosts as are rodents and insectivores or if

they represent a susceptible dead-end host (Yanagihara and Gajdusek 1988, Bennett et al. 1990 and Weigler 1995).

1.6 Viral Maintenance in Rodent Reservoirs

Hantaviruses are associated with a primary reservoir host and in the United States *Peromyscus maniculatus* (deer mouse) has been identified as the primary rodent reservoir of SNV (Childs et al. 1994*b*). Several other murid rodents have been identified, along with their respective hantaviral strains however, those will be discussed later. This section will focus on the population dynamics of the deer mouse and how that relates to viral maintenance and intra- and interspecific transmission; concepts that are applicable to other host species.

Deer Mouse Biology.-- *Peromyscus maniculatus* have been responsible for most of the human HPS cases in the United States thus far. Deer mice are the most abundant small mammals in North America and are comprised of more than 60 subspecies. *Peromyscus spp*. are thought to be "chaparral" dwelling species although there is a wide variation of habitats that they occupy. Deer mice are found at elevations ranging from sea level to more than 4,200 meters and occupy terrains consisting of forest, prairie, and desert (McCabe and Blanchard 1950, Childs et al. 1994*b*, Joyner et al. 1998 and Mills et al. 1999*a*).

It is probably no mistake that *Peromyscus spp*. have been the primary maintenance hosts in the United States. It is a genera of antiquity and great adaptability that is able to thrive in diverse climates and terrains (McCabe and Blanchard 1950). The deer mouse is very much a terrestrial creature exhibiting a limited ability to climb, having a preference for burrows and rotted tree trunks. Deer mice will often use tunnels and

burrows abandoned by moles, gophers and even spiders' holes under the chaparral. The mice themselves will dig their own burrows if the ground is soft. In an Indiana study, these burrows averaged 16 feet in length and were up to 12 inches below the surface (McCabe and Blanchard 1950 and Hoffmeister 1989).

Deer mice are opportunists, invading and exploiting areas altered by natural disasters and human disturbances such as flood areas, fires, landslides, strip mining, over grazing and land development. Most importantly, deer mice will often build nests inside human dwellings and storage buildings such as grain silos, barns, and sheds. Evidence of migration into buildings is observed primarily in the fall of colder weather climates but frequently occurs at anytime the deer mice find the opportunity to do so. This is in contrast to other *Peromyscus spp.*, which are rarely, if ever found in human dwellings (Hoffmeister 1989 and Calisher et al. 1999).

Subspecies of *P. maniculatus* are most often separated both geographically and ecologically, being found in almost any habitat. In the Four Corners area alone, subspecies are found in montane, mixed coniferous and spruce-fir forests; Northern Great Basin pinyon-juniper and sage-grassland; woodlands and desert scrub. This region is of particular interest due to the variation of biomes and enzoonotic status of hantaviruses. What has been observed in this region and other areas in the United States with biome diversity spanning a relatively moderate geographical area are the unique population dynamics of *Peromyscus spp.* For instance, in Northern New Mexico subspecies of *P. maniculatus* populations (*P.m.rufinus*) are continuous throughout montane, woodland, and lowland zones whereas in Central and Southern New Mexico, *P. maniculatus* are the dominant species in montane zones and sympatric with *P. leucopus* in desert scrub but

virtually non-existent in woodland zones which are dominated by other *Peromyscus spp.* (Sevilleta 1998, Calisher et al. 1999 and Engelthaler et al. 1999).

The Great Smoky Mountains National Park (GSMNP) in Eastern Tennessee and Western North Carolina is another area with overlapping *Peromyscus spp.* home ranges. The deer mouse is generally found throughout the park but is more abundant at higher altitudes (≥1986m) and the white-footed mouse is readily found at lower elevations (<900m). There is extensive overlapping of the 2 species at approximately 900m and they may be found in close proximity to one another despite the different habitat preferences (Linzey 1995).

In other regions of the United States, *P. maniculatus* remains more disjunct and such overlapping amongst subspecies and species is not readily observed (Hoffmeister 1989, Sevilleta 1998 and Engelthaler et al. 1999). Such extensive habitat diversity of the deer mouse creates a particular risk factor for rodent-human contact and HPS cases as they will inhabit structures and are quite prolific (Calisher et al. 1999).

Deer mice are omnivorous and they are opportunistic feeders as well as dwellers. The deer mouse diet is highly variable depending upon regional biomes and seasonality; from spring until fall mice will consume herbaceous matter, insects, and insect larvae, the latter primarily in the spring. During the fall, diets are mostly berries near cultivated fields, and in prairies deer mice will consume seeds of crops and regional plant seeds. Montane and woodland species consume a vast array of material such as acorns, nuts, carrion, insects, plant matter, leaves, bark, tubers, and roots. Deer mice will sometimes engage in copraphagy (McCabe and Blanchard 1950, Hoffmeister 1989, Linzey 1995, Bunker 1997, Joyner et al. 1998, Sevilleta 1998 and Calisher et al. 1999).

Both *P. maniculatus* and *P. leucopus* will go into a state of torpor for several days or weeks at a time during cold temperatures. It is the *P. maniculatus* however, that will câche berries, seeds, and seedlings (often poached from birds and squirrels) more so than any other *Peromyscus spp.* The deer mouse habitat is often subject to washing, gullying and flooding and the mice will build elaborate drainage foundations of stones, twigs and hard leaves for food caches. This is supported by the fact that when the proper materials are provided, deer mice will construct the same agglomerations in traps and cages (McCabe and Blanchard 1950, Hoffmeister 1989, Linzey 1995, Bunker 1997, Joyner et al. 1998, Sevilleta 1998 and Calisher et al. 1999). *Peromyscus spp.* are extremely important to the ecologic hierachy, they consume insects and other invertebrates and are important for the dispersal of seeds and mycorrhizal fungi spores. They are a food source for reptiles and several small carnivores such as owls, fox and weasels (Bunker 1997 and Joyner et al. 1998).

Peromyscus females are reproductively prolific and can produce litters every month, year-round in both captivity and in sylvatic settings in more temperate climates (Hoffmeister 1989). Typically, females are seasonally polyestrous and breeding extends from early spring into late fall with an average of 4 litters produced each year. On average, the estrous cycle is 5 days and usually commences at around 49 days of age although female sexual maturity can begin as early as 35 days old. Females born in the spring will come into estrous as soon as sexual maturity occurs and those born in fall will first breed the following spring (Bunker 1997, Joyner et al. 1998 and Sevilleta 1998).

Gestation is usually 23-25 days but variation occurs depending upon lactation status; non-lactating females exhibit gestation periods of 21-25 days and lactating

females, 24-30 days. Females will come into estrous again shortly after parturition and the longer gestation period may be attributed to an embryonic implantation delay due to lactation. The life expectancy of wild *Peromyscus spp.* is short and lifetime litter production for females is rarely more than 2-3 (Bunker 1997, Joyner et al. 1998 and Sevilleta 1998).

Litter sizes are highly variable and are correlated with the mother's size, age, and weight. The range is 1-13 young per litter but average 3-5 per litter. Subsequent litter sizes will increase until the fifth or sixth litter and then declines. Post-natal *P. maniculatus* are altricial except for suckling instincts and develop rapidly with eyes opening around day 15. They are weaned between 25 and 35 days although weaning can be as early as days 18 or 19. Weaned juveniles will move to new nesting sites prior to sexual maturity and by 6 weeks old, both males and females appear sexually mature (Hoffmeister 1989, Bunker 1997, Joyner et al. 1998 and Sevilleta 1998). Depending upon the time of year females give birth, it is common for the parents and 1 or 2 litters to overwinter together; often, unrelated groups will occupy the same nest for the winter.

The male will assist in preparing the nesting site, grooming and protecting young and teaching juveniles to find food outside of the nesting site. The females will also aggressively defend her young against intruders; in fact, reproductive females are more territorially aggressive than males (Bunker 1997 and Sevilleta 1998).

When young mice leave their nests, they will disperse 100 yards or more from the original nest. This distributive instinct increases the survivability of young mice as competition and intolerance of mature animals established at the original nesting site would undoubtedly result in the demise of the smaller animals. There is also long term

ecologic importance of *P. maniculatus* dispersal; the burden on vegetation and food sources of the microenvironment grows as does the population and therefore, it is more beneficial for young mice to establish elsewhere (McCabe and Blanchard 1950).

Home ranges of *P. maniculatus* can vary greatly; one study estimated home ranges of 242 square meters – 3,000 square meters. These estimates will vary both temporally and spatially, study to study due to environmental changes of an area and recapture success that can be altered by die-offs and trap responsiveness. Male deer mice have greater home ranges than do females and there is greater intra- and interspecific territory overlap for males than females. This leads to greater territory defense and more conspecific aggressive encounters as well as with other species and genera thus creating another route of horizontal transmission of hantaviruses (i.e. fighting) (Korch et al. 1989, Bunker 1997 and Calisher et al. 1999).

Rodent-Rodent Hantavirus Transmission.--It is well documented that hantaviral transmission between rodents and insectivores is responsible for viral maintenance in its respective host as well as spillover into closely related species. Despite attempts to prove otherwise, vertical transmission has not been documented and is unlikely (Lee et al. 1981 and Mills et al. 1999*a*).

Virus in infected rodents can be detected in the lungs, kidneys, submaxillary glands and rectal tissue; subsequently, large quantities of virus are shed persistently or sporadically in urine, saliva and feces for extended periods of time, even up to the lifetime of the host. Despite high circulating antibody titres in the serum, virus can still be isolated from organ tissue (Lee et al. 1981, Kariwa et al. 1998 and Mills et al. 1999*a*).

Several studies have confirmed that rodent-rodent transmission of hantaviruses occurs much in the same way that rodent-human transmission occurs, that is aerosolization of virus infected excreta. Urine from Seoul virus infected rats, inoculated intranasally into susceptible rats resulted in viral infection and shedding. Horizontal intracage transmission has been demonstrated by simply placing susceptible animals in cages with infected animals (*Apodemus agrarius*). This study also revealed that transmission is not sex – related as infection rates for inter and intra-sex pairings were not significantly different. It is unknown if the virus may be transmitted venerally in a sylvatic setting (Lee et al. 1981 and Kariwa et al. 1998).

Aerosolization, grooming, and fighting are the modes of transmission amongst wild populations of rodent host. Infected excreta in a shared burrow system are thought to serve as a source for new infection of susceptible animals. Infant and juvenile mice have become infected by their mothers as a result of grooming and exposure to infected excreta present in nests. There has been no demonstration that hantaviruses can cross the placental barrier or are present in the milk of lactating females (Lee et al. 1981, Kariwa et al. 1998 and Mills et al. 1999*a*).

Several studies have noted significant correlations between hantavirus prevalence amongst male rodents and fight wounds. In late summer and early fall, during the period of population peaks, male fighting is increased due to declining availability of food, territorial aggression, and competition for breeding partners. Data regarding a gender correlation with hantavirus prevalence are inconsistent and vary amongst species tested, but most support a higher male to female ratio of hantavirus prevalence. Fighting is the primary mode of interspecific transmission amongst closely related, sympatric species

and may be important for viral maintenance (Korch et al. 1989, Boone et al. 1998, Calisher et al. 1999, Mills et al. 1999*a*).

The average lifespan of rodents is brief, perhaps a few months, but mark-recapture studies have shown the some rodents can live 1-2 years or more. This is important for transseasonal persistence of hantaviruses, particularly during periods of low population densities. Infected overwintering adults sharing nests are an important source of infection for susceptible young animals, thus even a small percentage of persistently infected animals serve as reservoirs during low population densities (Lee et al. 1981, Korch et al. 1989, Rand 1994, Schmaljohn and Hjelle 1997, Boone et al. 1998, Kariwa et al. 1998, Calisher 1999 and Mills et al. 1999*a*).

There are 3 intrinsic factors associated with hantavirus prevalence: age, weight, and gender. Most long-term mark and recapture monitoring programs have demonstrated that male mice have a higher seroprevalence than females (Mills et al. 1999*a*). One study conducted in Arizona concluded that antibody positive male Brush mice (*Peromyscus boylii*) had longer survivability than antibody negative *P. boyii* and in disjunct optimal habitats; resident mice were predominantly dominant, antibody positive males. More data will need to be collected in order to determine if this phenomenon truly exists or if it is merely a function of population density and/or recapture success. Another Arizona study found no difference in survivability of seropositive male and females but had much smaller sample sizes and a shorter trapping period (7 months versus 35 months) (Kuenzi et al. 1999). If seropositive male mice do have a longer survivability, they could serve as important maintenance reservoirs for both transseasonal infection and during low population densities (Abbott et al. 1999 and Kuenzi et al. 1999)

The other 2 intrinsic factors associated with hantavirus seroprevalence are age and weight and are discussed together because one is a function of the other and cannot easily be stratified. In most field studies, animal weight is used to determine age and thus, the 2 factors must be considered together. Even in studies where eye lens weight was used to determine age, there were no significant differences in hantavirus prevalence when controlling for age or animal weight (Childs et al. 1985, Mills et al. 1997, Glass et al. 1998 and Abbott et al. 1999).

Most studies of *Peromyscus spp.* and rat species have demonstrated that antibody prevalences were highest in the highest weight classes. Body weight classes for *Peromyscus spp.* can be assigned I (juvenile), II (young adult), and III (adult) and are 6.0-19.0g, 19.1-22.0g, and 22.1-30.0g respectively. The weight classes for rats vary from study to study, most likely due to higher weight variability amongst the different species. The trend of increasing seroprevalence as weight class increases is very consistent for *Peromyscus spp.* in the Four Corners region. Additionally, antibody positive *Peromyscus spp.* in the heaviest weight class were also males and presumably the oldest. Since most of these were not mark-recapture studies, it is unknown whether the higher sero-prevalence in class III males represents newly acquired or long-standing infections. In a Walker River Basin study (NV, CA), Boone and others (1998) found no significant difference in seroprevalence between juveniles and adult *Peromyscus spp.* They did however find that antibody positive males were heavier than antibody negative males. While 2 independent studies yielded heavier male biases and hantavirus prevalence correlations, they were not on mark-recapture sites (Tsai 1987, Childs et al. 1985, Mills et al. 1997, Glass et al. 1998 and Mills et al. 1999*a*).

While the seroprevalence in weight class I is the lowest, it is interesting to note that the youngest mice in this weight class have the highest seroprevalence. Mills et al. (1999*a*) conducted a survey of small mammals in the Southwestern United States and divided the deer mice weight class I into 3 subclasses. Antibody prevalence in the heaviest subclass was 1%; 4% in the middle subclass; and 14% in the lightest subclass. The reason for this is that maternal antibodies are conferred to infants and that passive antibody is lost as young adults, making them susceptible to subsequent hantavirus infection. This phenomenon could potentially skew seroprevalences and should be considered when conducting field studies (Boone et al. 1998 and Mills et al. 1999*a*).

The seroprevalence amongst Norway rats (*Rattus norvegicus*) in Baltimore, MD demonstrated a weight, and thus age bias but not a gender bias. Heavier rats (>300g) had the highest prevalence but no significant differences among male and female rats. Glass and others conducted a study of Cotton rats (*Sigmodon hispidus*) in Florida; *S. hispidus* is the primary rodent reservoir of Black Creek Canal virus (BCC) in the Southeastern U.S. Their findings were more consistent with deer mice than with studies of rats. There were both male and weight / age biases and a higher seroprevalence in the lightest (youngest) weight class than in intermediate weight class which again reflects maternal antibody present, the loss of passive immunity and then subsequent infection (Childs et al. 1985, Childs et al. 1987*b*, Mills et al. 1997, Boone et al. 1998, Glass et al. 1998, Abbott et al. 1999, Kuenzi 1999 and Mills et al. 1999*a*).

Rodent Hantavirus Pathology.--In general, the rodent host remains asymptomatically, systemically infected upon experimental inoculation or naturally acquired hantaviruses. Full characterization of the viral pathogenesis; immune response;

viral persistency; and hantavirus-host biological relationship is incomplete. Further investigation of viral pathogenicity in the natural rodent host needs to be examined (Kim and McKee 1985, Kariwa et al. 1996 and Netski et al. 1999).

Inferences regarding viral shedding, humoral and cell mediated immune responses; and to some degree, pathogenesis in wild populations can only be made from controlled laboratory experiments. Many of these studies have demonstrated that viral persistence and exhibition of clinical symptoms are age dependent (Morita et al. 1985, Yanagihara and Gajdusek 1988, Kariwa et al. 1996 and Hutchinson et al. 1998).

Experimentally infected young (6 weeks old) *Sigmodon hispidus* with BCC will have systemic viral complimentary RNA (vcRNA) between 7 and 14 days post-inoculation (p.i.). Infectious virus was present in all tissues, including salivary glands at this time and blood virus titres peaked on 14 days p.i. while titres in all other tissues peaked on day 21 p.i. Virus titres declined rapidly after peaking, between 21 and 50 days p.i. but could still be detected on day 150 even though vcRNA could only be found in brain tissue. IgG was detected 14 days p.i., peaked by day 50 p.i. and remained at high levels throughout the duration of the experiment (150 days). Shedding of infectious virus in the urine began on day 7 p.i. Viral titres peaked between 21 and 50 days p.i. and declined after that although infectious virus was present in the urine and feces on day 150 p.i. It is interesting to note that vcRNA was present in the testicles between days 7 and 50, which suggests the possibility of venereal transmission although that has yet to be documented. No clinical symptoms such as weight loss, meningoencephalitis or mortality were observed in any of the animals (Yanagihara and Gajdusek 1988 and Hutchinson et al. 1998).

A similar experiment, inoculating adult *A. agrarius* mice with both 76/118 and Lee strains yielded similar results. Viremia was present on day 7 p.i., subsequently transient, and infectious virus was present in all other tissues and excreta by day 12 p.i. Infectious virus isolated from the lungs, kidneys, and urine persisted for up to 180, 260, and >360 days respectively. High titres of neutralizing and low titres of immunofluorescent antibodies were present for at least 360 days p.i. (Lee et al. 1981). Both of these experiments demonstrated a semichronic pattern of tissue infection with an initial acute phase and viral shedding via rodent excreta persisting for longer periods (Lee et al. 1981 and Hutchinson et al. 1998).

Two studies using Wistar rats inoculated with either the KI-262 (Seoul virus) or SR-11 (laboratory derived) strains differed from studies previously mentioned but supported an age dependent factor involved in viral persistence and the ability to act as a source of secondary infection. Three or seven week old rats inoculated with Hantaan strains had either no viral antigen in any tissue (3 week olds) or viral genome in the lungs only (7 week olds). The 7-week-old rats had no virus in the lungs after 50 days p.i. IgG avidity (bond strength between an antibody and an antigen) increased linearly with infection whereas neutralizing antibodies peaked on day 50 p.i. and were maintained after that. Antibody responses persisted throughout the course of the study (Morita et al. 1985 and Kariwa et al. 1996).

Newborn rats, 24-48 hours old, when inoculated with the same strains had a much different response. Viral genome was detected in all tissues examined between 1 and 5 days p.i. viral antigen was detected by 3 weeks p.i. and persisted for at least 6 weeks in the lungs serving as a secondary source of infection for cagemates previously uninfected.

These data indicate that viral persistency is age dependent and that newborn rats may be a factor in viral maintenance in a natural setting (Morita et al. 1985 and Kariwa 1996).

Early attempts to discover an animal model for hantaviral infections and to understand the biological relationship of the host and hantavirus infection revealed that some pathology does occur in the reservoir host (Kim and McKee Jr. 1985 and Netski et al. 1999). The pathological changes that occur in experimentally infected animals differs greatly from naturally infected animals due to the amount of viral inoculums and use of serially diluted, passaged strains in experimental infections (Yamanouchi et al. 1984 and Netski et al. 1999).

Inoculation of newborn (<24 hours old) mice and rats with *Apodemus* or *Rattus* derived hantavirus isolates exhibit clinical symptoms that do not resemble human hantaviral infection. Animals will first exhibit hyperactivity and hyperexcitability, which lasts approximately 2-3 days. Initially, animals will not gain weight and over the disease course will lose weight. They will often have a ruffled coat, hunched posture and hind limb paralysis. Convulsions, coma and death usually occur shortly after inoculation. On postmortem examination, animals are runted, dehydrated, and ~50% the weight of normal controls. Animals that do survive will have growth retardation and significant atrophy and paralysis of the hindquarters. Histologically, small foci of necrotic neurons in brain tissue; mononuclear infiltration of the meninges, myocardium, epicardium, and liver; congestion, edema, and interstitial pneumonitis in the lungs; and medullary interstial congestion were observed (Kurata et al. 1983, Yamanouchi et al. 1984 and Kim and McKee Jr. 1985).

This is an age dependent phenomenon. With increasing age, the mortality rate decreases, clinical symptoms diminish and by young adulthood, clinical manifestations are inapparent and resemble the naturally infected state. The differences in immune responses in an age-dependent fashion may dictate clinical manifestations and viral clearing in the rodent host (Kurata et al. 1983, Yamanouchi et al. 1984, Kim and McKee Jr. 1985 and Yanagihira and Gajdusek 1988).

Naturally infected, wild-caught deer mice exhibited no outward clinical symptoms although pathological changes were observed histologically. The viral antigen load was positively correlated with the pathology observed. Septal edema in lung tissue with mononuclear infiltrates; immune infiltrates in liver portal zones; and infected kidney glomeruli endothelium were observed. These data are consistent with the pathological changes that occur in *P. leucopus* infected with New York virus and humans infected with SNV. The deer mice spleens, which contain numerous immune cell types, had viral antigen present, which suggests that SNV may infect immune cells (Netski et al. 1999).

The reservoir hosts' humoral immunity is important for neonatal protection from hantaviral infection. Rodent dams confer protective antibodies to their young in utero and via mammary transfer. Maternal immunoglobins G (IgG) and A (IgA) give neonatal resistance to infection when challenged with hantaviral isolates. Cross fostering experiments have shown that both IgG and IgA antibody titres in neonates peak 2-3 weeks after fostering and when challenged with low virus titres (lethal titres) infants developed no clinical symptoms or had signs of viral infection as compared to nonimmune controls. Neonates challenged with higher virus titres (also lethal dose) exhibited one of two antibody titre changes. One-half of the group had decreasing daily

titres of IgG and IgA with transient IgM, followed by an increase of IgG and IgA. This group became persistently infected as evidenced by recovered virus from organs. The other half of this group had a decrease in IgG and IgA, no virus recovered, and demonstrated complete dissipation of antibodies. Neither group had clinical symptoms. In the group that was challenged with the lower virus titres, it is believed that complete protection was acquired by the dams' immunity since IgM was not detected (Dohmae et al. 1993, Gavrilovskaya et al. 1993, Dohmae and Nishimune 1995 and Dohmae and Nishimune 1998).

There are few data addressing specific IgM circulation in infected animals other than one report of Wistar rats challenged with a B1 strain of hantavirus had detectable IgM for over 6 months (Dohmae et al. 1993, Gavrilovskaya et al. 1993, Dohmae and Nishimune 1995 and Dohmae and Nishimune 1998).

Neonates born to and/or suckling from immune dams have no detectable antibody after they are 3 months old and thus are susceptible to infection or reinfection in natural settings or maintained with infected cagemates (Dohmae et al. 1993).

A humoral response may be insufficient to clear virus after systemic infection. T-lymphocytes are thought to protect against and assist in recovery from hantavirus infection in mice. T-cell deficient nude rats are more susceptible to Seoul virus than immunocompetant rats. Infant BALB/c mice inoculated with serial dilutions of immune spleen cells 24 hours after challenge with Hantaan virus were protected 30%-100%, which correlated directly with the dilution of the immune spleen cells. Additionally, IgG, IgM and neutralizing antibody titres appeared earlier and higher titres in immune spleen

cell protected mice compared to unprotected controls (Nakamura et al. 1985 and Kariwa et al. 1996).

1.7 Hantavirus and Tennessee

Between December 1994 and April 1995, the Centers for Disease Control and Prevention (CDC) conducted a hantavirus serosurvey of 39 natural, Central and Eastern U.S. National Park Service sites. The 1993 Four Corners hantavirus outbreak prompted Mills and others to determine the extent of hantavirus activity outside the Southwestern endemic area of the U.S. (Mills et al. 1998).

Of particular interest are the results from GSMNP. As mentioned earlier, *P. maniculatus* and *P. leucopus* are sympatric in many areas of the park. Although both species were captured and tested, 2 *P. maniculatus* out of 27 (or 7.6%) were antibody positive for a hantavirus that cross-reacted with Sin Nombre antigen (Mills et al. 1998).

The characteristics of seropositive deer mice in this study were consistent with previously and subsequently published data, that is hantavirus prevalence was higher among males than females and a trend of increasing seroprevalence with increasing weight class (Mills et al. 1998).

Genetic analysis of hantavirus positive *P. maniculatus* in Tennessee reveals a distinct lineage that has been associated with a 1995 HPS case fatality in Eastern North Carolina. Within this distinct lineage is the Sevier County, TN strain that differs from the Tuckasegee, NC strain by 7.9% at the nucleotide level. The Tennessee lineage is 12.2%, 14.4%, and 15.8% different from the New York, Monogahela, and Sin Nombre lineages respectively (Monroe et al. 1999). Although antibody positive deer mice have been

captured in Eastern Tennessee, there have been no documented human cases thus far (Mills et al. 1998).

Based on the results of the 1994-1995 CDC survey, we sampled 18 sites with varying levels of potential human-rodent contact that included the 3 sites sampled by the CDC. Between September 13, 2000 and December 1, 2000; November 28 and 29, 2001; May 27, 2002 through July 24, 2002; and May 1, 2004 through May 8, 2004 trapping was conducted in GSMNP. A total of 310 rodents and 35 insectivores (a total of 345 animals) were captured and sampled by 2 methods of RT-PCR. Blood samples were obtained from 305 animals and subsequently tested for anti-hantavirus antibodies with enzyme linked immunosorbent assays (ELISAs) and IFA.

1.8 Molecular Methods for the Detection of Hantaviruses

Serology.--The need for improved serologic and virologic diagnostic assays for clinical and epidemiological utility continues to motivate investigators to devise methods of disease detection that are rapid, efficient, sensitive and specific. The formation of the web-based sequence repository GenBank and its collaborating databases (National Institute of Health, Bethesda, MD); software designed for nucleotide and amino acid alignment and phylogenetic tree construction have provided investigators with resources to identify and classify new sequences. For our purposes, sequence identification and phylogeny allow us to predict the potential for human pathogenicity of newly identified hantaviruses and develop rational approaches to diagnostic assay genesis and refinement.

Serologic assays based on specific antibody detection are useful for clinical diagnosis and epidemiological investigations of hantaviruses. Sensitive and specific serodiagnostics in a clinical setting can result in earlier detection and appropriate medical

treatment of HCPS particularly due to the disappearance of viremia at the onset of the cardiopulmonary phase when many patients seek medical treatment (Feldmann et al. 1993, Terajima et al. 1999, Bharadwaj et al. 2000, Galeno et al. 2002 and Schmidt et al. 2005).

Patients elicit a strong humoral response against the N and G_1 proteins during the acute phase of HCPS, while animals have not demonstrated an antibody response against the G_1 protein. Moreover, the N protein is more highly conserved and elicits a stronger initial response than G_1 and thus has been the primary focus for serology (Feldmann et al. 1993, Hjelle et al. 1995 and Elgh et al. 1996 and 1997). Many assays such as immuno-fluorescence (IFA), hemagglutination inhibition assay (HIA) and plaque and focus-reduction neutralization assays (PRNT and FRNT, respectively) require the manipulation of laboratory-cultivated viruses (Hjelle et al. 1995, Elgh et al. 1996 and 1997 and Takakura et al. 2003). Hantaviruses are difficult to grow in cell culture, expensive, time-consuming and require a minimum of BSL-3 containment laboratories (Schmaljohn et al. 1995, Elgh et al. 1996 and Takakura et al. 2003). Where viral isolates are available, IFA has been a standard serologic assay to which most others are often compared due to the high sensitivity and specificity of the test (Niklasson and Lundkvist 1999). In addition to the requirement of whole viral isolates, IFA has other disadvantages. Detection of IgM by IFA is insensitive; numerous viral isolates remain unavailable at this time; IFA's are prone to observers' error and are not practical for screening large numbers of samples (Nikklason and Lundkvist 1999 and Takakura et al. 2003).

These limitations prompted a molecular approach to the refinement of hantavirus serology (Feldmann et al. 1993). The production of recombinant N proteins can be

accomplished comparatively quickly and in the absence of viral isolation (Feldmann et al. 1993 and Elgh et al. 1997). The expression and purification of full length N proteins appears to be variable between viral strains and often result in aggregates with functional losses of the protein, therefore it is often preferable to produce truncated N proteins that span the immunodominant regions of their respective viral strains (Elgh et al. 1996 and 1997 and Jonsson et al. 2001). Recently, some investigators have overcome this problem with the selection of novel expression vectors such as Semliki Forest virus amplicon in mammalian cells and *Saccharomyces cerevisiae* in lieu of the more commonly utilized insect cells or *Escherichia coli* (Billecocq et al. 2003, Razanskiene et al. 2004 and Schmidt et al. 2005). Sensitivities and specificities of either full length or truncated recombinant N proteins used in ELISA, Western blot or IFA format often meet or exceed those of assays utilizing whole viruses (Elgh et al. 1997, Billecocq et al. 2003, Razanskiene et al. 2004, Maes et al. 2004 and Schmidt et al. 2005). Other advantages to recombinant N protein assays, particularly ELISA's are the abilities to screen large sample sizes and to do so with heterologous antigen preparations. Several distinct hantavirus genotypes have been described that are circulating in geographical regions such as Europe, North and South America (Hjelle et al. 1995, Ahlm et al. 1997, Elgh et al. 1997, Gavrilovskaya et al. 1999, Hujakka et al. 2003 and Chu et al. 2003).

Other serologic assays based on either recombinant protein antigens and/or synthetic peptides have been developed. Recently, Hujakka et al. (2003) tested a rapid combination test that contained recombinant N antigens from Puumula, Dobrava-Belgrade and Hantaan virus. While preliminary studies of the assay demonstrated lower sensitivity and specificity as compared to single antigen tests, with refinement a

combination test can be useful in areas where multiple hantaviruses are endemic (Hujakka et al. 2003). The strip immunoblot assay (SIA) is similar in principle to Western blot and incorporates "slot blotted" recombinant proteins and synthetic peptides. The SIA appears to demonstrate high sensitivity and specificity and is suitable for field diagnosis (Hjelle et al. 1997, Hjelle et al. 1999 and Bharadwaj et al. 2000).

There are numerous impediments to recombinant protein development however. As mentioned earlier, the purification of recombinant proteins can be problematic; target proteins are difficult to remove from insoluble fractions and no protein purification processes can completely remove degradation products (Elgh et al. 1996 and 1997 and Jonsson et al. 2001). Moreover, impure protein preparations reduce the efficacy of the seroassay with non-specific reactions and affect antigenicity or immunogenicity due to decreased stability and degradation over time (Nikklason and Lundkvist 1999 and Razanskiene et al. 2004). Recently, some investigators have discovered that proteins dialyzed and subsequently lyophilized and stored at either –20°C or 4°C do not lose antigenicity for "a long time", however the time frame was not defined (Razanskiene et al. 2004).

Another alternative antigen format that has emerged that eliminates the potential for nonspecific reactions resulting from protein contamination. Synthetic peptides are free of degradation products that confer long-term stability and no detectable loss of antigenicity (Gonzalez et al. 1997, Greijer et al. 1999 and Li et al. 2002). Current serological techniques such as Western blot, SIA and ELISA for IgG and IgM reactivities are easily adapted to utilize synthetic peptide antigens and have been shown to produce

sensitivities and specificities concordant with commercial assays or "gold standard" assays (Gonzalez et al. 1997 and Greijer et al. 1999).

Real-Time Detection and Quantitative PCR.--The advent of other molecular tools such as RT-PCR have enabled investigators to detect and identify numerous hantavirus genomic sequences that were previously uncharacterized due to the difficulty of isolation in cell culture. RT-PCR assays have been particularly advantageous for epidemiological surveys for the ability to differentiate viral variants with multiple primer sets and phylogenetic comparisons.

Modifications of RT-PCR have emerged such as real-time detection-PCR (RTD-PCR) and quantitative PCR (qPCR) that are rapid, sensitive, specific and able to quantitate virus in samples which make these assays suitable for clinical applications as well (Abe et al. 1999, Garcia et al. 2001 and Drosten et al. 2002). The general principle of RTD-PCR is as follows: The target sequence is selected, preferably in a highly conserved region but unique to the viral genome of interest. Also, RTD-PCR performs better with nucleotide sequences less than 400 bp but optimally with sequences ranging from 50-150 bp. The fluorogenic hybridization probe is synthesized with a 5' reporter dye and a 3' quencher and as the RTD-PCR reaction progresses, the probe anneals to the target sequence between the forward and reverse oligonucleotides and the 5' exonuclease activity of *Taq* polymerase cleaves the probe between the reporter and quencher. Cleavage of the probe generates the reporter fluorescence, proportional to the accumulated amount of product. The reporter fluorescence increases as the target amplicon increases during PCR amplification (log phase) until the reaction plateaus (stationary phase). The real-time analysis is qualified in the mode of a threshold cycle

(C_T), which is the point at which the amplification plot crosses the threshold of sequence detection (Abe et al. 1999 and Garin et al. 2001). A standard curve may be generated to quantify viral copies by plotting the C_T values of known viral concentrations versus the log of viral titres (Garin et al. 2001).

RTD-PCR has afforded a spectrum of applications particularly in a clinical setting. Many viral hemorrhagic fevers (VHF's) that include Ebola, Marburg, Crimean-Congo hemorrhagic fever, Rift Valley fever, Dengue and Yellow fever viruses are endemic in tropical or sub-tropical regions. VHF's cause numerous clinical manifestations and are difficult to distinguish. RTD-PCR has made it possible to run parallel assays with sequence specific primers for rapid testing of numerous pathogens thus facilitating proper clinical treatment and epidemiological data (Drosten et al. 2002). RTD-PCR has also been evaluated for the detection of Hepatitis B virus and Rift Valley during the acute phase of illness and monitoring the efficacy of anti-viral drug therapies as well as serum viral titres in patients (Abe et al. 1999 and Garcia et al. 2001).

Hantavirus research has been incorporating the use of RTD-PCR for numerous applications. Epidemiological surveys can be conducted with greater efficacy due to the high levels of sensitivity and specificity that RTD-PCR can provide. The detection limits of these assays are often lower than those of RT-PCR/nested PCR assays and in naturally infected animals and patients (Garin et al. 2001 and Aitichou et al. 2005). Viral titres in patients and experimentally infected animals can be detected and quantified. Additionally, a recent preliminary study examined antibody-bound virus and unbound virus titres in HCPS patients with RTD-PCR and found a trend of increased severity of disease along with higher percentages of viral RNA in immune complexes. This was a

limited study but provides a basis for a larger-scale study and perhaps an HCPS pathogenesis model (Hutchinson et al. 1998 and Terajima et al. 2003). High levels of viremia are present in acute phase HCPS patients and can be detected prior to clinical symptoms or antibody response (Galeno et al. 2002). RTD-PCR can provide rapid, quantitative, sensitive and specific diagnosis of HCPS as compared to RT-PCR/nested PCR or virus isolation. RTD-PCR can be performed in a single reaction eliminating the need for an additional nested reaction that can introduce contamination leading to false positives (Abe et al. 1999, Garin et al. 2001 and Aitichou et al. 2005).

Phylogenetic Analysis.--Prior to molecular techniques, antigenically distinct viruses were identified with FRNT or PRNT which required live virus cell cultures (Xiao et al. 1994, Chu et al. 1994 and Li et al. 1995). Phylogenetic studies are capable of estimating the evolutionary relatedness of hantaviruses that allows investigators to identify genetically distinct hantaviruses and possible gene segment reassortment events (Li et al. 1995 and Baldauf 2003).

The very basic premise of nucleotide or amino acid phylogenetic tree construction is as follows: A phylogenetic tree has branches and the point at which two or more branches diverge is a node. The last common ancestor gives rise to an internal node while the derivative sequences used to construct the tree end in terminal nodes. The clade or monophyletic grouping is all the branches that emerge from an internal node and have diverged a unique common ancestor. A scale is displayed along with proportional branch lengths that correspond to the evolutionary rate of change or percent sequence difference between the nodes the branches emerge from. Thus, the longer the branches, the more sequence divergence there is. A dataset of related sequences is generated by

searching for and selecting sequences from nucleotide databases or placing unreposited sequences in the appropriate format (such as FASTA) and adding to the dataset. The multiple sequence alignments are constructed in a stepwise fashion, this is, the most similar (convergent) sequences are aligned first progressing to the more dissimilar (divergent) sequences. The alignment of multiple sequences must be refined to remove gap regions; correct for multiple substitutions for sequences with less than 95% homology and select a distance calculation (e.g. neighbor-joining) and statistical analysis (e.g. bootstrapping), all which can be done with an alignment program. Bootstrapping assigns a probability, expressed as a percentage, based on the frequency at which random sub samples of the dataset can be reproduced on "test trees". The consensus tree can then be viewed with a tree-viewing program with branch lengths to scale and bootstrap values displayed at the nodes (Baldauf 2003).

Phylogenetic analysis of hantavirus nucleotide and amino acid sequences have demonstrated the relationship of previously uncharacterized viruses to existing ones (Xiao et al. 1994). This analysis has also allowed investigators to visualize examples of geographic clustering of genetically distinct hantaviruses and identify potential reassortment events (Spiropoulou et al. 1994, Rowe et al. 1995, Li et al. 1995 and Rawlings et al. 1996).

In conclusion, the scope of our study includes the application and evaluation of a novel synthetic peptide-based ELISA on all 305 rodent and insectivore sera collected from GSMNP. We also analyzed all 345 tissue samples with nested PCR and RTD-PCR and selected positive samples were sequenced to identify, virologically, the most likely maintenance host(s) of the hantaviral strain circulating in GSMNP. Finally, we

phylogenetically analyzed partial S and M genome segments to determine the homology of our strain, Newfound Gap virus (NGV) to other New World hantaviruses.

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Part 2

THE DEVELOPMENT OF A SYNTHETIC-PEPTIDE BASED ELISA FOR SCREENING SERA FOR ANTIBODIES AGAINST NEWFOUND GAP VIRUS-A NEWLY IDENTIFIED HANTAVIRUS

This chapter is a revised version of a paper by the same name to be submitted to the Journal of Virology by Shawn Lewis, Stephen Kania, Dorcas O'Rourke, John New, Jr., Yong-Kyu Chu, Colleen Jonsson, Kathy Thomas and Michael McDonald.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) most of the collection of samples, (2) most of the preliminary serological processes preceding the development of a synthetic peptide format, (3) most of the synthetic peptide assay optimization, (4) most of the reference serology and its interpretation, (5) collection and organization of all statistics, (6) acquisition of all the relevant literature and its interpretation and (7) composition of the data into a single paper and most of the editing.

1. INTRODUCTION

Hantaviruses are classified within the family *Bunyaviridae* along with *Phlebovirus, Nairovirus* and *Uukuvirus* (Martin et al. 1985 and Fauquet 2000). New world hantaviruses are the etiologic agents causing hantavirus pulmonary syndrome (HPS) which has a high mortality rate (Hjelle et al. 1995 and 1997). The disease rapidly progresses from febrile illness to noncardiogenic pulmonary edema. The hallmark lesion associated with HPS is vascular endothelial cell damage, and death is caused by respiratory insufficiency and/or hemodynamic collapse in about 50% of cases (Hjelle et al. 1994 and Weigler 1995).

Each hantaviral strain is associated with a specific rodent/insectivore host and geographical region (Monroe et al. 1999). Reservoir hosts are asymptomatically, persistently infected and shed virus particles intermittently in urine, saliva and feces. Thus, the primary transmission route for hantaviruses from infected small mammals to

humans is inhalation of aerosolized excreta (Gavrilovskaya et al. 1999 and Hjelle and Glass 2000).

Hantaviruses are negative-sense RNA viruses that possess an envelope and a single stranded tripartite genome. This tripartite genome consists of large (L), medium (M) and small (S) segments that correspond to single large open reading frames (Hjelle et al. 1995, Yamada et al. 1995).

The L segment encodes the viral transcriptase. The M segment encodes the G_1 and G_2 surface glycoproteins and is transcribed as a single mRNA. Wild and laboratory-infected rodents produce neutralizing antibodies to the G_1 and G_2 proteins, possibly protecting them from infection. The G_1 and G_2 glycoproteins may participate in host cell surface receptor(s) recognition. The antigenic differences that occur among hantaviruses are most likely due to the less conserved immunodominant region near the amino terminus of the G_1 protein, corresponding to amino acids 59-89. Thus, antibody responses to G_1 proteins are type specific (Jenison et al. 1994, Feuer et al. 1999, Gavrilovskaya et al. 1999, and Kaukinen et al. 2001).

The N protein, translated from the S segment encapsulates the viral genome by wrapping genomic RNA segments. Recombinant N proteins are more highly conserved and cross-reactivity occurs between IgG and IgM antibodies produced against this protein. To date, there are 23 identified different hantaviral types and several more quasispecies that have a specific reservoir host association (Jenison et al. 1994, Feuer et al. 1999, and Kaukinen et al. 2001).

1.1 Hantaviruses in North America

Hantavirus pulmonary syndrome (HPS) in the United States was first recognized in June of 1993 following the deaths of previously healthy, adult Navajo Americans. By November 1993, there were 42 confirmed HPS cases with a 62% mortality rate in the Four Corners area of the United States (Arizona, Colorado, New Mexico, and Utah). The etiologic agent responsible for these HPS cases is referred to as Four Corners or Sin Nombre virus (SNV) and is the prototype etiologic agent of HPS (Weigler 1995, Hjelle et al. 1997 and Kitsutani et al. 1999). The primary rodent reservoir of SNV is *Peromyscus maniculatus* (deer mouse) although other North American hantaviruses, representing unique viral strains, have been found in association with other rodent hosts (Hjelle et al. 1997). As of July 2005, 396 HPS cases in 30 states, with a 36% mortality rate have been confirmed since reporting began in 1993 (CDC 2005).

1.2 CDC Survey of the Great Smoky Mountains National Park (GSMNP)

Between December 1994 and April 1995, the Centers for Disease Control and Prevention (CDC) conducted a hantavirus serosurvey of 39 U.S. National Park Service sites located in the central and eastern United States. Two *P. maniculatus* out of 27 (7.6%) tested in GSMNP were antibody positive for a hantavirus that cross-reacted with SNV nucleocapsid antigen (Mills et al. 1998).

Genetic analysis of 139 base pair (bp) fragments of the G_2 region isolated from *P. maniculatus* in Tennessee reveals a distinct lineage that has been associated with a 1995 HPS case fatality in western North Carolina. Within this distinct lineage is the Sevier County, TN strain that differs from the Tuskasegee, NC strain by 7.9% at the nucleotide level. The Tennessee lineage is 12.2%, 14.4% and 15.8% divergent from the New York,

Monongahela and Sin Nombre lineages respectively. (Monroe et al. 1999). Although antibody positive deer mice have been captured in eastern Tennessee, there have been no documented human cases thus far (Mills et al. 1998).

1.3 Nucleocapsid and Glycoprotein Epitopes

Epitope mapping studies of full length and partial Four Corners virus (FCV) nucleocapsid proteins have determined the reactivities to the linear epitope(s) in the deer mouse and humans (Jenison et al. 1994 and Yamada et al. 1995). In both human and deer mouse studies, the dominant epitope lies between amino acids 17 and 59 (inclusive) which corresponds to:

QLVTARQKLKDAERAVELDPDDVNKSTLQSRRAAVSALETKLG (Jenison et al. 1994 and Yamada et al. 1995). However, the inclusion of the first 16 amino acids, MSTLKEVQDNITLHEQ, increased antibody reactivity in deer mice (Yamada et al. 1995). The nucleocapsid region is highly conserved and thus, cross-reactivity with other closely related hantavirus antibodies to N proteins is not type specific (Jenison et al. 1994 and Yamada et al. 1995).

Conversely, the G_1 antibody-reactive epitope is type specific and heterologous proteins do not elicit cross-reactivity. Furthermore, hantavirus infected humans, but not rodents will develop both IgG and IgM responses against the G_1 glycoprotein (Jenison et al. 1994, Hjelle et al. 1995 and Yamada et al. 1995). Epitope mapping studies of the G_1 glycoprotein have identified a linear immunodominant region spanning amino acids 58-88 and 59-89, although it is unclear if this is the only immunogenic region of G_1 (Jenison et al. 1994 and Hjelle et al. 1995). Wang et al. (1993) have reported a region between nucleotides 489 and 661 consisting of 57 residues that is recognized by non-neutralizing

G_1 Monoclonal antibodies (MAbs). The G_2 glycoprotein appears to elicit a negligible, initial antibody response in humans but appears to generate a strong antibody response during the convalescent phase of infection and thus, human studies have focused on G_1 and N antibody responses (Jenison et al. 1994).

1.4 Current Serologic Tests

Enzyme-linked immunosorbent assays (ELISAs) can be highly sensitive and specific for detection of either IgG or IgM antibodies. IgM antibodies are also extremely useful for determining acute phase infections that are particularly important for human diagnostics (Niklasson and Lundkvist 1999).

The indirect immunofluorescent antibody (IFA) test is routinely used for determination of infection with a hantavirus (Takakura et al. 2003). However, IFAs have disadvantages such as the poor growth of hantavirus in cell culture, the requirement of a biosafety level 3 containment facility for antigen preparation, misinterpretation of positives and negatives by the reader, high cross-reactivity with geographically diverse hantaviruses, and is unsuitable for large sample sizes (Niklasson and Lundkvist 1999, Billecocq et al. 2003 and Takakura et al. 2003).

These limitations led to the development of recombinant antigen ELISAs. Recombinant antigens may be synthesized from relatively short S segments of the N-open reading frame; nucleocapsid proteins are used as antigenic targets for anti-hantavirus antibodies due to its high immunogenicity (Feldmann et al. 1993 and Padula et al. 2000). Recombinant proteins can be propagated in higher concentrations and with greater purity than native proteins and at less cost (Padula et al. 2000 and Takakura et al. 2003). Direct IgG and capture IgM ELISAs that employ the use of recombinant N

antigens have demonstrated higher sensitivities than IFA. rN IgG ELISAs have higher sensitivities than native antigen tests, hemagglutination inhibition tests, and complement fixation tests due to non-denaturing recovery methods, higher purity and greater yield (Feldmann et al. 1993, Niklasson and Lundkvist 1999, Padula et al. 2000, Billecocq et al. 2003, and Takakura et al. 2003). Both homologous and heterologous antigens may be used. While the former is more sensitive and specific than the latter, heterologous antigens can be useful for screening samples from diverse geographical areas (Feldmann et al. 1993 and Yamada et al. 1995). Large numbers of samples can be screened quickly and efficiently with recombinant protein ELISAs as compared to other serodiagnostic tests (Takakura et al. 2003).

ELISAs can be easily adapted for multiple species use with the wide range of commercially available conjugated secondary antibodies. For example, horseradish peroxidase (HRP) conjugated anti-rat, anti-*Peromyscus leucopus*, and anti-mouse was recommended for use by Lee et al. (2003) when testing samples from many sigmodontine and murine rodents. Anti-mouse IgG conjugate is used in serodiagnostics for arvicoline rodents (voles), *Heteromyidae* (kangaroo rats and pocket mice), and *Sciuridae* (squirrels). Protein A *Staphylococcus aureus* and group G streptococci cell wall components, were recently found to have better binding affinity to *Critinae* (hampsters and Gambian rats) immunoglobulins than anti-hampster IgG conjugate (Ahlm et al. 1997, Bennett et al. 1999 and Lee et al. 2003).

1.5 Synthetic Peptide ELISAs

Despite the advantages of recombinant protein ELISAs propagation and purification of recombinant proteins is costly and time-consuming. Longer functional N protein can

be lost due to conformational changes from denaturing conditions during the purification step (Elgh et al. 1996 and Jonsson et al. 2001). Cross-reactivity can occur as a result of non-specific interactions between antibodies and cellular proteins remaining in recombinant protein preparations (Gonzalez et al.1997 and Li et al. 2002).

These general limitations, in addition to virus-specific limitations have led to the use of synthetic peptides as analogues for recombinant protein based serodiagnostic tests (Gonzalez et al. 1997, Hjelle et al. 1997, Greijer et al. 1999 and Li et al. 2002). A hantavirus strip immunoblot assay (SIA) that incorporates a synthetic SNV N peptide spanning the immunodominant region of aa residues 17-59 has been field-tested and demonstrates complimentarity to the SNV N recombinant protein SIA (Hjelle et al. 1997 and Chu et al. 2003).

A synthetic peptide based ELISA that incorporates 2 synthetic peptides corresponding to the highly conserved envelope regions of human immunodeficiency virus (HIV)-1 and HIV-2 has demonstrated 100% sensitivity and 99.72% specificity as compared to commercially available, whole viral lysate ELISAs, recombinant protein ELISAs, and western blotting kits (Gonzalez et al. 1997). Another successful application of a synthetic peptide based ELISA has been with human cytomegalovirus (HCMV), a herpesvirus. The "gold standard" virion antigen-based ELISA was problematic because it consists of complex viral lysates with exact compositions that were difficult to standardize. Additionally, propagation of lysate cultures consists of HCMV in fibroblasts that can result in cellular protein contamination. Greijer et al. (1999) developed an ELISA with a heterologous preparation of defined, synthetic, immunodominant peptides

with sensitivities of 98.9% and 96.4% for IgG and IgM assays, respectively as compared to the virion antigen-based ELISA (Greijer et al. 1999).

Most recently, a synthetic peptide ELISA was designed for application in the serodiagnosis of anti-Hantaan virus (HV) antibodies. Computer-assisted epitope prediction software was used to identify the major antigenic epitope spanning aa residues 17-66, the 50-mer was subsequently synthesized and purified. The resulting synthetic peptide ELISA was used to screen patients' sera with hemorrhagic fever with renal syndrome (HFRS), non-HFRS patients and healthy individuals. Both IgM and IgG responses of all 3 groups indicated appropriate reactivities to the synthetic N peptide antigen but the sensitivity and specificity were indeterminate (Li et al. 2002).

1.6 UTCVM Synthetic Peptide ELISA

The derivation of a synthetic peptide antigen that will provide the most sensitive and specific, rapid diagnostic assay is required for accurate seroprevalence estimations. The application of an ELISA incorporating regionally derived antigen would be particularly useful for trap-mark-recapture surveys in detecting temporal and spatial fluctuations of seropositive rodents. Profound weather events would impact rodent populations that would, in turn, impact hantavirus prevalence. Such information would allow us to identify high-risk scenarios.

Synthetic nucleocapsid protein, elucidated from the deduced amino acid sequence of mRNA extracted from regionally collected tissues will provide the strongest serological reactivity and provide increased sensitivity and specificity in subsequent ELISAs. The immunodominant region of the N recombinant protein is contained within our construct; will be recognized by anti-N protein antibodies from *Peromyscus spp.*, other rodents,

insectivores, and humans. Conservation of this protein will allow cross-reactivity with antibodies against the most pathogenic hantavirus strains in North America.

The small (S) segment of the hantavirus genome encodes the nucleocapsid protein which functions in the viral anti-sense genomic RNA assemblage and encapsidation (Yamada et al. 1995 and Jonsson et al. 2001). Hantavirus nucleocapsid antigen elicits a strong humoral response in both acutely laboratory-infected and wild caught *P. maniculatus* mice (Yamada et al. 1995 and Elgh et al. 1996). The goal of this project is to produce antigen that will be used for the serologic detection of antibodies against hantavirus produced by rodents and insectivores captured in this geographic region. A rapid and sensitive assay that will detect anti-hantavirus antibodies is essential for estimating seroprevalence in a given area. GSMNP is the most visited park in the country and we wish to be able to provide park staff with an accurate assessment of the potential for human contact with potentially hantavirus infected rodents. Moreover, a synthetic peptide-based ELISA can provide area clinicians with a rapid, sensitive and specific method for the diagnosis of a pathogenic hantavirus.

2. MATERIALS AND METHODS

2.1 Trapping and Processing

Based on the results of the 1994-1995 CDC survey, we sampled the same sites in addition to several more with varying levels of potential human-rodent contact. Between September 13, 2000 and December 1, 2000; November 28 and 29, 2001; May 27, 2002 through July 24, 2002; and May 1, 2004 through May 8, 2004 trapping was conducted in GSMNP. A total of 310 rodents and 35 insectivores (a total of 345 animals) were

captured. Blood samples were obtained from 305 animals and subsequently tested for anti-hantavirus antibodies with enzyme linked immunosorbent assays (ELISAs) and IFA.

In addition to blood collection, spleen, liver, lung and kidneys were collected from every animal. Embryos were collected from 15 females and tail tips from animals trapped during the 2002 survey. After euthanasia and sample collection, the animals were tagged with identification numbers and placed in 10% formalin. For a complete description of trapping methods and sample collection, please refer to "Seroprevalence of Hantavirus in the Great Smoky Mountains National Park", Materials and Methods section (Lewis 2002).

2.2 Nucleocapsid Epitope Region PCR

mRNA was extracted from spleen tissue obtained from a serologically positive *P. maniculatus*. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using primers for the G_2 region of the M gene and genetic sequencing was performed on this sample. Nucleotide comparisons of this 201 bp fragment of the G_2 region have revealed a unique viral strain that is closely related to both Monongahela and New York viruses. These strains have been associated with 3 HPS case fatalities (1 in North Carolina and 2 New York). This strain has been designated "Newfound Gap virus" (NGV) and registered in GenBank (Accession #AF406788). Preliminary PCR and sequencing analysis of the G_1 region of the M gene has yielded similar results i.e. a unique strain with the closest homology to New York virus based on a 296 bp fragment (Genbank accession #AY396717).

RT-PCR and nested PCR performed on the G_1 and G_2 regions of the M gene amplified gene fragments of 296 and 201 base pairs, respectively. Sequencing of these

amplicons was performed at the University of Tennessee's Core Sequencing facility. Nucleotide sequence comparison using BLAST software indicates that these envelope glycoproteins amplified from tissues collected in GSMNP represent a novel genetic variation of the glycoproteins in SNV. The G_1 and G_2 glycoproteins have the closest homology to New York hantavirus, 80 and 85% respectively. Based on these data, oligonucleotide primers were designed to align with the S segment cDNAs from RI-R virus (GenBank accession # UO9488).

The nucleotide sequences are as follows: outer position 1,5' TAGTAGTAGACTTCGT(AG)AA(GA)AGCTAC; outer position 980,5' CGATCTGGAGCACA(TC)GCAAA(GT)ACCCA; inner position 20,5' AAGCTACTACGACTAAAGCT; inner position 476,5' CCTCTAGTTGACAACATAT. Primers were reconstituted with RNAse/DNAse free H_2O for a final concentration of 5.00×10^{-5}M.

2.3 mRNA Extraction

mRNA was extracted from kidney tissue based on a previously positive serological assay (sample 43) and using TRIzol® extraction (Gibco Co., Invitrogen Corp., Carlsbad, CA). Ten mg of kidney tissue was excised and placed in a petrie dish with 1000µL of TRIzol® Reagent. The tissue was cut into smaller pieces and homogenized by drawing through an 18 gauge needle and 3 mL syringe. After several passages, the homogenized tissue was placed in an Eppendorf 2.0 mL microcentifuge tube (Eppendorf AG, Hamburg, Germany). Phase separation was achieved by first incubating homogenized sample at 25° C for 5 minutes, then by adding 200µL chloroform; shaken vigorously for 15 seconds; incubated at 25°C for 3 minutes and

centrifuged at 4°C, 12,000 x g for 15 minutes. To precipitate RNA, the aqueous phase was transferred to a fresh Eppendorf 2.0 mL microcentrifuge tube and 500µL of isopropyl alcohol was added. The sample was incubated at 25°C for 10 minutes and centrifuged at 4°C, 12K x g for 10 minutes, the RNA precipitate could be visualized as a small pellet at the bottom of the tube. The RNA was washed by removing the supernatant, adding 1000µL of 75% ethanol, vortexing and then centrifuging at 4°C, 7,500 x g for 5 minutes. The supernatant was drawn off and the pellet was air dried for 10 minutes at 25°C. The RNA was redissolved in 50 µL of DEPC treated water and incubated at 55°C for 10 minutes.

2.4 RT-PCR/PCR Profiles

RT-PCR was performed using Superscript One-Step RT-PCR with Platinum Taq® (Invitrogen Corp., Carlsbad, CA). The 25 µL RT-PCR reaction mixture consisted of 12.5 µL 2x reaction mix, 40 units RNAse Out (Invitrogen Corp., Carlsbad, CA), 1.25×10^{-9} M (final concentration) forward outer primer, 1.25×10^{-9} M (final concentration) reverse outer primer, 1.0 unit RT taq polymerase, 1.25×10^{-11} g/L (final concentration) RNA and 4.25 µL DEPC treated water. A 30-minute RT step at 50°C was followed by a 94°C pre-denaturation step for 2 minutes, 30 cycles of denaturing (94°C for 1 minute), annealing (50°C for 30 seconds) and extension (72°C for 1 minute). There was a final extension for 10 minutes at 72°C and samples were held at 4°C. Five µL of the RT-PCR reaction mixture was used as a template for the 25 µL nested reaction which also included 12.5 µL PCR Master Mix with Taq polymerase™ (Promega Corp., Madison, WI), 1.25×10^{-9} M each of the forward and reverse nested primers, and 6.5 µL nuclease-free H_2O. The

thermal profile for the nested reaction was the same for the first round PCR except there was a 5-minute final polymerization step.

Ten μL of the nested PCR product plus 2 μL of gel loading solution and a 1 Kb DNA ladder was loaded onto a 1.4% agarose gel with 10x TBE, 3 μL ethidium bromide, and electrophoresed for approximately 40 minutes at 100 volts. The gel was viewed on a transilluminator and a band was visible at ~980 bp, which was consistent with the band size that should be produced by the outer primer pairs (Figure 1).

Three μL of template was used for a 100 μL 3rd round nested PCR reaction with the same reagents and thermal profile described for the nested round. Seventeen and one-half microliters of the 2nd round PCR reaction mixture, that yielded the ~980 bp amplicon earlier, plus 3.5 μL gel loading solution were loaded into 3 wells and 15 μL of the 3rd round nested PCR reaction mixture plus 3 μL of gel loading solution were loaded into 6 wells of a 1.4% agarose gel, electrophoresed, and transilluminated as described earlier. Bands corresponding to the expected size of 457 bp were visualized (Figure 2) and bands corresponding to the expected size of ~980 bp were visualized (Figure 3).

2.5 Gel Purification

The bands were excised from the gel placed in 2.0 mL Eppendorf microcentrifuge tubes. Using QIAGEN QIAquick gel extraction kit® (Invitrogen Corp., Carlsbad, CA), amplicons were extracted from the gels in accordance with the manufacturer's instructions.

2.6 Ligation into Cloning Vector

Purified DNA products were ligated into the linearized PCR 2.1 Cloning Vector® (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol with the

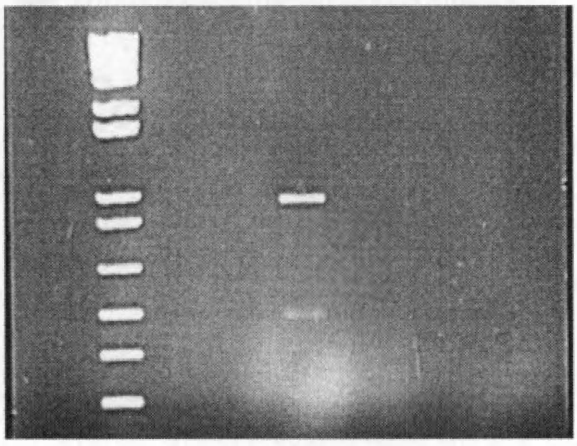

Figure 1. 980 bp amplicon produced from outer primer sets amplified by RT-PCR and PCR visualized on an agarose gel (1.4%) containing ethidium bromide. A 1 Kb ladder was used as a standard.

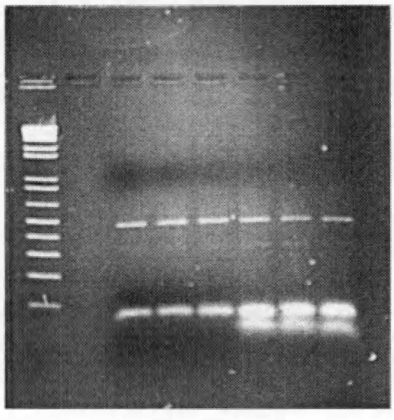

Figure 2. 457 bp amplicon produced with nested primer sets after RT-PCR and 2 rounds PCR visualized on an agarose gel (1.4%) with ethidium bromide. A 1 Kb ladder was used as a standard.

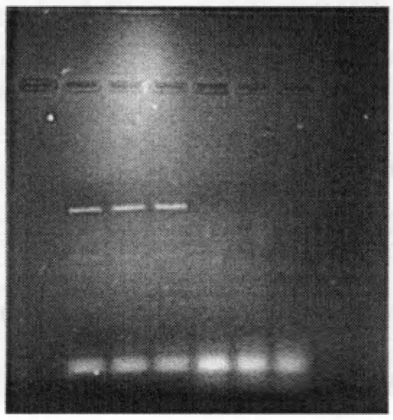

Figure 3. 980 bp amplicon produced with outer primer sets as in Figure 1. Excised for gel purification. A 1 Kb ladder was used as a standard.

following modifications: 4 µL sterile H_2O and 2 µl of 100ng of fresh PCR product were added.

The ligation reactions were incubated overnight (~14 hours) in 0.65 mL microcentrifuge tubes in a 14°C water bath. The ligation reaction containing the 980 bp fragment was placed in a –20°C freezer for future transformation and the ligation reaction containing 457 bp fragment was placed on ice for immediate transformation.

2.7 Transformation

Transformation was performed using One Shot INVαF' Competent Cells® (Invitrogen Corp., Carlsbad CA) in accordance with the manufacturer's protocol.

2.8 Analysis

Ten and fifty microliter amounts of the transformation reaction were spread onto Luria Bertani agar containing 50µg/ml ampicillin (LB-AMP) for selection of bacteria containing plasmids. The plates were incubated at 37°C for 2 hours, inverted and incubated overnight at 37°C. Colonies were subcultured on LB-AMP agar and

MacConkey agar plates. MacConkey agar is a selective medium that inhibits the growth of gram-positive microorganisms. White colonies are formed on MacConkey agar if they fail to produce β-galactosidase due to disruption of the *LACZα* fragment. Twenty singular colonies were numbered and selected from the previously incubated LB-AMP plates and streaked onto the corresponding numbered grid of the LB-AMP plate and then MacConkey plate. The plates were inverted and placed in a 37°C incubator overnight.

2.9 PCR Screen

Six 0.65 mL microcentrifuge tubes were filled with 10 µL of RNAse free H_2O. Six colonies (that were white on the MacConkey agar) were streaked from the corresponding numbered LB-AMP agar plate and placed in a microcentrifuge tube. The tubes were boiled at 100°C for 10 minutes to lyse cells and release genomic material. The tubes were then centrifuged for 1 minute at 14K x g to pellet cellular debris (plasmid DNA is contained in the supernatant).

A PCR master mix containing 100 µL Promega PCR Master Mix (Promega Corp., Madison, WI), 5.36 x 10^{-6} M each of forward and reverse nested primer, and 10µL nuclease free H_2O was prepared. Seven µL of the PCR master mix and 3 µL of each template were added to a thin-walled tube and placed in the thermocycler (protocol as described previously). A 1.4% agarose gel was prepared; samples were loaded, and electrophoresed (as described previously). Four of the colonies contained the 457 bp PCR product.

2.10 Sub-Culture/Mini-Prep and Sequencing

Colonies that were positive from PCR screening were sub-cultured into 10 mL LB-AMP broth containing 1 mg ampicillin. Caps were placed loosely on the conical tubes and taped, tubes were placed in a 37°C shaking incubator at 225-RPM overnight.

Using a Wizard plus SV mini-prep DNA purification system (Promega Corp., Madison, WI), samples were prepared for sequencing as follows: Five mL each of the bacterial cultures were harvested by centrifugation for 5 minutes at 10,000 x g. The supernatant was poured off and pellets of bacteria remained in the microcentrifuge tubes. Two hundred and fifty µL of cell resuspension solution was added to each tube and vortexed to completely resuspend the pellets. Two hundred and fifty µL of cell lysis solution was added and tubes were inverted 4 times. The lysate was allowed to clear for 4 minutes and 10µL alkaline protease solution was added. The tubes were inverted 4 times and incubated for 5 minutes at room temperature. Three hundred and fifty µL of neutralization solution was added to the tubes and inverted 4 times. The tubes were incubated for 10 minutes at room temperature. Spin columns were inserted into 2 mL collection tubes and cleared lysate samples were added to spin columns without disturbing the white precipitate. The spin columns were centrifugated at 14,000 x g for 1 minute at room temperature. Contents of the collection tubes were discarded and 750 µL of column wash solution (previously diluted with 35 mL of 95% ethanol) was added to each tube. The spin columns were centrifuged at 14,000 x g for 1 minute at room temperature. Contents of the collection tube were discarded and this wash step was repeated with 250 µL of column wash solution and a 2-minute centrifugation. The spin column was transferred to a new 2.0 mL microcentrifuge tube, the plasmid DNA was

eluted with 100 µL of RNAse/DNAse free H_2O, and centrifuged for 1 minute at 14,000 x g at room temperature.

Two of the samples were stored in a –20°C freezer and 2 samples were submitted for sequencing at the University of Tennessee's Core Sequencing facility. M13 forward and reverse primers were used for the sequencing reactions to identify sites flanking the inserted PCR product (Figure 4). Basic Local Alignment Search Tool (BLAST) queries demonstrated 92 and 97% alignments to SNV at the nucleotide level and Monongahela virus at the amino acid level, respectively.

2.11 Synthetic Peptide ELISA

The deduced amino acid sequence of the 59 residue epitope region from the N-protein was:

MSTLKEVQDNITLHEQQLVTARQKLKDAERAVEVDPDDVNKSTLQSRRAAVSA LETKLG (aa 1-59). 10 mg of the 6576D 59-mer was synthesized by BIOPEPTIDE Co., LLC (San Diego, CA) and purified to 95% by high performance liquid chromatography (HPLC) with a phenomenex proteo C18 column (Beckman Gold, Beckman Coulter, Inc., Fullerton, CA). The synthetic peptide was diluted to 10mg/mL with Gibco RNAse/DNAse free H_2O (Invitrogen Corp., Carlsbad, CA).

The optimal dilution for the application of the synthetic peptide as an ELISA antigen was determined to be 2.5µg/µL in 0.2M carbonate bicarbonate (pH 9.4) (Pierce, Rockford, IL). 100 µL of the diluted antigen was adsorbed into each well of Immulon 2HB flat-bottom 96 well ELISA microplates (Thermo Electron Corp., Milford, MA) and kept at 4°C until use (1-14 days). All volumes were 100 µL unless otherwise noted.

1 AAGCTACTAC GACTAAAGCT GGA**ATGAGCA CCCTCAAAGA AGTGCAGGAC AACATCACAC**
60 TACACGAACA ACAGCTCGTG ACTGCCAGGC AGAAGCTTAA AGATGCAGAA AGAGCAGTGG
120 AGGTGGACCC CGATGATGTT AACAAGAGCA CATTACAGAG CAGACGGGCA GCTGTGTCTG
180 CATTGGAGAC CAAACTCGGA GAACTCAAGC GGCAGTTGGC TGATCTTATT GCAGCTCAGA
240 AGTTGGCTTC AAAACCTGTT GATCCAACAG GGATTGAACC TGATGACCAT CTAAAGGAGA
300 AATCATCGTT AAGATATGGT AATGTCCTTG ATGTGAATTC AATTGACTTG GAAGAACCAA
360 GTGGGCAAAC AGCAGACTGG AAGTCAATCG GCCTCTACAT CTTAAGTTTT GCAATACCGA
420 TCATCTTAAA AGCACTGTAT ATGTTGTCAA CTAGAGG 457

Figure 4. 457 nt sequence of the Nucleocapsid region of the S gene. The bolded portion encodes the linear epitope (333 bp)(Genbank Accession #AY396718).

The plates were washed 3 times (300 µL dispensed) with PBS-0.05% Tween-20 (PBST) (Tween-20, Fisher Bio Tech, Fairlawn, NJ) using a mechanical plate washer (BIO-TEK Inst., Inc., Winooski, VT). Any free binding sites were saturated by incubation with 2% fetal bovine serum (FBS) (Sigma Chem. Corp., St. Louis, MO) in PBST for 30 minutes at 37°C. Plates were washed as previously described.

Conjugate Optimization.--To determine the optimal conjugate for multiple species screened and working concentration we selected four secondary antibodies, along with protein A/G, all of which were horseradish peroxidase (HRP) conjugates. Goat anti-mouse (γ) IgG (Kirkegaard and Perry, Gaithersburg, MD), goat anti-*P. leucopus* (h+l) IgG (Kirkegaard and Perry, Gaithersburg, MD), goat anti-cat (h+l) IgG (Kirkegaard and Perry, Gaithersburg, MD), chicken anti-mouse (h+l) IgG (Bethyl Labs, Inc., Montgomery, TX) and protein A/G conjugate (Pierce Rockford, IL) were selected for this study.

An ELISA format was used to evaluate the binding of secondary antibodies and protein A/G to Immunoglobulins derived from 7 species that included: *Peromyscus*

maniculatus, Peromyscus leucopus, Mus musculus, Sigmodon hispidus, Clethrionomys gapperi, Microtus ochrogaster and *Blarina brevicauda*. All volumes added to microtitre plate wells were 100µl; all dilutions were performed with Gibco PBS-0.05% and all washes were performed three times with PBS-0.05% Tween-20 (PBST). PBS and PBST were used as controls for all plates.

Ninety-six well Immulon 2HB flat-bottom microtitre plates (Thermo Electron Corp., Milford, MA) were adsorbed with the following: Rabbit anti-mouse (h+l) IgG unconjugated capture antibody (Pierce Rockford, IL) $1:10^3$-$1:10^9$ 10-fold dilutions; Goat anti-mouse (h+l) IgG unconjugated capture antibody (Bethyl Labs, Inc., Montgomery, TX) $1:10^3$-$1:10^9$ 10-fold dilutions; species sera-$1:10^3$-$1:10^9$ 10-fold dilutions and species sera-1:1000. Plates were wrapped in plastic and stored overnight at 4°C. Plates were washed with PBST. Species sera diluted 10-fold, $1:10^3$-$1:10^9$ were added to the goat anti-mouse and rabbit anti-mouse adsorbed plates in duplicate along with controls.

Plates were incubated at room temperature for 1.5 hours then washed with PBST. Goat anti-mouse (γ) IgG HRP diluted to 1:400 was added to all wells and incubated at room temperature for 1 hour then washed with PBST. ABTS was added to wells and incubated at room temperature for 30-40 minutes.

The 10-fold serially diluted species sera adsorbed plates were washed with PBST. Protein A/G HRP 1:5000 and goat anti-*P. leucopus* (h+l) IgG HRP 1:400 were added to wells along with controls. Goat anti-*P. leucopus* (h+l) IgG HRP diluted 2-fold 1:100-1:400 and goat anti-cat (h+l) IgG HRP diluted 1:300, 1:600 and 1:900 were added to plates in a checkerboard fashion along with controls. The plates were incubated at room

temperature for 1 hour then washed with PBST. ABTS was added to wells and incubated at room temperature for 30-40 minutes.

The plate containing species sera diluted 1:1000 was washed with PBST. Chicken anti-mouse (h+l) IgG HRP was diluted 2-fold from 1:500-1:24,000 and added to all wells and incubated at room temperature for 1 hour then washed with PBST. ABTS was added to wells and incubated at room temperature for 30-40 minutes.

The optimal dilutions of both sera and IgG conjugate were determined by checkerboard dilutions and found to be 1:400 and 1:500 respectively. Pooled normal mouse sera from *Mus musculus* served as the negative control and sera from an anti-hantavirus IgG antibody and virus positive deer mouse served as a positive control. Sample sera, negative, and positive controls were diluted 1:400 with 2% FBS in PBST; 2% FBS served as a blank. The sample sera, controls, and blank were plated in quadruplicate and incubated for 2 hours at 37°C.

Checkerboard dilutions with numerous conjugates demonstrated that only chicken anti-mouse IgG heavy and light chains (h+l) reacted with both rodent and insectivore spp. Immunoglobulin. Washed plates were incubated with HRP conjugated chicken anti-mouse IgG (h+l) (Bethyl Labs, Inc., Montgomery, TX) diluted to 1:500 with 2% FBS in PBST for 1 hour at 37°C. The plates were washed 3x as previously described and developed with 2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS) in the dark at room temperature until the raw positive control OD was between 1.9 and 2.3. Plates were read at 405 nm with KC Jr. Software and an ELX 800 universal microplate reader (BIO-TEK Inst., Inc., Winooski, VT).

Statistical Analysis.-- SPSS software (Version 13.0, Chicago, IL) was used to generate the cut-off values for positive samples. First, samples and controls were blanked by subtracting the blank wells from the sample and control wells. Then, the means and standard deviations of the negative controls of each plate were calculated. The negative control mean was then subtracted from each of the sample OD values to give a sum adjusted OD for each quadruplicate. A mean and standard deviation were calculated for each sample's sum adjusted OD value. A sample was considered positive if the mean of the sum adjusted OD was greater than 4 standard deviations above the corresponding negative control mean.

2.12 Recombinant Protein ELISA

A complete description of the recombinant N (rN) antigen ELISA is referenced in Feldmann et al. (1993) and "Seroprevalence of Hantavirus in the Great Smoky Mountains National Park", Materials and Methods section (Lewis, 2002). Samples from the 2002 collection were not included in this assay, however the 2004 collection samples were. We could not acquire a sufficient amount of antigen to assay all of our samples.

2.13 IFA

Infection of Cells.--CC107-SNV infected cells were propagated as described elsewhere and performed in a Biosafety Level 3 laboratory at the Southern Research Institute, Birmingham, Alabama (Schmaljohn et al. 1983). Briefly, CC107-SNV were propagated in Vero E6 cell cultures with 10% FBS diluted in DMEM and 100U/mL penicillin and 100µg/mL streptomycin as growth medium. The growth media with virus-infected cells was incubated for a minimum of 7 days at 37°C.

Slide Preparation.--Complete DMEM was prepared with 10% FBS diluted in DMEM with 1% penicillin/streptomycin. Infected cells were trypsinized with Gibco 0.25% Trypsin-EDTA (Invitrogen Corp., Carlsbad, CA). The cell media was discarded leaving the cells in the flask, the remaining cells were washed with ~1/2 of the trypsin. The trypsin wash was discarded and repeated with the 2nd 1/2 of the trypsin with ~1 mL remaining in the flask. This was incubated for 5-10 minutes, swirling occasionally to release attached cells. Approximately 9mL of complete DMEM was added and pipetted up and down (~30x) to break up clumped cells. The remainder of complete DMEM (~50mL) was added to the flask for a yield of ~10^5 cells/mL DMEM. One drop of media containing cells was added to each well of 10-well Teflon coated slides (Erie Scientific, Portsmouth, NH); presence of cells was verified microscopically; slides were covered with foil; and incubated overnight at 37°C, 5% CO_2.

Fixing Slides.--Slides were removed from the incubator and cell adhesion was verified. Excess media was removed from the slides and dipped briefly in 0.01M PBS, gently rocked, repeated 2X with fresh PBS, and air-dried. The dried slides were placed in a glass, open-bottom slide holder, fixed with pure anhydrous acetone, previously equilibrated to –20°C and placed in a –20°C freezer for 10-15 minutes. Acetone-fixed slides were air-dried and stored at –80°C until use.

Staining Slides.--Slides were equilibrated at room temperature and a 1:32 dilution (1µL sera + 31µL 0.01M PBS) of sample, positive, and negative control sera was prepared in a biological safety cabinet. 30µL of samples and controls were placed in each well and incubated for 1 hour at 37°C in a moisture chamber. The slides were washed with PBS; placed in a slide-washing chamber with ice-cold stock PBS; shaken

gently for 30 seconds; let stand for 2 minutes; and the wash step was repeated with fresh PBS. The slides were given a final wash with dH_2O. The slides were air-dried and 15µL of FITC conjugated chicken anti-mouse IgG (h+l) (Bethyl Labs, Inc., Montgomery, TX) diluted to 1:50 with 0.01 M Gibco PBS was placed in each well and the slides were incubated for 30 minutes at 37°C in a moisture chamber. The wash step was repeated as before. The slides were dried in a 37°C incubator for ~5 minutes and read at 200X and 400X with a fluorescent microscope (Nikon, Melville, NY). Positive samples were diluted 2-fold, 1:32-1:2048 to determine titres. The samples were considered positive if the titres exceeded 1:64 and there were 2 observers' agreement.

3. RESULTS

3.1 IgG Immune Responses Against NGV Synthetic Peptide Antigen

Sera from a total of 305 rodents and insectivores from 6 genera and 7 species were tested for IgG antibodies against NGV (Table 1). We detected 27 (8.9%) samples that were positive by the synthetic peptide ELISA and 27 (8.9%) by IFA however there was not 100% concordance (Table 2).

Only *Peromyscus spp.* were seropositive by the synthetic peptide ELISA. Eighty-seven white-footed mice and 165 deer mice were tested. Three (3.4%) of white-footed mice and 24 (14.5%) of deer mice were seropositive for anti-NGV antibodies (Table 1).

Sera from the same 305 rodents and insectivores tested by the synthetic peptide ELISA were also tested by IFA. Again, considering the 2 species found to be seropositive, 4.6% (4/87) and 13.9% (23/165) were white-footed mice and deer mice, respectively.

Table 1. – Species tested for anti-hantavirus IgG antibodies by the synthetic peptide ELISA, IFA and a recombinant protein.

Species	Synthetic Peptide Positive/Tested (%[a])	IFA Positive/Tested (%[a])	Recombinant Positive/Tested (%[a])
Blarina brevicauda	0/5 (0)	0/5 (0)	0/5 (0)
Clethrionomys gapperi	0/45 (0)	0/45 (0)	0/44 (0)
Microtus ochrogaster	0/1 (0)	0/1 (0)	0/1 (0)
Neotoma magister	0/1 (0)	0/1 (0)	0/0 (0)
Peromyscus leucopus	3/87 (3.4)	4/87 (4.6)	3/57 (5.3)
Peromyscus maniculatus	24/165 (14.5)	23/165 (13.9)	16/101 (15.8)
Sigmodon hispidus	0/1 (0)	0/1 (0)	0/1 (0)
All animals Positive/Tested (%)	**27/305 (8.9)**	**27/305 (8.9)**	**19/209 (9.1)**

a. Within species % Positive/Tested

Table 2. Comparison of IFA titres and synthetic peptide and rN ELISA OD_{405} values.

ID	Species	IFA titre	Synthetic peptide ELISA OD	RN ELISA OD
40	*P. maniculatus*	>2048	2.05	1.75
43	*P. maniculatus*	512	1.39	0.82
45	*P. maniculatus*	128	0.49	1.22
49	*P. maniculatus*	512	1.31	1.15
57	*P. maniculatus*	0	0.58	0
66	*P. maniculatus*	256	1.04	0.65
120	*P. maniculatus*	2048	2.00	1.39
123	*P. maniculatus*	>2048	2.04	1.44
145	*P. leucopus*	>2048	1.83	1.65
175	*P. leucopus*	512	1.16	1.32
229	*P. maniculatus*	1024	2.11	Not Tested
289	*P. leucopus*	2048	1.76	0.26
291	*P. maniculatus*	512	0.93	0.10
295	*P. maniculatus*	2048	1.93	0.11
297	*P. maniculatus*	>2048	2.05	0.11
298	*P. maniculatus*	512	0.58	0
299	*P. maniculatus*	>2048	1.94	0.59
301	*P. maniculatus*	0	0.46	0
303	*P. maniculatus*	2048	1.51	0.19
311	*P. maniculatus*	0	0	0.10
313	*P. maniculatus*	512	0	0
323	*P. maniculatus*	>2048	2.22	0
339	*P. maniculatus*	256	0.94	0
347	*P. maniculatus*	2048	1.56	0
348	*P. leucopus*	128	0	0
351	*P. maniculatus*	0	0	0.14
353	*P. maniculatus*	2048	2.02	0.48
354	*P. maniculatus*	>2048	2.96	0.14
356	*P. maniculatus*	>2048	1.67	0
357	*P. maniculatus*	>2048	2.33	0
358	*P. maniculatus*	128	1.01	0

Sera from 209 rodents and insectivores were tested for anti-NGV IgG antibodies. Samples from the 2002 collection were not included for testing with the recombinant protein ELISA. We could not obtain a sufficient amount of recombinant N antigen to perform assays on these samples. Only *Peromyscus spp.* were seropositive; 3/57 (5.3%) of white-footed mice and 16/101 (15.8%) of deer mice were positive by the rN ELISA (Table 1).

3.2 Epidemiology

All 305 rodents and insectivores tested by the synthetic peptide ELISA were categorized into 3 habitats based on trapsite and the potential for human-rodent contact. Each of the 18 trapsites were evaluated for the potential for close human-rodent contact and categorized into habitat 1: low potential, areas such as trails, fields, streambeds and heavily wooded areas that have little or no human visitation; habitat 2: moderate potential, areas such as observation sites, pave parking lots and paved walking trails, and picnic areas that would bring humans in open-air, casual contact with rodents and their excreta; and habitat 3: high potential, areas such as shelters, cabins, and campgrounds where exposure to potentially infected rodents and risk of hantavirus transmission can be high.

The results shown in table 3 are for the synthetic peptide ELISA alone. Of the 24 *Peromyscus spp.* captured and tested in habitat 1, 3 (12.5%) were sero-reactive by the synthetic peptide ELISA and IFA. This habitat designation has the least human disturbance and visitation and thus, poses the lowest risk for human exposure to potentially infected animals. There were 129 *Peromyscus spp.* captured and tested in habitat 2, and 9 were sero-reactive by synthetic peptide ELISA and IFA, 7% tested from

Table 3. Synthetic peptide ELISA seroprevalence by habitat.

Species	Habitat 1 #positive/#tested (%positive)[a]	Habitat 2 #positive/#tested (%positive)[a]	Habitat 3 #positive/#tested (%positive)[a]
Blarina brevicauda	0/0 (0)	0/5 (0)	0/0 (0)
Clethrionomys gapperi	0/2(0)	0/30 (0)	0/13 (0)
Microtus ochrogaster	0/0 (0)	0/1 (0)	0/0 (0)
Neotoma magistier	0/0 (0)	0/0 (0)	0/1 (0)
Peromyscus leucopus	0/12 (0)	1/39 (2.6)	2/36 (5.6)
Peromyscus maniculatus	3/12 (25)	8/90 (8.9)	13/63 (20.6)
S. hispidus	0/1 (0)	0/0 (0)	0/0 (0)
Total[b]	3/27 (11.1)	9/165 (5.5)	15/113 (13.3)

a. within species
b. within habitat

this habitat. This habitat designation had the most human disturbance, visitation, and animals captured but infected animals were considered a moderate risk as any contact with potentially infected animals and excreta is unlikely in a manner that would facilitate viral transmission.

Lastly, there were 15 *Peromyscus spp.* out of 99 (15.2%) tested that were sero-reactive by synthetic peptide ELISA and IFA. However, the numbers of seropositive white-footed mice and deer mice were incongruent between the 2 tests. Two white-footed mice and 13 deer mice were positive by synthetic peptide ELISA and 3 white-footed mice and 12 deer mice by IFA. The risk of human exposure to infected rodents and excreta is potentially very high in this habitat that is comprised of campgrounds, shelters, and cabins. Visitors routinely enter closed cabins that are infested with rodents and sleep in close proximity to rodents and their excreta in campgrounds and shelters. Incidentally, this habitat designation has the highest hantavirus seroprevalence of the 3 habitats (Table 3).

3.3 Sensitivity

A total of 305 serum samples were tested for anti-NGV IgG antibodies using a synthetic peptide ELISA. This assay had 92.6% sensitivity as compared to IFA. Both IFA and synthetic peptide ELISA had 25 positive samples in accordance with each other and the synthetic peptide ELISA had 2 additional positive serum samples, ID numbers 57 and 301 (Table 2). These samples had 2 of the lowest OD_{405} values, 0.576 and 0.463 respectively. Conversely, samples 45 and 298 were positive by both IFA and synthetic peptide ELISA and had the other 2 lowest OD_{405} values, 0.488 and 0.583 respectively (Figure 5). Increasing the cut-off point to 0.900 for the synthetic peptide ELISA would actually cause the sensitivity and specificity to remain unchanged as the 4 lowest OD_{405} values would reverse classifications of 2 true positives and 2 false negatives based on the reference IFA (Table 2 and Figure 5). A total of 209 serum samples were tested for anti-NGV IgG antibodies using a recombinant protein ELISA. The sensitivity as compared to IFA using the same serum samples was 65.4%. The rN ELISA had 17 positive samples in accordance with IFA, which had 26 positive samples for this sample set.

3.4 Specificity

The 305 serum samples tested by the synthetic peptide ELISA and IFA yielded 276 samples that were non-reactive by the synthetic peptide ELISA and 278 samples that were non-reactive by IFA. The 2 additional samples that were positive by IFA were 313 and 348. These samples were also positive by real-time PCR for an amplicon of the nucleocapsid region that supports an active host hantaviral infection (data not shown). Thus, the specificity of the synthetic peptide ELISA was 99.3%. The 209 serum samples tested by the rN ELISA and IFA yielded 181 samples that were non-reactive by the rN

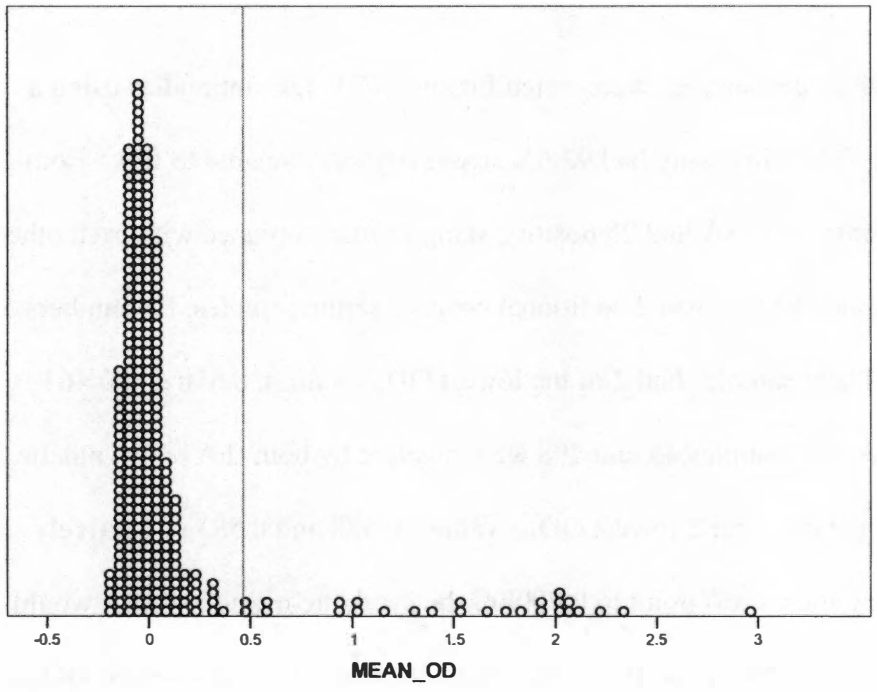

Figure 5. Cut-off for positive serum samples[a]

[a]There were no values less than zero, this figure was presented in this fashion to aid visualization

ELISA and 183 samples that were non-reactive by IFA. Thus, the specificity of the rN ELISA was estimated to be 98.9%. The 2 additional samples that were positive by the rN ELISA were 311 and 351. Neither of these samples was positive by the synthetic peptide ELISA (Table 2). Again, there was 100% concordance with IFA when the 2004 samples are excluded from the data set.

4. DISCUSSION

The utility of synthetic peptide based serodiagnostics has been reported for successful field and laboratory applications (Gonzalez et al. 1997, Hjelle et al. 1997, Li et

al. 2002 and Chu et al. 2003). This is the first reported application of a synthetic peptide based ELISA for the detection of new world hantavirus IgG antibodies. The sensitivity of the synthetic peptide ELISA was estimated to be 92.6% (25/27) and specificity was estimated to be 99.3% (276/278) as compared to IFA.

The ELISA positive cut-off values were extremely conservative but validated by positive IFA. The goal of this study was to evaluate a new serodiagnostic technique for the detection of anti-NGV IgG antibodies in rodent hosts with a homologous antigen. The positive cut-off may be adjusted to accept lower OD values for determination of a past exposure to a hantavirus or presence of maternal antibodies in animals for epidemiological surveys. The efficacy of this test may be further elucidated by comparison to western blot (WB) and plaque reduction neutralization test (PRNT) that may demonstrate a much greater sensitivity of the synthetic peptide ELISA.

4.1 IFA Antigens

The IFA antigen used for a standard comparison to the synthetic peptide ELISA was Convict Creek (CC107) virus isolated from California deer mice (Schmaljohn et al. 1995). A nucleotide and amino acid comparison of the NGV epitope to that of CC107 demonstrated 93 and 96% identities respectively (Table 4). Serological and phylogenetic studies have suggested that nucleotide divergence between hantaviruses that are less than about 25% are serologically indistinguishable due to the considerable conservation of deduced amino acid sequences (Chu et al. 1994, Xiao et al. 1994, and Schmaljohn et al. 1995). NGV diverges from Four Corners virus (FCV), Sin Nombre virus (SNV), and Monongahela virus (MV) by 6-7% at the nucleotide level and 0-4% at the amino acid level and thus, NGV may be classified as a separate isolate of a single virus (Table 4)

Table 4. Nucleotide and deduced amino acid sequence identities of the epitopes for NGV, CC107, FCV, SNV and MV

	CC107	FCV	SNV	MV
NGV	93/96	93/96	94/98	94/100

(Schmaljohn et al. 1995). Consequently, a comparison of the NGV synthetic peptide ELISA to an IFA utilizing any of these closely related viruses as antigens should be adequate to determine, at least initially, the efficacy of this assay.

4.2 Advantages of the Synthetic Peptide ELISA vs. IFA

Studies comparing the biological reactivities of full-length or native N proteins with partial recombinant N antigens have determined serological methods employing the use of truncated, partial N proteins can work as well or better than those that utilize whole cell lysates or full length N proteins without many of the encumbrances (Ahlm et al. 1997, Tang et al. 2001, Billecocq et al. 2003, Takakura et al. 2003 and Maes et al. 2004). Whole cell lysate IFA requires antigens prepared by replication of live virus in cell culture that requires a biosafety level 3 containment laboratory and quality controlled mass production in order to test large quantities of samples and reduce interbatch variations that would affect antigenicity (Elgh et al. 1997 and Takakura et al. 2003). Additionally, IFA is an insensitive method for the detection of IgM antibodies and particularly for serum samples subjected to repeated freeze-thaw cycles (Settergren et al. 1987 and Niklasson and Lundkvist 1999).

4.3 Recombinant and Full Length N Protein Purity and Stability

Recombinant N antigens containing the major immunodominant region have often achieved greater sensitivities and specificities than full-length recombinant proteins when used as ELISA antigens. It has also been observed that lower quantities of recombinant cell extracts are required for microtitre plate adsorbtion as compared to the native protein (Elgh et al. 1996 and 1997, Kaukinen et al. 2001 and Tang et al. 2001 and Billecocq et al. 2003). The production and efficacy of truncated recombinant proteins are not without disadvantages however. The production of recombinant antigens is costly and time consuming. Many expression systems have low yields and target proteins are often contaminated with host cell proteins, resulting in inferior activity and non-specific reactions (Tang et al. 2001, Billecocq et al. 2003 and Takakura et al. 2003). Recombinant protein ELISAs require the use of negative antigens to control for background produced by cross-reactions to host cell contaminants, thus reducing the number of samples screened by half (Feldmann et al. 1993).

The seemingly stochastic nature of recombinant and native protein extraction and purification has become another inducement for alternate antigen preparations. Denaturing purification strategies are often used due to the low solubility of full length and recombinant N proteins in aqueous solutions. However, native conformation is often lost resulting in functional impairment of the N protein (Elgh et al. 1996, Jonsson et al. 2001 and Billecocq et al. 2003). Non-denaturing methods used for purification of N proteins result in a greater yield but reduced purity (Jonsson et al. 2001). Recently, Razankiene et al. (2004) reported the expression of Puumula virus nucleocapsid protein

in *S. cerevisiae* with greater yields and purity. Since degradation products cannot be completely eliminated from recombinant proteins during expression, extraction, and purification processes, additional measures must be taken to maintain stability. Recombinant nucleocapsid proteins may be stabilized by dissolving in urea and stored at 4°C but it has been conjectured that this particular treatment may affect protein antigenicity and immunogenicity (Razankiene et al. 2004). Protein dialysis against PBS, lyophilisation, and storage at –20°C also had varying affects on stability (Razanskiene et al. 2004). This particular detriment may have been evidenced in the ELISA results for the 2004 samples using the recombinant antigen. It can be inferred that the lower sensitivity of the recombinant protein ELISA was due to degradation and loss of functional N epitope. If the 2004 samples are excluded, the sensitivity of the rN ELISA increases to 100% (9/9 positive samples). This discrepancy may be explained by the degradation of the recombinant protein over time. The aliquot of antigen used had been stored at a constant –20°C and not subjected to repeated freeze/thaw cycles.

The 59 amino acid synthetic peptide containing the full length N epitope has demonstrated complete solubility in aqueous solution; excellent reactivity as an ELISA antigen; and long-term stability. The synthetic peptide was stored at –20°C and some aliquots were subjected to numerous freeze-thaw cycles. Despite these conditions, the synthetic peptide had no apparent loss of reactivity more than 2 years subsequent to hydration (Table 2). Presumably, the synthesis and purification of this peptide was such that no degradation artifacts were present as is often the case with recombinant protein expression and purification. The stability of the synthetic peptide is further evidenced by

microtitre plates adsorbed with the synthetic peptide had no appreciable loss of antigenicity with use after several weeks of storage at 4°C (data not shown).

4.4 Conclusion and Future Studies

This synthetic peptide ELISA performed well as compared to IFA and had overall greater reactivity than the recombinant N antigen (Table 2). The promising results of a synthetic peptide N antigen in a direct IgG ELISA; high sensitivity and specificity; purity, and ease of production demonstrates the feasibility of this approach for both seroepidemiological surveys and clinical diagnostics. The production of synthetic peptides is rapid and cost effective. Microtitre plates may be adsorbed with multiple antigens and used for heterologous reactions that would be ideal for screening geographically diverse sera. The favourable performance of this assay makes it a suitable candidate as a screening method for serodiagnosis of hantaviruses.

The scope of this study for the validation of a new screening method was limited. Future studies should include comparisons of the synthetic peptide ELISA to both plaque reduction neutralization test and Western Blot for a more accurate determination of this assay's efficacy. This ELISA method should also be run with an IgM conjugate with known SNV or closely related hantavirus infected sera to examine this test's potential as a clinical diagnostic method. Additionally, the synthetic peptide should be analyzed via SDS-PAGE to ascertain degradation with long-term storage.

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Part 3

THE EVALUATION OF A REAL-TIME PCR METHOD FOR THE DETECTION OF A NOVEL HANTAVIRUS IN RODENTS AND INSECTIVORES OF THE GREAT SMOKY MOUNTAINS NATIONAL PARK

This chapter is a revised version of a paper by the same name to be submitted to the Journal of Virology by Shawn Lewis, Stephen Kania, John New, Jr., Dorcas O'Rourke and Rebecca Penrose-Wilkes.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) most of the collection of samples, (2) most of the optimization of the RT-PCR/nested PCR assays, (3) some of the RTD-PCR optimization, (4) performed all of the experimental analysis, (4) collection of all the statistics, (5) acquisition of all the relevant literature and its interpretation and (6) compilation of the data into a single paper and most of the editing.

1. INTRODUCTION

The *Hantavirus* genus is in the family Bunyaviridae and consists of at least 22 distinct species associated with hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Chizhikov et al. 1995, Bharadwaj et al. 2000, Vapalahti et al. 2003 and Schmidt et al. 2005). Hantaviruses are rodent-borne pathogens that have putatively co-evolved with their respective rodent and insectivore hosts for millions of years (Dekonenko et al. 1997, Vapalahti et al. 2003 and Schmidt et al. 2005).

The prototype etiologic agent of HCPS in the Americas is Sin Nombre virus (SNV) carried predominantly by deer mice, *Peromyscus maniculatus* although secondary rodent hosts are not uncommon (Hjelle et al. 1995*a*, Feuer et al. 1999 and Bharadwaj et al. 2000). Other distinct viruses within the *Hantavirus* genus responsible for HCPS are Black Creek Canal virus (BCCV) carried by cotton rats, *Sigmodon hispidus*; Bayou virus

(BAYV) carried by rice rats, *Oryzomys palustris*; New York virus (NYV) carried by white-footed mice, *Peromyscus leucopus*; and Andes virus (ANDV) carried by long-tailed pygmy rice rats, *Oligoryzomys longicaudatus* (Chizhikov et al. 1995, Galeno et al. 2002, Aitichou et al. 2005 and Chu et al. 2003). Numerous other distinct hantavirus genotypes and their respective rodent hosts from North, Central, and South America continue to be characterized (Hjelle et al. 1995*a,b*, Galeno et al. 2002 and Chu et al. 2003).

1.1 Molecular Characterization

Hantaviruses are spherical enveloped viruses that consist of a tripartite negative sense single-stranded genome that encodes at least 4 structural proteins (Hutchinson et al. 1998, Feuer et al. 1999 and Hujakka et al. 2003). The three genomic segments are large (L), medium (M), and small (S) that is each complexed with the nucleoprotein (N) to form individual nucleocapsids (Hjelle et al. 1994*a,b,* 1995, Chizhikov et al. 1995, and Hutchinson et al. 1998).

The L segment encodes the L protein, the putative viral RNA polymerase and is approximately 250 kilo Daltons (kDa). There appears to be high conservation among hantavirus L protein domains particularly within motifs located in the amino terminal half of the protein and in the carboxy terminal half (Chizhikov et al. 1995 and Feuer et al. 1999).

The M segment encodes the 2 envelope glycoprotein precursors G_1 and G_2 and is approximately 125-127 kDa. The variability observed between hantaviruses is due primarily to the highly mutable amino terminus region of the G_1 glycoprotein (Spiropoulou et al. 1994 and Chizhikov et al. 1995). Additionally, mutation frequencies

of G_1 viral clones have been observed to differ between deer mouse organs (Feuer et al. 1999).

The S segment encodes 2 proteins, the nucleocapsid (N) protein that is approximately 48 kDa and a smaller nonstructural (NS_s) protein of unknown function although it has been hypothesized that this protein may be involved in alterations of viral pathogenicity or play a role in viral adaptation to the maintenance host (Chizhikov et al. 1995, Feuer et al. 1999 and Ulrich et al. 2002). There is high conservation amongst N proteins of different hantaviruses thus cross-reactivity of antibody responses is frequently observed. The N protein the basis for serological assays because of the heterogeneity of the N protein among hantaviruses; the appearance of antibodies against the N protein during the acute phase of illness in humans; and the lack of antibody response of rodent hosts to G_1 or G_2 glycoproteins (Jenison et al. 1994 and Yamada et al. 1995).

1.2 Human Pathogenesis

Hantavirus pulmonary syndrome (HPS) has been used by investigators to describe clinical manifestations of patients infected with a new world hantavirus. However, since the majority of hantavirus case fatalities are a result of cardiac depression rather than hypoxia, many investigators prefer the term hantavirus cardiopulmonary syndrome (HCPS) (Bharadwaj et al. 2000 and Schmidt et al. 2005).

Since the initial outbreak of HCPS in May 1993 the case fatality rate has declined to ~36% from ~80%. This decline in mortality may be due, in part, to the recognition of milder cases of HCPS (Zavasky et al. 1999 and Schmidt et al. 2005). The incubation period of HCPS is approximately 8-28 days from the time of contact with infected rodent excreta, the primary mode of transmission (Zavasky et al. 1999 and Bharadwaj et al.

2000). The syndrome usually proceeds in 4 stages: the febrile prodrome that typically lasts 1-11 days; the cardiopulmonary phase that may last 2-10 days, diuresis, and convalescence (Hjelle et al. 1994*a,b*, Zavasky et al. 1999, Bharadwaj et al. 2000, and Galeno et al. 2002).

The febrile prodrome is characterized by fever, myalgia, nausea, headache, and gastrointestinal symptoms (Duchin et al. 1994, Hjelle et al. 1994*a,b* and Bharadwaj et al. 2000). Specific Immunoglobulin (Ig) G and IgM antibodies to N and G_1 antigens are present in almost all patients upon hospital admission (Hallin et al. 1996, Bharadwaj et al. 2000, and Galeno et al. 2002). Viremia is also present during the prodrome phase but after fever resolution and at the onset of pulmonary edema, viral RNA cannot be detected. Additionally, the level of viremia appears to correlate with the severity of disease (Hjelle et al. 1994*a*, Terajima et al. 1999, Hujakka et al. 2003 and Terajima and Ennis 2003). The onset of the cardiopulmonary phase is usually abrupt and it is at the onset of respiratory symptoms that patients usually seek medical attention. If death occurs during the cardiopulmonary phase, it does so within 1-3 days after onset due to cardiogenic shock and respiratory failure (Hjelle et al. 1994*b*, Zavasky et al. 1999, and Bharadwaj et al. 2000). Some patients have exhibited mild illness during the cardiopulmonary phase and experienced rapid clinical improvement (Duchin et al. 1994, Hallin et al. 1996, and Galeno et al. 2002). The convalescent phase is variable in duration and clinical symptoms and patients have detectable levels of IgG antibodies against hantavirus N proteins (Galeno et al. 2002 and Schmidt et al. 2005).

Conversely, reservoir hosts have been described as persistently infected asymptomatic carriers that excrete virus in urine, feces, and saliva (Schmaljohn and

Hjelle 1997, Hutchinson et al. 1998, Botten et al. 2000, and Vapalahti et al. 2003). However, the infection dynamics of maintenance hosts is more complex.

Studies of experimentally and naturally infected deer mice have demonstrated that the mice experience an initial acute phase followed by low-grade chronic infection (Netski et al. 1999 and Boone et al. 2002). Viral RNA dissemination throughout various tissues in response to cellular and humoral immune responses and mild pathology has also been observed during host infections (Feuer et al. 1999, Netski et al. 1999 and Boone et al. 2002).

During the acute phase viral titres appear to be at their highest levels, are detectable in various tissues such as bladder, liver and lung and viremia is present. It is thought that at this time, infected animals shed virus in blood, urine, feces, and saliva and that rodent-human and rodent-rodent hantavirus transmission occurs at this time (Feuer et al. 1999 and Boone et al. 2002). In experimentally infected animals, viral loads in the heart, kidneys, liver, lung, brown fat and white fat peaked at about 21 days and began decreasing at about 28 days with the appearance of high titres of neutralizing antibodies (Botten et al. 2000). Coincident with these findings were naturally infected deer mice that were acutely infected (i.e. they had mounted a detectable immune response and were viral antigen positive) that demonstrated morphological changes in tissues with SNV antigen. Observed pathologies included alveolar septal edema in the lungs and periportal hepatitis (Netski et al. 1999).

During the persistent chronic phase, viral loads in both blood and tissues drop in response to humoral and cellular responses but low-grade, intermittent viral shedding still occurs (Feuer et al. 1999 and Boone et al. 2002). It has been suggested that as the

infected animal's immune response progresses viremia is cleared and septal edema resolves, however viral persistence and low levels of viral shedding continues (Feuer et al. 1999 and Netski et al. 1999). Lungs, heart and brown fat have high viral titres even in the presence of a strong immune response suggesting that these tissues may serve as depots for viral replication and/or viral maintenance. It has been hypothesized that latent virus in brown fat may exploit host thermoregulatory signals during torpor stimulating the production of virions as has been suggested for the maintenance of rabies and murine cytomegaloviruses (Netski et al. 1999 and Botten et al. 2000).

1.3 Molecular Testing

The diagnosis of suspected hantavirus cases as well as epidemiological studies have relied primarily on serological assays such as immunofluorescence and ELISA (Yamada et al. 1995 and Schmidt et al. 2005). Almost all patients have a detectable level of IgM antibodies against the N protein upon presentation and most acutely infected and convalescent patients have detectable levels of IgG antibodies against N, G_1, and G_2 proteins (Hjelle 1994*a* and Bharadwaj et al. 2000). Thus far, epidemiological and laboratory studies have failed to detect antibody responses in rodent hosts to either G_1 or G_2 glycoproteins (Yamada et al. 1995).

More recently, molecular tests such as RT-PCR and quantitative (qPCR) or real-time detection PCR (RTD-PCR) have demonstrated utility as highly specific and sensitive assays that are complimentary to hantavirus diagnosis and monitoring disease progression. Additionally, virus isolation is time consuming, hazardous, and hantaviruses grow poorly in cell culture indicating the need for alternative rapid diagnostics (Dekonenko et al. 1997 and Billecocq et al. 2003).

The applications of RT-PCR, qPCR, and RTD-PCR for other viral infections have included rapid detection of etiologic agents and monitoring the efficacy of antiviral drugs and experimental vaccines (Abe et al. 1999 and Kehl et al. 2001). The use of a multiplex RT-PCR assay in an enzyme hybridization probe system has been described for diagnosis of respiratory viruses such as Respiratory Syncytial Virus (RSV), influenza, and parainfluenza. Routine diagnostics include tissue culture that often provides results after the patient is discharged and ELISA's that have widely varying sensitivities (Kehl et al. 2001). The multiplex RT-PCR assay demonstrated better sensitivities than traditional diagnostics; faster results than tissue culture; and detection of dual infections that would allow clinicians to develop an improved response to respiratory infections (Kehl et al. 2001).

Conventional PCR or RT-PCR has some limitations that include poor quantitation, potential contamination, and variable sensitivities (Abe et al. 1999 and Drosten et al. 2002). Quantitative PCR or RTD-PCR have the ability to detect a lower number of viral copies; quantification of virus; and can be performed in one step or in two steps (Garcia et al. 2001 and Drosten et al. 2002). Quantitative PCR has been successfully applied to the diagnosis of Hepatitis B virus; numerous viral hemorrhagic fever viruses such as Ebola, Crimean-Congo Fever, and Dengue; herpesvirus, and cytomegalovirus. Investigators have reported improved sensitivities and specificities of viral identification as compared to conventional PCR and RT-PCR; the ability to quantify viral loads in patients during treatment; and reduce the need for cell cultures that are often time consuming and require biosafety level 3 facilities (Abe et al. 1999, Garcia et al. 2001, Drosten et al. 2002 and Billecocq et al. 2003).

The application of qPCR and RTD-PCR to hantavirus research has demonstrated utility for diagnostics; surveillance of the efficacy of anti-viral therapies and experimental vaccines; and epidemiological surveys. Patients with HCPS or HFRS have high viral serum titres during the acute phase of the disease (Terajima et al. 1999). Early diagnosis of HCPS is crucial for facilitating immediate care. Galeno et al. (2002) described AND virus isolation from a patient's blood prior to exhibiting any symptoms or detectable IgM and IgG responses (Bharadwaj et al. 2000 and Galeno et al. 2002). Quantitative PCR is both highly sensitive and specific as compared to conventional RT-PCR amplification and is rapid, efficient, and able to provide quantitative analysis (Garin et al. 2001).

1.4 UTCVM Analysis

We collected blood and other tissues from 345 rodents and insectivores in selected sited of the Great Smoky Mountains National Park (GSMNP) during trapping sessions conducted in 2000, 2001, 2002, and 2004. RNA's extracted from kidneys were submitted to conventional RT-PCR and RTD-PCR amplifications. All applications of hantavirus RTD-PCR described thus far require a separate reverse transcription synthesis step. In our study, we describe the development of a highly sensitive and specific one-step RTD-PCR assay based on Superscript III reverse-transcriptase-Platinum *Taq* polymerase enzyme mixture. PCR amplicons were detected in real time in a Cepheid Smart Cycler® II System with the use of a 5'hybridization probe and results were compared to RT-PCR amplification products.

2. MATERIALS AND METHODS

2.1 Tissue Sample Collection

Between September 13, 2000 and December 1, 2000; November 28 and 29, 2001; May 27, 2002 through July 24, 2002; and May 1, 2004 through May 8, 2004 trapping was conducted in GSMNP. A total of 310 rodents and 35 insectivores (a total of 345 animals) were captured. Blood samples were obtained from 305 animals and subsequently tested for anti-hantavirus antibodies with enzyme linked immunosorbent assays (ELISAs) and IFA.

In addition to blood collection, spleen, liver, lung and kidneys were collected from every animal. Embryos were collected from 15 females and tail tips from animals trapped during the 2002 survey. After euthanasia and sample collection, the animals were tagged with identification numbers and placed in 10% formalin. For a complete description of trapping methods and sample collection, please refer to "Seroprevalence of Hantavirus in the Great Smoky Mountains National Park", Materials and Methods section (Lewis 2002).

2.2 RNA Extraction

mRNA was extracted from kidney tissue using TRIzol® extraction (Gibco Co., Invitrogen Corp., Carlsbad, CA). RNA isolation was carried out in a biosafety level-3 facility located at the UTCVM. Approximately 10 mg of kidney tissue was excised and placed in a petrie dish with 1000µl of TRIzol® Reagent. The tissue was cut into smaller pieces and homogenized by drawing up through an 18 gauge needle and 3 mL syringe, after several passages, the homogenized tissue was placed in an Eppendorf 2.0 mL microcentifuge tube (Eppendorf AG, Hamburg, Germany). Phase separation was

achieved by first incubating homogenized sample at 25° C for 5 minutes, then by adding 200µl chloroform; shaken vigorously for 15 seconds; incubated at 25°C for 3 minutes and centrifuged at 4°C, 12,000 x g for 15 minutes. To precipitate RNA, the aqueous phase was transferred to a fresh Eppendorf 2.0 mL microcentrifuge tube and 500µl of isopropyl alcohol was added. The sample was incubated at 25°C for 10 minutes and centrifuged at 4°C, 12,000 x g for 10 minutes, the RNA precipitate could be visualized as a small pellet at the bottom of the tube. The RNA was washed by removing the supernatant, adding 1000µl of 75% ethanol, vortexing and then centrifuging at 4°C, 7,500-x g for 5 minutes. The supernatant was drawn off and the pellet was air dried for 10 minutes at 25°C. The RNA was redissolved in 50 µl of DEPC treated water and incubated at 55°C for 10 minutes.

2.3 Primer Design and RT-PCR Amplification of the G_1 Fragment

Two pairs of primers were selected for the partial G_1 region of the M gene. The first outer set were degenerate primers based upon North American Sigmodontine-associated hantaviruses that would produce an expected amplicon size of 596 nt. The 5' G_1 and 3' G_1 outer oligonucleotides were MG1FST (ACAATGGGITCIATGGTITGTG A) and MG1RFST (TTIAATITIICATCCATCCA) respectively. The nested 5' and 3' oligonucleotides were G1FWD (GGGTGGGAGACAGCAAAAGAG) and G1REV (CCCAGATGATCAATTCTGTTG) respectively and would produce an expected amplicon size of 296 nt. Oligonucleotides MG1FST and MG1RFST were synthesized by GibcoBRL (Invitrogen Corp., Carlsbad, CA) and oligonucleotides G1FWD and G1REV were synthesized by Sigma Genosys (Sigma Corp., The Woodlands, TX).

Primers MG1FST and MG1RFST were used to generate cDNA and DNA using Superscript One-Step RT-PCR with Platinum *Taq*® (Invitrogen Corp., Carlsbad, CA). The 10 µl total volume RT-PCR reaction mixture consisted of 5.0 µl of 2x Reaction Mix, 0.5 µl of RT Platinum *Taq*® polymerase, 2.0 µl of RNA, 1.5 µl of RNAse free H_2O, and 2.5 µM of each MG1FST and MG1RFST. The thermal profile for the one-step RT-PCR reaction consisted of a 30 min RT step at 50°C followed by a 94°C pre-denaturation step for 2 minutes, 30 cycles of denaturing (94°C for 1 min), annealing (50° C for 30 sec), and extension (72°C for 1 min), there was a final extension for 10 minutes at 72°C. Two microlitres of the resultant PCR product was added to a nested PCR reaction mixture (10 µl final volume) along with 5.0 µl of Takara Premix Taq® 2x PCR Solution (Takara BIO Inc., Otsu, Shiga, Japan), 2.0 µl of RNAse free H_2O, and 2.5 µM of each G1FWD and G1REV nested primers. The thermal profile for the nested reactions was the same for the first round PCR except there was a final 5-minute elongation step. Five microlitres of the amplified DNA was loaded onto a 1.4% agarose gel stained with ethidium bromide in TBE buffer (Tris 0.9 M, Borate 0.02 M, and EDTA 0.02 M, pH 8.3) and electrophoresed for approximately 20 minutes and the gel was then transilluminated.

2.4 Primer Design and RTD Amplification of the N Segment

The primers and fluorogenic hybridization probe were designed with Primer 3© software version 0.2 (Whitehead Inst. For Biomedical Research, Cambridge, MA) to align with highly conserved regions of the Nucleocapsid fragment of the S gene (Figure 1). The 5' and 3' oligonucleotides and the 5' Cy5™ (Carbocyanine) fluorescent hybridization probe with Black Hole Quencher 2™ were synthesized by Integrated DNA

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63 CACGAACAAC AGCTCGTGAC TGCCAGGCAG AAGCTTAAAG ATGCAGAAAG AGCAGTGGAG
   >>>>>>>>>> >>>>>>>>>>                                    ^^^^^^^^^
         N-RT-5'                                          N-RT-PROBE

   GTGGACCCCG ATGATGTTAA CAAGAGCACA TTACAGAGCA GACGG 167
   ^^^^^^^^^^ ^               <<<<< <<<<<<<<<< <<<<<
                                    N-RT-3'
```

Figure 1. Primer and hybridization probe alignment with target sequence of the nucleocapsid region[a].

[a]Target sequence alignment with NGV (GenBank accession number AY396718)

Technologies (IDT Inc., Coralville, IA). The sequences of the 5' oligonucleotide (N-RT-5'), 3' oligonucleotide (N-RT-3'), and 5' hybridization probe (N-RT-PROBE) were CACGAACAACAGCTCGTCAC, CCGTCTGCTCTGTAATGTGC, and GCAGTGGA GGTGGACCCCGA respectively and should produce an amplicon 104 nt in length.

RTD-PCR reactions were performed using Superscript™ III Platinum© One-Step Quantitative RT-PCR System (Invitrogen Corp., Carlsbad, CA) and Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA). The 25µl total volume RTD-PCR reactions contained 12.5 µl of 2x reaction mix, 0.5 µl of Superscript™ III RT/Platinum® *Taq* mix, 2.0 µl of RNA, 5.5 µl of RNAse free H_2O, 0.5 µl of RNase OUT™ (Invitrogen Corp., Carlsbad, CA), 0.2µM of N-RT-PROBE, and 0.3µM each of N-RT-5' and N-RT-3'. The thermal profile for RTD-PCR reactions was 50°C for 30 min for cDNA synthesis followed by a 95°C pre-denaturation step for 2 min and 50 cycles of denaturing (95°C for 15 sec), annealing (56°C for 30 sec), and extension (72°C for 30 sec).

2.5 Sequencing

Preparation of a representative positive sample was performed by combining 4-25µl RTD-PCR reactions of the same sample for a total volume of 100µl. The 100µl of PCR product was purified by the addition of 40µl of ExoSAP-IT® (USB Corp., Cleveland, OH), incubation at 37°C for 15 min, then heat inactivation for 15 min at 80°C. The sample was prepared for sequencing at the University of Tennessee's Core Sequencing facility. A Basic Local Alignment Search Tool (BLAST) query was performed on the N amplicon to determine homology to known hantaviral sequences.

3. RESULTS

3.1 RT-PCR/Nested PCR

The RT-PCR and nested PCR assays yielded 21 positive samples and 13 indeterminate samples. PCR amplification products that were considered positive yielded a single clear band of the expected size of ~296 bp or slightly lower than the 300bp marker of a 1-Kb ladder. Samples were considered indeterminate if the expected band size was lightly visible or multiple bands were clustered around the expected ~296 nt amplicon. Sample 43 was not positive in this experiment but had been positive by RT-PCR/nested PCR in previous experiments and thus was considered positive (Table 1).

3.2 RTD-PCR

The RTD-PCR assays yielded 18 positive samples with a mean threshold cycle (C_T) of 33.29, range = 24.75-47.61. Any sample that recorded a C_T value was accepted as positive (Table 1). Sample 325, *Sorex fumeus* (smoky shrew) was selected for sequencing. BLAST analysis confirmed 100% identity of the 50bp amplicon sequenced to that of the NGV partial nucleocapsid sequence (GenBank accession AY396718).

Table 1. RT-PCR and RTD-PCR results for samples found to be positive or indeterminate.

		RT-PCR		RTD-PCR			RT-PCR		RTD-PCR
ID#	Species	Positive	Indeterminate	C_T	ID#	Species	Positive	Indeterminate	C_T
18	*C. gapperi*	+	-	-	295	*P. maniculatus*	-	-	30.54
32	*P. leucopus*	-	+	-	296	*P. maniculatus*	+	-	-
34	*C. gapperi*	-	+	-	297	*P. maniculatus*	+	-	26.74
43	*P. maniculatus*	+	-	35.23	299	*P. maniculatus*	+	-	28.31
66	*P. maniculatus*	-	-	31.21	300	*P. maniculatus*	-	+	-
77	*C. gapperi*	-	-	39.58	301	*P. maniculatus*	-	+	-
98	*P. leucopus*	+	-	-	303	*P. maniculatus*	+	-	-
153	*P. leucopus*	-	+	-	304	*P. maniculatus*	-	-	41.60
154	*B. carolinensis*	-	+	-	313	*P. maniculatus*	+	-	30.88
166	*P. leucopus*	-	-	47.61	323	*P. maniculatus*	+	-	24.88
175	*P. leucopus*	-	-	27.67	325	*S. fumeus*	-	-	39.54
178	*P. leucopus*	-	+	-	344	*P. maniculatus*	+	-	-
179	*P. maniculatus*	-	-	38.16	345	*P. gapperi*	+	-	-
222	*P. maniculatus*	-	+	-	346	*P. leucopus*	+	-	-
229	*P. maniculatus*	-	-	35.48	347	*P. maniculatus*	+	-	-
251	*P. leucopus*	-	+	-	348	*P. leucopus*	-	+	24.75
270	*P. leucopus*	+	-	-	350	*P. leucopus*	+	-	-
272	*P. leucopus*	+	-	-	351	*P. maniculatus*	+	-	-
279	*P. maniculatus*	-	+	-	353	*P. maniculatus*	+	-	37.80
289	*P. leucopus*	-	+	-	354	*P. maniculatus*	-	-	25.30
290	*P. maniculatus*	-	+	-	355	*P. maniculatus*	+	-	-
294	*P. maniculatus*	+	-	-	357	*P. maniculatus*	+	-	33.96
Totals		6	10	7	Totals		15	3	11

3.3 Viral Prevalence

The total viral prevalence estimated by RT-PCR and nested PCR was 6.1% (21/345). The total viral prevalence estimated by RTD-PCR was 5.2% (18/345) (Table 2). Two *Clethrionomys gapperi* (Southern red-backed voles) were positive and 1 was indeterminate by RT-PCR/nested PCR. One *Clethrionomys gapperi* was positive by RTD-PCR but there was no sample in concordance with any of those from the RT-PCR/nested PCR assays (Table 1). One *Sorex fumeus* (smoky shrew) was positive by RTD-PCR. Nineteen *Peromyscus spp.* (7.4%) were positive and 11 (4.3%) were indeterminate by RT-PCR/nested PCR as compared to 16 (6.2%) positive *Peromyscus spp.* estimated by RTD-PCR (Table 2).

4. DISCUSSION

We have developed a rapid, one-step RTD-PCR assay for the detection of NGV. The primers and hybridization probe were designed for a highly conserved region of the S gene residing within the nucleocapsid epitope. Thus, this assay should be able to detect several strains of New World hantaviruses.

Table 2. Viral prevalence by species

	RT-PCR		RTD-PCR
Species	# positive/ # tested (%)	# indeterminate/ # tested (%)	# positive/ # tested
B. carolinensis	0/9 (0)	1/9 (11.1)	0/9 (0)
S. fumeus	0/13 (0)	0/13 (0)	1/13 (7.7)
C. gapperi	2/48 (4.2)	1/48 (2.1)	1/48 (2.1)
P. leucopus	5/90 (5.6)	6/90 (6.7)	3/90 (3.3)
P. maniculatus	14/167 (8.4)	5/167 (3.0)	13/167 (7.8)
All Other Species	0/18 (0)	0/18 (0)	0/18 (0)
Total Viral Prevalence	21/345 (6.1)	13/345 (3.8)	18/345 (5.2)

This assay may be refined to allow for the quantification of viral genomic copies in samples by the development of a standard curve with known virus concentrations (Garin et al. 2001). Other investigators have reported the increased accuracy of quantification with real-time PCR assays allowing for detection of small changes in viral levels over classical virologic quantification methods. Additionally, the viral detection limits of RTD-PCR often exceed those of nested PCR assays and of naturally infected subjects (Abe et al. 1999, Garin et al. 2001, and Drosten et al. 2002).

The efficacy and rapidity of our RTD-PCR assay may be used in conjunction with serology such as IFA, ELISA and Western Blot for the diagnosis and treatment of HCPS. Viremia is present during the acute phase of HCPS and can be detected before clinical manifestations are observed and an antibody response is mounted (Galeno et al. 2002). A preliminary study of the quantification of viral genomic RNA in immune complexes has demonstrated a trend of disease severity and increased viral RNA in immune complexes (Terajima et al. 1999 and Terajima and Ennis 2003).

The RTD-PCR assay can be performed in about 3 h, inclusive of RNA extraction as compared to RT-PCR/nested PCR, which can be performed in about 7 h. Hantaviruses are difficult to propagate in cell culture and require BSL-3 containment facilities. Additionally, the process is expensive, time-consuming and not suitable for clinical diagnosis. Numerous hantavirus gene sequences are readily available for comparison making nucleic acid based assays more feasible and efficient to perform (Hjelle et al. 1994*a*, Dekonenko et al. 1997). These and the pathological features described qualify the

RTD-PCR assay as a complimentary method for early clinical diagnosis of HCPS and disease progression (Hjelle et al. 1994*a* and Galeno et al. 2002).

The reduced efficacy of RTD-PCR in clinical diagnosis of HCPS occurs due to the disappearance of viremia at the onset of the cardiopulmonary phase. It is at this time that neutralizing antibodies and other immune responses are reducing plasma viremia; it is also at this time that the majority of HCPS patients seek medical treatment (Hjelle et al. 1994*a*, Terajima et al. 1999, Bharadwaj et al. 2000 and Galeno et al. 2002).

Our RTD-PCR assay detected the target sequence of the N region in a shrew; this is the first report of a New World hantavirus detected in an Insectivore. The RT-PCR/nested PCR assay failed to detect this sample, which supports other investigators findings, that RTD-PCR sensitivity is equal to or greater than that of nested PCR assays (Abe et al. 1999, Garcia et al. 2001 and Garin et al. 2002). Additionally, both RT-PCR/nested PCR and RTD-PCR assays produced amplicons that annealed with our respective primer sites from *Clethrionomys gapperi*. Again, this is the first reported case of hantavirus evidence found in a *Clethrionomys spp.* among rodents harboring New World hantaviruses. Other investigators have found Arvicoline spp. infected with and believed to be the primary or secondary hosts of distinct hantaviruses. Prospect Hill virus (PHV), the prototypic New World hantavirus is harbored by the meadow vole (*Microtus pennsylvanicus*) (Hjelle et al. 1995*b* and Rowe et al. 1995). Other *Microtus spp.* hosted hantaviruses include Isla Vista virus, harbored by the California vole (*M. californicus*); PH-like viruses found in meadow voles (*M. pennsylvanicus*), mountain voles (*M. montanus*) and prairie voles (*M. ochrogaster*); and an EL Moro Canyon-like virus found in *M. montanus* (Rowe et al. 1995 and Bennett et al. 1999). None of these viruses have

been associated with human illness although PHV reactive antibodies have been detected in humans (Hjelle et al. 1995*b*, Rowe et al. 1995 and Bennett et al. 1999).

At this time, we believe that the deer mouse is the primary reservoir host and that the white-footed mouse, smoky shrew and Southern red-backed vole are spillover hosts as combinations of these and other species were captured on the same trap-lines (data not shown). It may be conjectured however, that *P. leucopus* may represent a co-maintenance host with *P. maniculatus* based on phylogenetic analysis of partial N, G_1 and G_2 sequences (GenBank accession numbers: AY396718, AY396717 and AF406788, respectively). The G_1 and G_2 amplicons had 96% and 100% identities respectively at the aa level to New York virus and the N amplicon had a 97% identity at the aa level to Monongahela virus. New York virus is harbored by *P. leucopus* and Monongahela, an SNV-like virus, is harbored by *P. maniculatus*. Both *Peromyscus spp.* are sympatric in GSMNP, thus one possible explanation for this unique isolate is a genetic reassortment event has occurred where these 2 species home ranges overlap and the virus is co-maintained (See Chapter 4).

In conclusion, RTD-PCR assays are fairly simple, quantitative, sensitive, specific and reproducible and are less prone to false positive results that may occur due to contamination during second-round amplification of nested PCR (Dekonenko et al. 1997 and Garin et al. 2001). This study was a pilot experiment of the application of an RTD-PCR method to determine the viral prevalence of a hantavirus in GSMNP. This assay was performed as a qualitative rather than quantitative one and thus our interpretation of results is limited. We were able to estimate that viral prevalence based on RT-PCR/nested PCR was 6.1% (21/345) and based on RTD-PCR was 5.2% (18/345) and that

4 species from 3 genera were positive for our target sequences. These findings warrant further investigation such as genome sequencing to determine if the same virus is co-circulating among the different species.

We could not elucidate the sensitivity and specificity of our assays since we did not sequence all of our positive samples nor run the RTD-PCR assays with a standard curve. This limitation may be overcome by developing a standard curve generated by plotting the C_T of known viral concentrations versus the log of the viral titre (Garin et al. 2001). We would also like to determine specificity with multiple primer sets and hantaviruses.

With the appropriate validation, this RTD-PCR assay may be a superior method for clinical diagnosis and epidemiological identification of hantaviruses. This method is already used for Hepatitis B and Herpes virus 8 quantification in patient sera and for the evaluation of anti-viral drug efficacy (Abe et al. 1999, Garcia et al. 2001 and Garin et al. 2001). RTD-PCR validation would allow the circumvention of laborious, hazardous and unreliable virus isolation methods particularly when large numbers of samples are tested.

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Part 4

NEWLY CHARACTERIZED HANTAVIRUS ISOLATED FROM *PEROMYSCUS MANICULATUS* (DEER MOUSE) IN EAST TENNESSEE AND THE PHYLOGENETIC RELATIONSHIP WITH OTHER PATHOGENIC HANTAVIRUSES

This chapter is a revised version of a paper by the same name to be submitted to the Journal of Virology by Shawn Lewis, John New, Jr., Moges Woldemeskel and Stephen Kania.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) most of the collection of samples, (2) the selection of this topic and the experimental design, (3) most of the molecular work required for data acquisition, (4) all of the phylogenetic analysis, (5) acquisition of all the relevant literature and its interpretation and (6) compilation of the data into a single paper and most of the editing.

1. INTRODUCTION

Hantaviruses are in the family *Bunyaviridae* along with *Phlebovirus, Nairovirus,* and *Uukuvirus* (Martin et al. 1985 and Fauquet 2000). New world hantaviruses are the etiologic agents causing hantavirus pulmonary syndrome (HCPS) and have a high mortality rate (Hjelle et al. 1995*a,b*, 1997). Each hantaviral strain is associated with a specific rodent/insectivore host and geographical region (Monroe et al. 1999). Reservoir hosts are asymptomatically, persistently infected and shed virus particles intermittently in urine, saliva, and feces. Thus, the primary transmission route is inhalation of aerosolized excreta (Gavrilovskaya et al. 1999 and Hjelle and Glass 2000).

Hantaviruses are negative-sense RNA viruses that possess an envelope and a single stranded tripartite genome. This tripartite genome consists of large (L), medium (M) and small (S) segments that correspond to single large open reading frames (Hjelle et al. 1995*a,b* and Yamada et al. 1995). The L segment encodes the viral transcriptase. The M segment encodes the G1 and G2 surface glycoproteins and is transcribed as a single

mRNA. The antigenic differences that occur among hantaviruses are most likely due to the less conserved immunodominant region near the amino terminus of the G1 protein. The N protein encapsulates the viral genome. The nucleocapsid protein is highly conserved and cross-reactivity occurs between IgG and IgM antibodies produced against this protein. To date, 23 different hantaviral types and several more quasispecies that have a specific reservoir host association have been identified (Jenison et al. 1994, Feuer et al. 1999, Gavirilovskaya et al. 1999, and Kaukinen et al. 2001).

HCPS in the U.S. was first recognized in 1993, the responsible etiologic agent was identified as Sin Nombre virus (SNV). The primary host reservoir of SNV is *Peromyscus maniculatus* (deer mouse). As of July 2005, 396 HCPS cases with a 36% mortality rate have been confirmed in 30 states (Hjelle et al. 1994, Weigler 1995, Kitsutani et al. 1999 and CDC 2005).

We have made comparisons of these viral isolates and other New World hantavirus variants to demonstrate the relatedness of Newfound Gap virus to pathogenic strains that have been implicated in HCPS cases. We can infer a possible evolutionary link between Western and Eastern United States variants based on phylogenetic analysis, that genetic reassortment may be occurring, and that the genetic differences that occur among hantaviruses may vary both geographically and topographically.

2. MATERIALS AND METHODS

2.1 Sample Collection and Rodent Processing

From September 13, 2000 to July 24, 2002, 265 rodents and insectivores were collected from 18 sites in GSMNP. Three of these sites were those previously surveyed

by Mills et al. (1998). The other 15 sites were selected on the basis of potential human-rodent contact and reports by park staff of heavily rodent infested areas.

Most sites were visited for 2 nights and an average of 35 traps (range=16-50) were set each trapnight. Sherman live traps (3x3.5x9", Sherman live traps, Tallahasee, FL) were baited with a mixture of rolled oats, peanut butter, and apple and placed on the trapline in the early evening. Cottonballs or 4x4 gauze pads were also placed in traps for nesting material to reduce trap related deaths during colder months (Mills et al. 1999).

Early the following morning, sprung traps were checked to verify that they contained animals, placed in a plastic biohazard bag, and double-bagged in a plastic garbage bag. If a second trapnight was planned, traps were sprung to ensure animals did not enter during the day, otherwise they were picked up. The animals were transported back to a central processing area from all but 1 site in which case, field processing was carried out in the same manner.

The processing area was opened on 1 side with a laminar flow to the outside, away from investigators conducting animal processing. Investigators donned disposable Tyvek® (E.I. du Pont de Nemours and Company, Wilmington, DE) coveralls, boot covers, hair bonnets, latex gloves, and face shields with powered air purifying respirators equipped with HEPA filters (3M™ Air-Mate, St. Paul, MN). Investigators conducting data collection remained behind the laminar flow. Processing was performed according to standardized protocols (Mills et al. 1995) with the following exceptions: Isofluorane™ (Rhodia Asia Pacific, Singapore) was used as the anesthetic agent.
Animals were first placed in a Ziploc™ bag with an Isofluorane™ soaked cottonball to induce anesthesia. A Falcon™ (BD-Becton, Dickinson and Company, Franklin Lakes,

NJ) 15 ml conical tube containing an Isofluorane™ soaked cottonball placed over the animal's nose and used to maintain anesthesia during cardiac puncture (Mills et al. 1995). Blood was then transferred into a serum separator microtainer® (BD-Becton, Dickinson and Company, Franklin Lakes, NJ). After euthanasia by cervical dislocation, tissue samples were collected for RT-PCR analysis and placed in an appropriately marked 2.0 mL cryovials (Fisherbrand®, Suwanee, GA) containing Trizol™ reagent (GibcoBRL Life Technologies, Gaithersburg, MD) (150µl for kidneys and 75µl for all other tissues). Specimen identification was made using morphological characteristics as described by Laerm and Boone (1997). Traps were cleaned, disinfected, re-baited, and returned to the trapline, all other traps were re-set for a second night. On the last morning of trapping at each trapsite, all traps were collected and cleaned according to standardized protocols (Mills et al. 1995).

2.2 Molecular Testing

mRNA was extracted from kidney tissue based on a previously positive serological assay (data not shown), using TRIzol® extraction and performed according to manufacturer's protocol (Gibco Co., Invitrogen Corp., Carlsbad, CA). First-round and semi-nested degenerate primers for the G2 region of the M gene were based on SNV (5'outer/nested: TGTGAITATCAAGGIAAIAC; 3'outer: ACIGWIGCICCATAICACAT; and 3'nested: CCCCAIGCICCITCATT). G1 outer and nested primer sets were degenerate primers based upon North American Sigmodontine-associated hantaviruses (Johnson et al. 1997). Forward outer: ACAATGGGITCIATGGTITGTGA; Reverse outer: TTIAATITIICATCCATCCA; Forward nested: GAITGIGAIACAGCAAAAGA; and Reverse nested:

CCCCAIGCICCITCATT. RT-PCR and nested PCR performed on the G1 and G2 regions of the M gene amplified gene fragments of 296 and 201 base pairs, respectively. Sequencing Analysis on these amplicons was performed at the University of Tennessee's Core Sequencing facility. Nucleotide sequence comparison using BLAST software indicated that these envelope glycoproteins amplified from tissues collected in GSMNP represent a novel genetic variation of the glycoproteins in SNV. The G1 and G2 glycoproteins have the closest homology to New York hantavirus, 80 and 85% respectively. Based on these data, N oligonucleotide primers were designed to align with the S segment cDNAs from RI-R virus (GenBank accession # UO9488). The nucleotide sequences are as follows: outer position 1,5' TAGTAGTAGACTTCGT(AG)AA(GA)AGCTAC; outer position 980,5' CGATCTGGAGCACA(TC)GCAAA(GT)ACCCA; inner position 20,5' AAGCTACTACGACTAAAGCT; inner position 476,5' CCTCTAGTTGACAACATAT.

RT-PCR was performed using Superscript One-Step RT-PCR with Platinum Taq® (Invitrogen Corp., Carlsbad, CA). The 25 µl RT-PCR reaction mixture consisted of 12.5 µl 2x reaction mix, 1.25 µl RNAse inhibitor, 0.5 µl forward outer primer, 0.5 µl reverse outer primer, 1.0 µl RT taq polymerase, 5.0 µl RNA and 4.25 µl DEPC treated water. A 30-minute RT step at 50°C was followed by a 94°C pre-denaturation step for 2 minutes, 30 cycles of denaturing (94°C for 1 minute), annealing (50°C for 30 seconds) and extension (72°C for 1 minute), there was a final extension for 10 minutes at 72°C and held at 4°C. Five µl of the RT-PCR reaction mixture was used as a template for the 25 µl nested reaction which also included 12.5 µl PCR Master Mix with Taq polymerase™ (Promega Corp., Madison, WI), 0.5 µl each of the forward and reverse nested primers,

and 6.5 µl nuclease-free H_2O. The thermal profile for the nested reaction was the same for the first round PCR except there was a 5-minute final polymerization step. Ten µl of the nested PCR reaction plus 2 µl of gel loading solution was loaded onto a 1.4% agarose gel with 10x TBE, 3 µl ethidium bromide, and electrophoresed for approximately 40 minutes at 100 volts. The gel was transilluminated and bands were visible at ~296 bp, ~201 bp, and ~947 for the G2, G1, and N regions, respectively.

Gel Purification was performed using QIAGEN QIAquick gel extraction kit® (Invitrogen Corp., Carlsbad, CA). Purified DNA products were ligated into the linearized PCR 2.1 Cloning Vector® (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. Transformation was performed using One Shot INVαF' Competent Cells® (Invitrogen Corp., Carlsbad CA).

Ten and Fifty µl of the transformation reaction were plated onto Luria Bertani agar with Ampicillin (50 µg/L)(LB-AMP). The plates were incubated at 37°C for 2 hours, inverted and incubated overnight at 37°C. Another LB-AMP agar plate and a MacConkey agar plate were divided into 20 grids. Twenty singular colonies were numbered and selected from the previously incubated LB-AMP plates and streaked onto the corresponding numbered grid of the LB-AMP plate and then MacConkey plate. The plates were inverted and placed in a 37°C incubator overnight.

Six 0.65 mL microcentrifuge tubes were filled with 10 µl of RNAse free H_2O. Six colonies (that were white on the MacConkey agar) were streaked from the corresponding numbered LB-AMP agar plate and placed in a microcentrifuge tube. The tubes were boiled at 100°C for 10 minutes to release plasmids. The tubes were then centrifuged for 1 minute at 14,000 x g to pellet cellular debris.

A PCR master mix with 100 µl Promega PCR Master Mix (Promega Corp., Madison, WI), 15 µl each of forward and reverse nested primer, and 10µl nuclease free H_2O was prepared. Seven microlitres of the PCR master mix and 3 µl of each template were added to a thin-walled tube and placed in the thermocycler (nested protocol as previously described). Transformants were resolved on a 1.4% agarose gel. Colonies that were positive from PCR screening were sub-cultured into 15 mL conical tubes containing 10 mL LB-AMP broth and 20 µl Ampicillin (50 µg/mL). Caps were placed loosely on the conical tubes and taped, tubes were placed in a 37°C shaking incubator at 225 RPM overnight.

Using a Wizard plus SV mini-prep DNA purification system (Promega Corp., Madison, WI), plasmids were isolated for sequencing. Samples were submitted to the University of Tennessee's Core Sequencing facility. M13 forward and reverse primers were used to identify sites flanking the inserted PCR product. We have named this strain "Newfound Gap" and registered the G1, G2, and N partial sequences in GenBank (accession numbers AY396717, AF406788, and AY396718, respectively).

2.3 BLAST Analysis, Sequence Alignments and Phylogenetic Analysis

Basic Local Alignment Search Tool (BLAST) queries performed on the one antibody positive sample has revealed a unique viral strain that is closely related to Monongahela and responsible for a human HCPS fatality in North Carolina (Monroe et al. 1999). Consensus sequences were determined and submitted for BLAST queries. Sequences from BLAST queries in addition to New World hantavirus sequences accessed from GenBank and Monroe et al. (1999) were used to construct phylogenetic trees for

nucleotide and deduced amino acid sequences. GenBank accession numbers (where applicable) are included in the phylogenetic analysis.

Sequences were aligned with CLUSTAL® alignment software (European Bioinformatics Institute, Cambridge, England). Phylogenetic trees were constructed using the distance based Neighbor-Joining (NJ) method with MEGA 2.1 (Kumar et al. 2001). Kimura 2-parameter distance corrections were used for nucleotide trees and Poisson distance corrections were used for deduced amino acid trees. Bootstrap confidence values were calculated by 100 search repetitions for all phylogenetic trees (Figures 1-6).

3. RESULTS

3.1 Nucleocapsid Region of the S Gene

The 947 nt nucleocapsid fragment from coordinates 20 to 967 of the S gene was isolated and encodes a predicted protein 306 aa in length. A nucleotide comparison of our N isolate to other New World hantaviruses demonstrated that the closest homologies were to Monongahela, Sin Nombre, and New York strains (86, 85, and 85% respectively). Deduced amino acid comparisons of Newfound Gap virus differed from Monongahela, Sin Nombre, and New York viruses by only 3, 7, and 5% respectively despite the differences found at the nucleotide level.

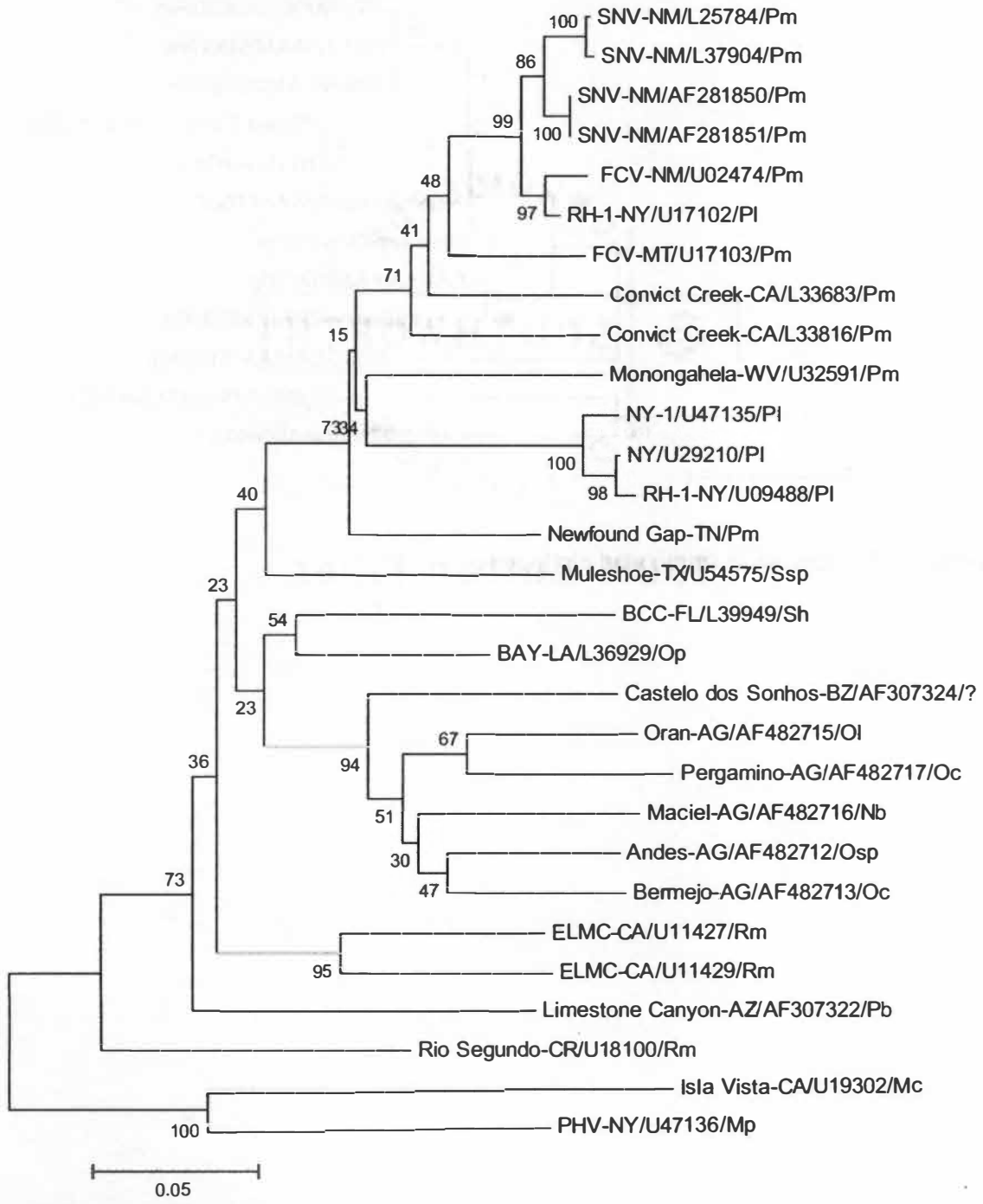

Figure 1. Nucleocapsid nucleotide phylogeny

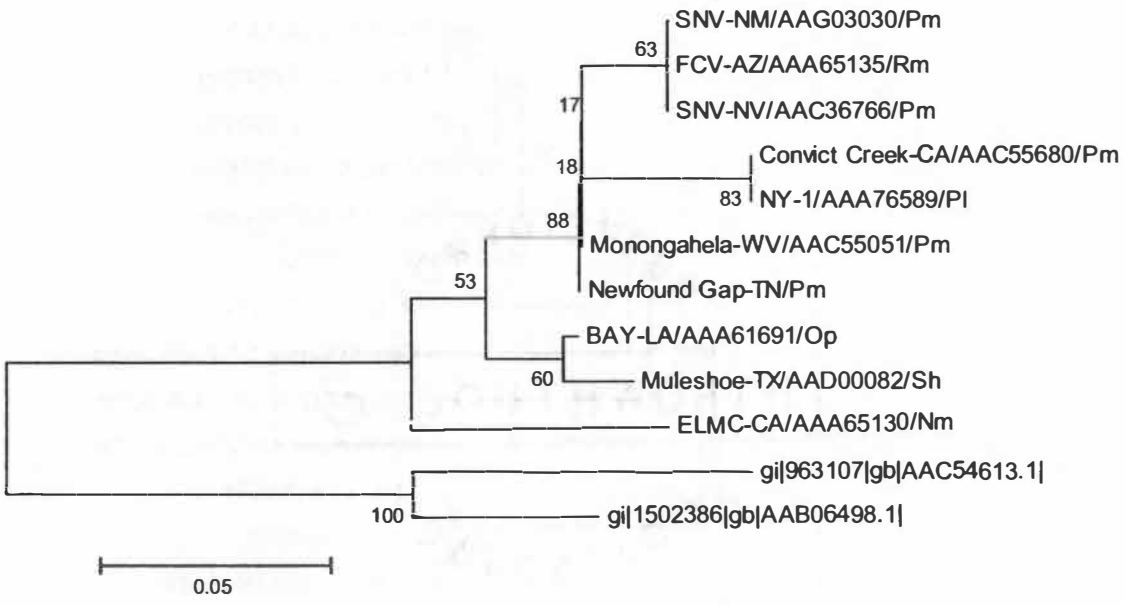

Figure 2. Nucleocapsid amino acid phylogeny

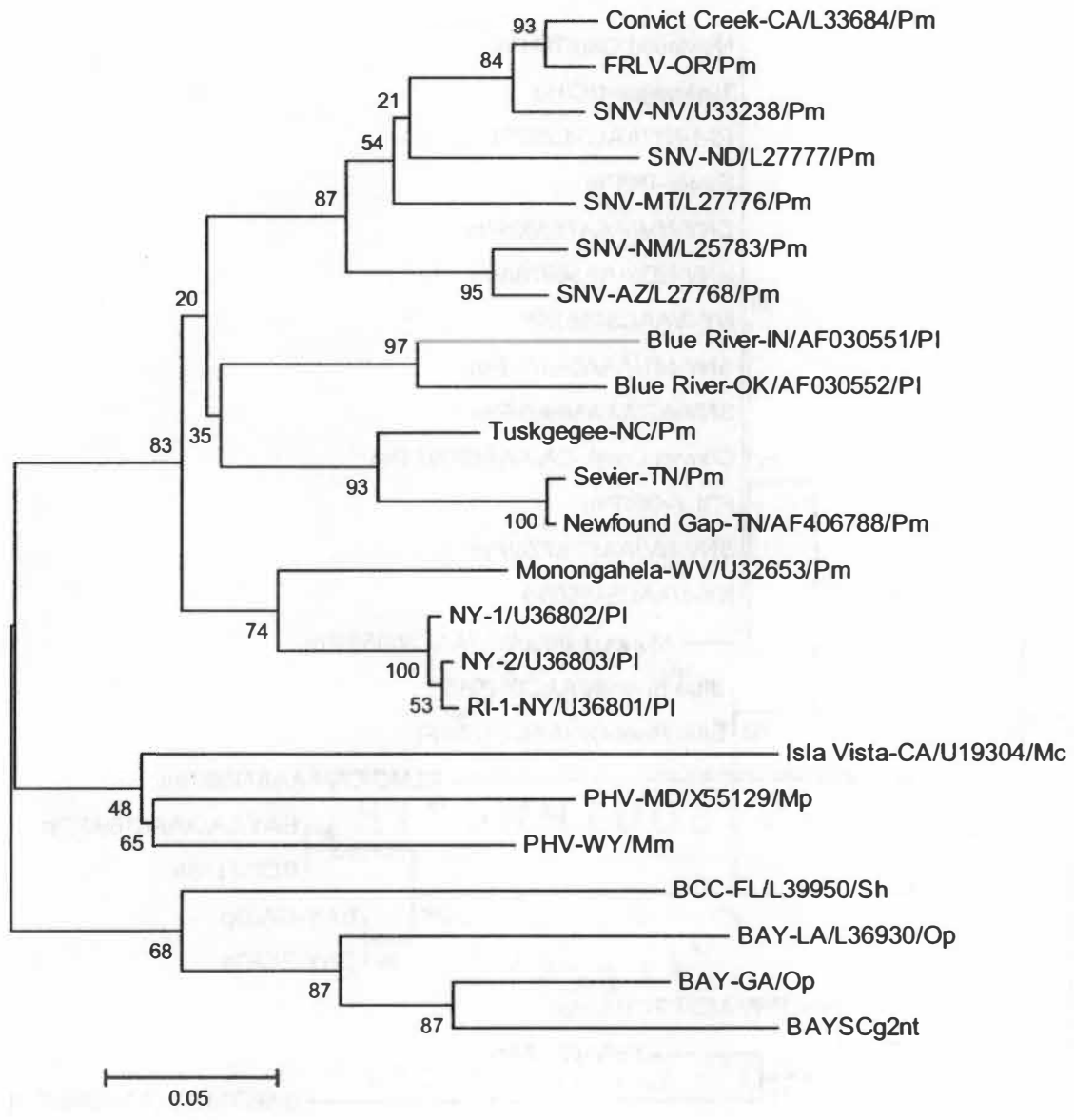

Figure 3. G_2 nucleotide phylogeny

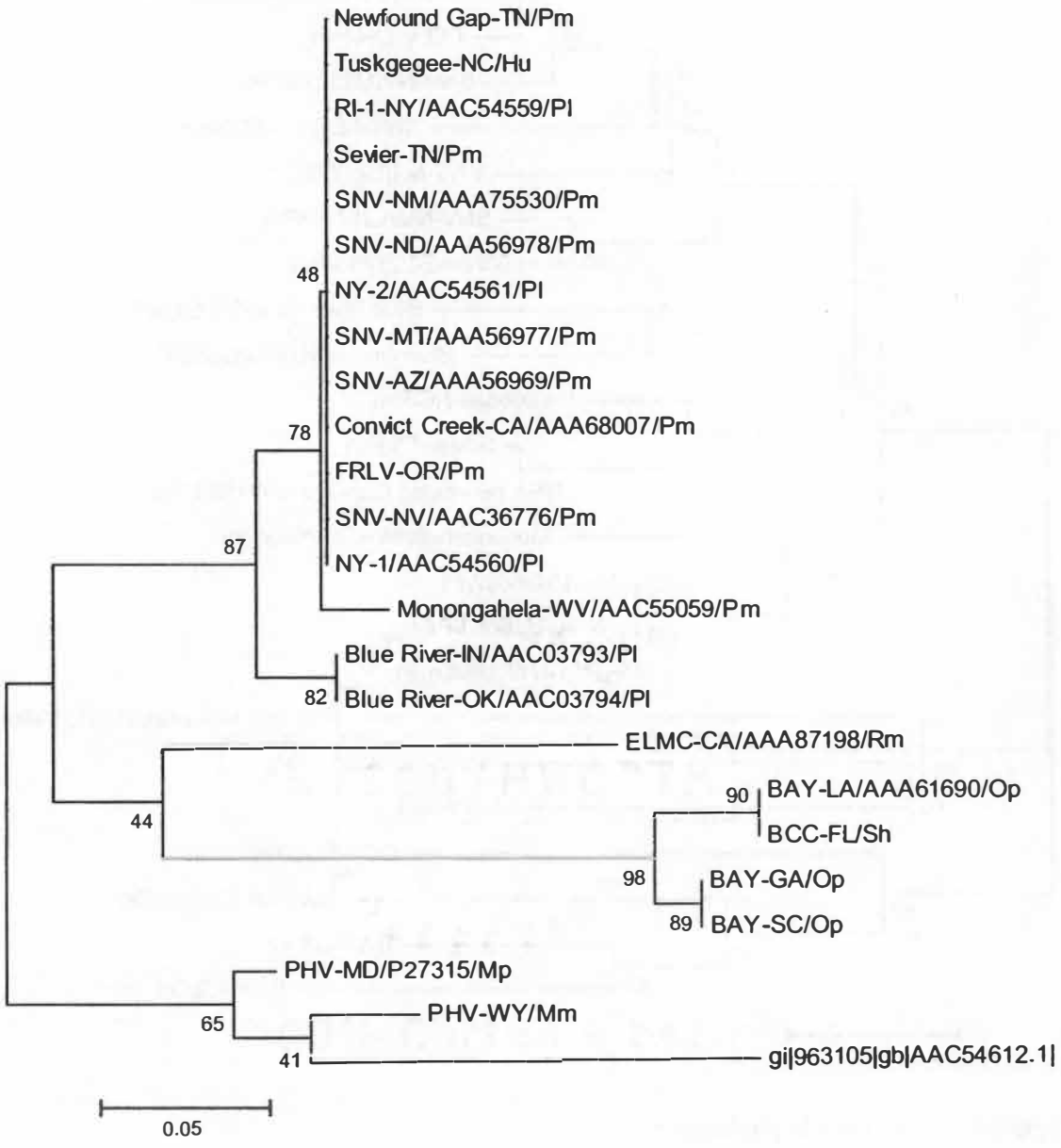

Figure 4. G_2 amino acid phylogeny

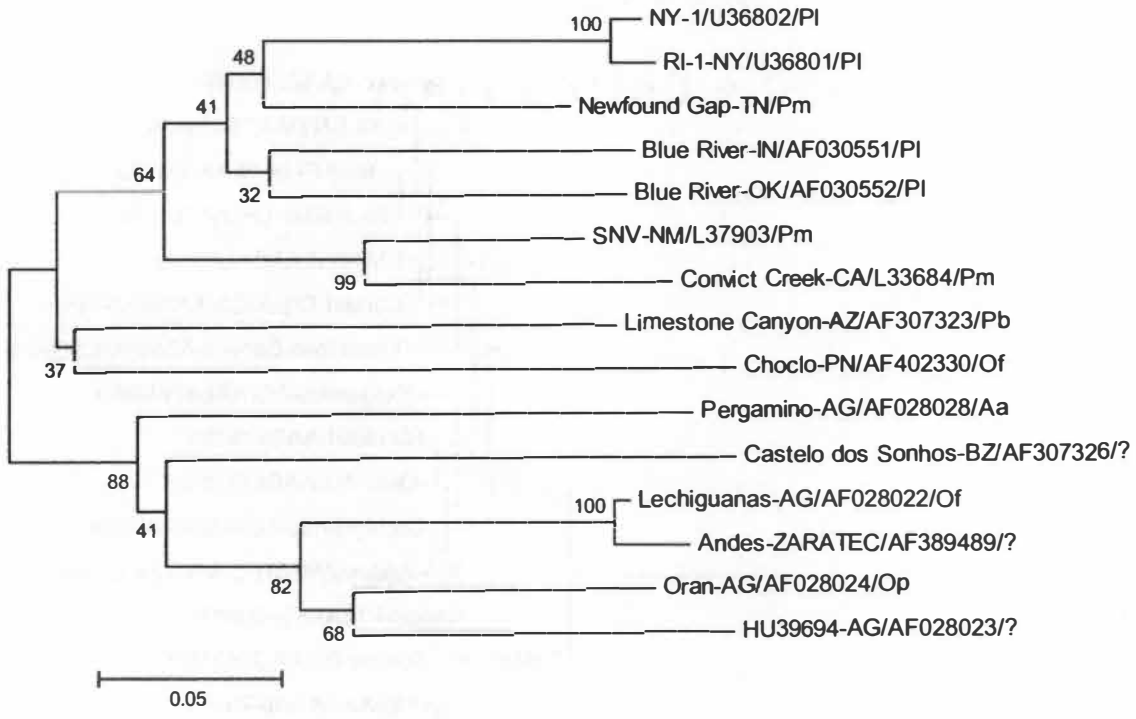

Figure 5. G_1 nucleotide phylogeny

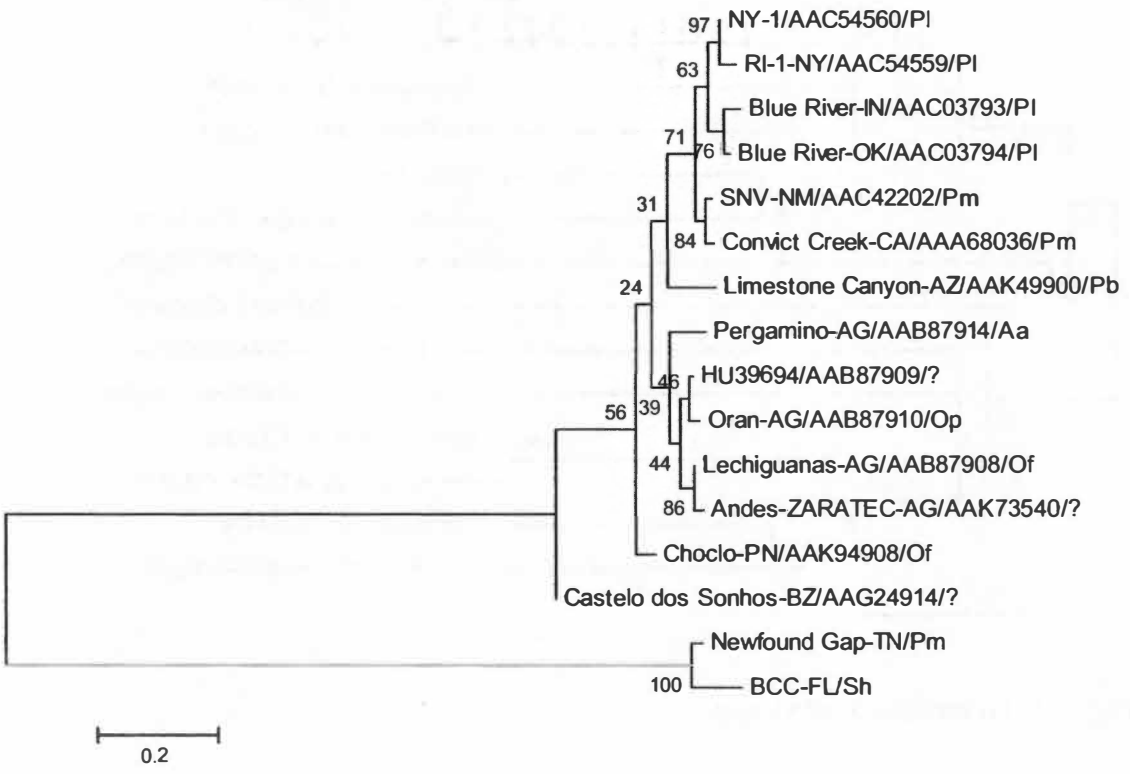

Figure 6. G_1 amino acid phylogeny

The nucleotide phylogenetic tree shows Newfound Gap virus represents a distinct viral lineage (Figure 1) while the amino acid tree shows a supported clade containing Monongahela and Newfound Gap viruses (Figure 2). Both trees demonstrate that Newfound Gap, Monongahela, and New York viruses have diverged from a common ancestor that is also shared by Sin Nombre and Four Corners Viruses.

3.2 G_2 Region of the M Gene

A 201 nt G2 fragment from positions 2789 to 2990 of the M gene, encoding a predicted protein 66 aa in length was compared to U.S. hantaviral strains. The nucleotide analysis of Newfound Gap G2 isolate diverges from NY-2 by 15%; Blue River-IN, NY-1, RI-1, and Convict Creek-CA by 16%; and Monongahela by 18%. Additional nucleotide sequences were available for G2 (Monroe et al. 1999). Comparisons made to isolates from Sevier County-TN and Tuskegee-NC demonstrate 100% and 92% homology to Newfound Gap virus respectively. The deduced amino acid alignments with New York viruses were highly conserved (100%). Alignments with Convict Creek-CA, SNV, Monongahela, and Blue River-IN were 99% homologous to Newfound Gap.

The nucleotide phylogenetic tree shows that Newfound Gap virus forms a well-supported (93% bootstrap value), distinct clade with geographically similar viral strains (Figure 3). The Blue River strains share the same ancestral node with the clade that includes Newfound Gap virus however this divergence is weakly supported (35% bootstrap value). Amino acid comparisons infer a possible evolutionary link of Newfound Gap, New York and Sin Nombre viruses although that clade is not well

supported (48% bootstrap value) (Figure 4). Monongahela forms a single branch that diverges from the same ancestral node as Newfound Gap.

3.3 G_1 Region of the M Gene

A 290 nt G1 fragment of the M gene from coordinates 1713 to 2003 was isolated and encodes a 95 aa protein that spans the immunodominant region. The differences between Newfound Gap and other hantavirus strains are similar to those of G2. Newfound Gap differs from NY-1 and NY-2 by 17% at the nucleotide level and only 1% at the amino acid level; 20% and 2-3% from the Blue River strains at the nt and aa levels respectively; and interestingly, 22% and 1% at the nt and aa levels respectively, from Southwestern Sin Nombre strains.

Newfound Gap virus forms a separate branch that diverged from an ancestral node that gave rise to NY-1 and RI-1 although this is not a well supported clade (48% bootstrap value) (Figure 5). There was disparity in the amino acid phylogenetic analysis; Newfound Gap formed a well-supported distinct clade with Black Creek Canal-FL virus (100% bootstrap support) (Figure 6). This clade and the clade that is comprised of the remainder of the strains used for this analysis have diverged from the same ancestral node. The disparity seen in this tree may involve the variability that is seen in the G1 region.

4. DISCUSSION

The results of this study expand knowledge of the dichotomy between genetic diversity and relatedness of North American Hantaviruses. Phylogenetic analysis using partial S and M sequences has demonstrated the relationship of a newly characterized

hantavirus, from an area where HCPS cases have not been reported, to pathogenic strains that have been associated with case fatalities.

The results of nucleotide comparisons of the S and M segments to other hantaviral strains were unexpected. Newfound Gap G1 precursor protein formed a distinct, robustly supported clade with BCCV G1 protein precursor. No other phylogenetic analyses including BCCV demonstrated this relationship.

The Newfound Gap G1 and G2 partial sequences of the M gene were most closely related to New York and Blue River strains, which are *P. leucopus* hosted hantaviruses. The partial N sequence of the S gene shares greater homology with Monongahela and SN viruses that are hosted primarily by *P. maniculatus*. Nucleotide comparisons of S and M segments of one viral strain to another have generally yielded analogous results. Differences in homologies of S and M segments have been observed by Li et al. (1995), postulating that genetic reassortment could potentially result in a hybrid virus. These results suggest genetic reassortment; possibly deriving from a host-switching event since *P. maniculatus* and *P. leucopus* are sympatric and synchronistic throughout the Appalachian region (Song et al. 1996 and Rhodes et al. 2000).

The Appalachian Mountains are comprised of contiguous ranges that span 1500 miles from Alabama to Newfoundland and are up to 200 miles wide. A study of Canadian hantaviruses found that eastern and western Canadian haplotypes were divergent from one another and the eastern Canadian strains clustered with New York-1 and Monongahela (Drebot et al. 2001). The Monongahela strain has been associated with HCPS cases in Pennsylvania, West Virginia, and Virginia (Rhodes et al. 2000). Our studies have demonstrated an ancestral divergence of Newfound Gap and Monongahela,

comparing partial S sequences. There is also considerable amino acid homology between Newfound Gap and Monongahela nucleocapsid proteins, which form a clade that also includes western viruses. A 139-bp fragment of the G2 region isolated from lung tissue of an HCPS patient in eastern North Carolina was only 8% different at the nucleotide level from Newfound Gap virus (Monroe et al. 1999). There has also been an HCPS case in Washington County, Vermont, which is within the northern Appalachian range, although there is no sequence data available on hantavirus isolates (Craig et al. 2001).

Based on these data, we hypothesize that not only do viral variants have a geographical correlation, which has been well established, but also a topographical dependence that would be consistent with particular species' host range. We can infer that a nexus of genetically similar variants fluxes along a topographic continuum and the viral homologies present in discrete rodent hosts may represent recent spillover events or evolutionary pressure to maintain protein sequence integrity. This may be evidenced by phylogenetic analysis of Rhode Island-1 virus and FCV sequences of infected *P. leucopus* captured in New Mexico. Hjelle et al. (1995 *a,b*) demonstrated that RI-1 aligned better with viruses from peromyscine rodents from the northeastern U.S. than with the *P. leucopus* hosted viruses from the southwest.

Our study has provided a unique opportunity to characterize a potentially pathogenic zoonotic organism. We have identified seropositive *P. leucopus* in GSMNP and plan to increase what is already known of hantavirus evolutionary relationships. Detailed genetic characterization of Newfound Gap virus, along with antigenic reactivity assays should further elucidate the pathogenic potential of this newly found virus. Additional sequence

data from the Appalachian region will be required to validate the interrelatedness of geographic and topographic hantaviruses.

LEGEND

Viral Strain-U.S State or Country of Origin/GenBank Accession #/Host

Aa = *Akodon azarac* (Cardales (field) mouse)
Mc = *Microtus californicus* (California meadow vole)
Mm = *Microtus montanus* (Montane vole)
Mp = *Microtus pennsylvanicus* (meadow vole)
Nb = *Neotoma benefactus* (black digger mouse)
Nm = *Neotoma mexicana* (Mexican woodrat)
Oc = *Oligoryzomys chacoensis* (crimson-nosed rat)
Of = *Oligoryzomys fulvescens* (pygmy rice rat)
Ol = *Oligoryzomys longicaudatus* (long-tailed rice rat)
Op = *Oryzomys palutris* (rice rat)
Pb = *Peromyscus boylii* (brush mouse)
Pl = *Peromyscus leucopus* (white-footed mouse)
Pm = *Peromyscus maniculatus* (deer mouse)
Rm_1 = *Reithrodontomys megalotis* (western harvest mouse)
Rm_2 = *Reithrodontomys mexicanus* (Mexican harvest mouse)
Sh = *Sigmodon hispidus* (cotton rat)
Ssp = *Sigmodon spp.*
? = Unidentified host

AG = Argentina
BZ = Brazil
CR = Costa Rica
PN = Panama

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Part 5

SUMMARY DISCUSSION

We evaluated two methods for the detection of hantavirus in small mammals in GSMNP and attempted to characterize the genetic variant found in rodents and insectivores by phylogenetically comparing NGV to other New World hantaviruses. We were able to estimate both sero and viral prevalences; determine the most likely reservoir of NGV and finally were able to conjecture that NGV may be a novel strain and has evolutionary relatedness to some of the most pathogenic American hantavirus species.

We elected to evaluate a synthetic peptide based ELISA due to the ease and purity with which target peptides may by synthesized. Recombinant antigens are still time consuming to prepare and extract and the purity and antigenicity can be highly variable resulting in decreased sensitivities and specificities (Billecocq et al. 2003, Takakura et al. 2003 and Razanskiene et al. 2004). The sensitivity and specificity of our synthetic peptide IgG ELISA were 92.6% and 99.3%, respectively. The cut-off for positive value acceptance was very conservative and may be adjusted to increase sensitivity.

Homologous N antigen ELISA's provide increased sensitivities but considerable cross-reactivity of antibodies to the N protein occur and thus cannot distinguish antigenic differences (Hjelle et al. 1995*a* and 1997, Elgh et al. 1997 and Schmidt et al. 2005). Hantavirus infected humans, but not rodents elicit IgM and IgG responses against the G_1 protein and the G_1 epitope is very type specific (Hjelle et al. 1995*a*, Elgh et al. 1997 and Gavrilovskaya et al. 1999). An ELISA may be easily formatted to accommodate multiple N and/or G_1 synthetic peptide antigens and may also be used with IgG, IgM and even IgA conjugates (Elgh et al. 1997, Padula et al. 2000, Hujakka et al. 2003 and Maes et al. 2004). Some of these alterations can provide serologic discrimination between

hantavirus serotypes which now can only be achieved with neutralization tests or PCR (Elgh et al. 1997, Hujakka et al. 2003 and Maes et al. 2004).

We also evaluated the efficacy of a one-step real-time detection-PCR assay specific for an amino terminal region of the nucleocapsid segment. While we did not estimate the sensitivity and specificity of this assay, as compared to nested PCR and sequencing or neutralization tests, we were able to sequence at least one sample detected with RTD-PCR that we were unable to detect with RT-PCR/nested PCR despite numerous attempts to do so. Additionally, we obtained unambiguous results with RTD-PCR compared to nested PCR with which we often observed multiple banding patterns. Other investigators have described the superior sensitivity, specificity and reproducibility of RTD-PCR over standard methods of viral detection (Abe et al. 1999, Garin et al., 2001, Drosten et al. 2002 and Aitichou et al. 2005).

One of the most predominant features of RTD-PCR is the quantitative capability. Viral quantification has numerous applications; for diagnostics viral RNA detection and quantification of human hantavirus infections during viremia can facilitate appropriate medical intervention (Dekonenko et al. 1997 and Terajima et al. 2003). Viral quantification can be applied to anti-viral drug efficacy either in-vivo to monitor patients' response or in-vitro to screen anti-viral drug candidates which currently requires cell culture (Garcia et al. 2001). For epidemiological purposes, RTD-PCR has been used to evaluate viral loads in maintenance host tissues to examine host/virus infectivity dynamics (Botten et al. 2000).

We did not exploit the quantitative capability of our RTD-PCR assay but rather the qualitative aspect since this was a preliminary evaluation. Both synthetic peptide

ELISA and RTD-PCR performed exceedingly well, however, require further validation and refinement.

Of the 7 species in 6 genera tested, we estimated *Peromyscus spp.* had an 8.9% (27/305) seroprevalence of IgG antibodies against NGV, no other animals tested were reactive to the synthetic peptide ELISA. All 12 species of 8 genera were tested for a nucleocapsid segment of the S gene and 5.2% (18/345) were determined to be virus positive (Table 1). Not all animals that were virus positive were antibody reactive to NGV antigen (Table 2). This finding was not unexpected since maintenance hosts have demonstrated periods of viral intermittence; newly infected animals may not have developed an IgG response and juveniles may have maternal antibodies (Hutchinson et al. 1998 and Botten et al. 2000). Both serologically and virologically positive animals were found with a greater frequency in areas with the highest potential for reservoir-human contact such as cabins, shelters and campgrounds.

We have determined that *P. maniculatus* is the primary reservoir of NGV since 14.5% (24/165) were seropositive and 7.8% (13/167) were virus positive. Additionally, no seropositive or virus positive white-footed mice were found in areas devoid of deer mice. *P. leucopus* most likely represents a spillover host; 3.4% (3/87) and 3.3% (3/90) were seropositive and virus positive, respectively (Table 1). Again this finding is not unexpected since maintenance host spillover has been documented with Puumula and Monongahela viruses where numerous species are sympatric and synchronistic (Rhodes et al. 2000 and Weidmann et al. 2005).

Table 1. Reactivities of rodent and insectivore sera with NGV synthetic peptide in an IgG ELISA and RNA detection with RTD-PCR.

Species	ELISA # Positive/# Tested	RTD-PCR # Positive/# Tested
B. brevicauda	0/5 (0)	0/9 (0)
B. carolinensis	0/0 (0)	0/9 (0)
C. gapperi	0/45 (0)	1/48 (2.1)
M. ochrogaster	0/1 (0)	0/2 (0)
N. magister	0/1 (0)	0/1 (0)
P. leucopus	3/87 (3.4)	3/90 (3.3)
P. maniculatus	24/165 (14.5)	13/167 (7.8)
S. cinereus	0/0 (0)	0/1 (0)
S. cooperi	0/0 (0)	0/1 (0)
S. fumeus	0/0 (0)	1/13 (7.7)
S. hispidus	0/1 (0)	0/1 (0)
S. longirostris	0/0 (0)	0/3 (0)
Totals: All animals Pos/Tested (%)	27/305 (8.9)	18/345 (5.2)

Table 2. Reactivities of positive rodents and insectivores with NGV synthetic peptide in an IgG ELISA and RNA detection with RTD-PCR

Sample ID	Species	ELISA	RTD-PCR
40	*P. maniculatus*	+	-
43	*P. maniculatus*	+	+
45	*P. maniculatus*	+	-
49	*P. maniculatus*	+	-
57	*P. maniculatus*	+	-
66	*P. maniculatus*	+	+
77	*C. gapperi*	-	+
120	*P. maniculatus*	+	-
123	*P. maniculatus*	+	-
145	*P. leucopus*	+	-
166	*P. leucopus*	-	+
175	*P. leucopus*	+	+
179	*P. maniculatus*	-	+
229	*P. maniculatus*	+	+
289	*P. leucopus*	+	-
291	*P. maniculatus*	+	-
295	*P. maniculatus*	+	+
Totals		14	8

Sample ID	Species	ELISA	RTD-PCR
297	*P. maniculatus*	+	+
298	*P. maniculatus*	+	-
299	*P. maniculatus*	+	+
301	*P. maniculatus*	+	-
303	*P. maniculatus*	+	-
304	*P. maniculatus*	-	+
313	*P. maniculatus*	-	+
323	*P. maniculatus*	+	+
325	*S. fumeus*	-	+
339	*P. maniculatus*	+	-
347	*P. maniculatus*	+	-
348	*P. leucopus*	-	+
353	*P. maniculatus*	+	+
354	*P. maniculatus*	+	+
356	*P. maniculatus*	+	-
357	*P. maniculatus*	+	+
358	*P. maniculatus*	+	-
Totals		13	10

One of the more interesting findings was the viral NGV amplicon detected by our RTD-PCR assay and subsequently sequenced from a *Sorex fumeus* (smoky shrew). We also amplified a target sequence from a *Clethrionomys gapperi* (Southern red-backed vole) but we were unable to obtain a sufficient DNA concentration for sequencing. This is the first reported case of a New World hantavirus being detected in a shrew and the first evidence of a hantavirus detected in a *Clethrionomys spp.* An antigenically distinct hantavirus, Thottapalayam virus has been isolated from *Suncus murinus* (Indian shrew) from India but has not been associated with human disease thus far (Chu et al. 1994).

Clethrionomys glareolus (bank vole) is the primary host reservoir of Puumula virus, endemic throughout Europe. New World Arvicoline rodent hosts include *M. pennsylvanicus*, *M. ochrogaster*, *M. montanus*, and *M. californicus*. Their respective viruses are Prospect Hill, Bloodland Lake, Prospect Hill-like and Isla Vista (Chu et al. 1994, Jenison et al. 1994, Hjelle et al. 1995*a,b* and Monroe et al. 1999). Antibody reactivity to Hantaan, Prospect Hill and Sin Nombre viruses has been demonstrated in *Clethrionomys gapperi* and a *Blarina brevidcauda* (short-tailed shrew) but virus has never been detected (Lee et al. 1982, Yanagihara et al. 1987 and Mills et al. 1998).

Finally, we constructed phylogenetic analysis to compare nucleotide and deduced amino acid sequences of partial N, G_1 and G_2 amplicons to other New World hantaviruses. The comparison of the nucleocapsid region of the S gene yielded amino acid sequences differences of 3, 5 and 7% to Monongahela, Sin Nombre and New York viruses, respectively. The deduced amino acid sequence comparisons of G_1 and G_2 had 99 and 100% identities to New York virus, respectively. Additional partial G_2 nucleotide sequences from Sevier County, TN and Tuskegee, NC were compared to NGV partial G_2

sequence and there were 100 and 99% homologies, respectively. These data are supported by other reports that Monongahela variant isolated from archived *P. maniculatus* from West Virginia is less than 8% different than the NGV strain and more similar to New York virus than SNV viruses (Monroe et al. 1999 and Rhodes et al. 2000). Monongahela and Tuskegee, NC strains have been associated with HCPS cases in Pennsylvania, West Virginia, Virginia and North Carolina (Monroe et al. 1999, Rhodes et al. 2000 and Rooney et al. 2004).

The incongruous homologies of the NGV S and M segments to other closely related hantaviruses suggest that genetic reassortment resulting in a hybrid virus may have occurred. Reassortment of hantaviruses in nature is rare but has been documented; additionally, in-vitro studies have demonstrated reassortment between distally related viruses (Hjelle et al. 1995*b*, Li et al. 1995, Schmaljohn et al. 1995 and Rizvanov et al. 2004).

This preliminary analysis may not be adequate to draw hard conclusions. We would like to sequence larger segments if not the entire genome for comparisons, which is what would be required for establishing reassortment (Li et al. 1995 and Schmaljohn et al. 1995). Xiao et al. (1994) demonstrated that smaller segment nucleotide trees yielded the same results as deduced amino acid sequences from the entire M segments of hantaviruses. It is also premature to conclude that NGV is a distinct virus species without additional analysis, although NGV possesses unique characteristics and is closely related to pathogenic strains that have resulted in HCPS case fatalities.

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Vita

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