FIELD AND LABORATORY BASED STUDIES ON THE TRANSMISSION AND PREVENTION OF NEW WORLD HANTAVIRUSES

BY

DAVID J. SAFRONETZ

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

The *Bunyaviridae* is a large group of viruses which contains several viruses of medical importance, the majority of which are transmitted to humans by arthropod vectors. The exception are the members of the genus *Hantavirus* which are maintained in nature and transmitted to humans by rodent reservoirs. Unique hantavirus species have been documented essentially worldwide and are associated with two distinct diseases in humans, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Currently, treatment and prevention options for HFRS and HPS are limited and mainly consist of education programs aimed at reducing contact with rodents and their excreta. The aim of the studies presented here was to investigate new strategies for preventing disease in humans through i). improved understanding of viral transmission from naturally infected rodents and ii). evaluating novel vaccine candidates in a lethal HPS disease model.

In an attempt to elucidate patterns of infection and transmission in rodents, field studies were conducted in southern Manitoba between August 2003 and October 2005. The findings of these studies provided new insight into potential mechanisms of transmission in the natural setting with respect to routes and timing of virus transmission in relation to the stages of infection and confirmed the existence of several patterns observed in studies conducted elsewhere (i.e., systemic infection in rodents, age, sex and seasonal bias of infection). Infection status in mice was determined using serological and molecular methods. To differentiate recently infected rodents from those infected in the distant past an antibody avidity assay was developed and determined to be 89.5% accurate in identifying recently (within 30 days) infected mice based on the presence of

low avidity antibodies. Initial application of the avidity assay revealed a greater proportion of mice with low avidity antibodies had detectable viral RNA in oropharyngeal fluid (OPF) or urine samples compared with those from mice with high avidity antibody (21% versus 6.8%), suggesting recently infected mice are more likely to transmit virus via these routes. In contrast, no difference in the detection of viral RNA in whole blood samples between the low and high avidity groups was observed. Retrospective analysis of samples collected as a part of a pilot in August 2003 demonstrated similar patterns. These data suggests, recently infected mice represent a greater risk to humans acquiring infection, however based on the low rate of detection of viral RNA in OPF and urine samples compared with that of blood, viral transmission among rodents may frequently occur via the blood-borne route, presumably as a part of wound to wound contact associated with aggressive behaviors.

The long-term goal for the prevention of hantaviral disease in humans is the development of effective vaccines capable of inducing complete, sterile immunity in naïve individuals. Currently no specific vaccine exists for HPS, and the study of the protective immune response has been hampered by the lack of animal models which reflect disease progression in humans. The recently described lethal hamster model of HPS was evaluated for the study of Andes virus (ANDV) induced HPS and found to be an accurate disease model which was 100% lethal within approximately 10-11 days and demonstrated several features of disease in humans including symptomolgy and pathophysiology. Two, recombinant, replication deficient, viral vector platforms (Vesicular stomatitis virus pseudo-particles bearing the ANDV glycoproteins, VSVΔG*AND-GPC, and Adenovirus constructs, recAd, individually expressing ANDV

G_N, G_C or N) were evaluated as potential vaccines in the hamster model. Immunization with VSVΔG*AND-GPC was protective in approximately 50% of immunized hamsters, however survivors had detectable N antibody titers, demonstrating seroconversion and indicating the presence of replicating ANDV. By contrast, immunization with any of the recAd vectors either alone, or in combination, was 100% protective from ANDV challenge. Based on low titers of neutralizing serum antibodies post-immunization, the protective immune response associated with the recAd vectors was likely due to a potent cellular immune response. Unlike with VSVΔG*AND-GPC, complete, sterile immunity was achieved in hamsters which received both the G_N and G_C expressing recAd vectors as determined by the lack of N specific antibodies, as well as no detectable viral RNA in tissue samples at 6 and 9 days post-challenge. The recAd vectors are useful tools for dissecting the protective immune response and warrant further investigation as vaccine candidates for HPS and HFRS.

ACKNOWLEDGMENTS

"Knowledge is in the end based on acknowledgement."

- Ludwig Wittgenstein

First and foremost, I'd like to thank my family and friends for their continued support during my PhD research. I am indebted to the staff members of the NML and the Department of Medical Microbiology at the University of Manitoba who have taken time to assist me in various aspects of my research. Over the last few years I have been privileged to work with numerous people in the Zoonotic Diseases and Special Pathogens department, all of which had a hand in the work presented here. My field studies projects could not have been completed without the expertise of Dr. Robbin Lindsay. I would also like to acknowledge the help of Tyler Cote and Kai Makowski with the fieldwork and Dr. Brian Hjelle and his laboratory (University of New Mexico) for numerous discussions and for providing the timed sera samples necessary for validating the avidity assay. A special debt of gratitude is also owed to the individuals who allowed me to collect deer mice from their private land.

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Safronetz, D., Lindsay, R., Hjelle, B., Medina, R.A., Mirowsky-Garcia, K. & Drebot, M.A. 2006. Use of IgG avidity to indirectly monitor epizootic transmission of Sin Nombre virus in deer mice (*Peromyscus maniculatus*). Am. J. Trop. Med. Hyg. **75:**1135-1139.

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Safronetz, D., Drebot, M.A., Artsob, H., Cote, T., Makowski, K. & Lindsay, L.R. 2008. Sin Nombre Virus shedding patterns in naturally infected deer mice (*Peromyscus maniculatus*) in relation to duration of infection. Vector Borne Zoonotic Dis. **8:**97-100.

aa	amino acid(s)
Ad-ANDV-G_N	recombinant Adenovirus vector expressing the ANDV G_N protein
Ad-ANDV-G_C	recombinant Adenovirus vector expressing the ANDV G_C protein
Ad-ANDV-N	recombinant Adenovirus vector expressing the ANDV N protein
Ad-EMPTY	empty, recombinant Adenovirus vector (control virus)
AdHu-5	human adenovirus serotype 5
Ampicillin	(2S,5R,6R)-6-([(2R)-2-amino-2-phenylacetyl]amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, ($C_{16}H_{19}N_3O_4S$)
ANDV	Andes virus
ANOVA	analysis of variance
ATCC	American Type Culture Collection
bp	base pair
BCCV	Black Creek Canal virus
BSL 2/3/4	biosafety level 2/3/4
°C	degrees Celsius
Carbenicillin	(2S,5R,6R)-6-[(3-hydroxy-3-oxo-2-phenylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, ($C_{17}H_{18}N_2O_6S$)
CDC	Centres for Disease Control
cDNA	complementary DNA
cm	centimeter (10^{-2} meters)
CMR	capture-mark-recapture
CO_2	carbon dioxide
CPE	cytopathic effect
cRNA	complementary RNA
CsCl	cesium chloride
CTL	cytotoxic T lymphocyte
DEA	diethylamine ($C_4H_{11}N$)
DMEM	Dulbecco's modified essential media
DNA	deoxyribose nucleic acid
dNTP	deoxynucleotide phophate(s)
dpi	days post-inoculation
df	degrees of freedom
DTT	dithiothreitol (1,4-Bis-sulfanylbutane-2,3-diol, $C_4H_{10}O_2S_2$)
E. *coli*	*Escherichia coli*
EDTA	ethylene diamine tetracetic acid 2-[2-(Bis(carboxymethyl)amino) ethyl-(carboxymethyl)amino]acetic acid ($C_{10}H_{16}N_2O_8$)
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ESP	estimated standing prevalence (= (MNI/MNA) x100)
EtBr	ethidium bromide (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide, $C_{21}H_{20}BrN_3$)
FAM	fluorescein aminohexyl

FBS	fetal bovine serum
FFU	focus forming units
g	gram
ga	gauge
GFP	green fluorescent protein
GPC	glycoprotein precursor
G_N/G_C	glycoproteins (formerly G1 and G2)
x g	times gravity
halothane	2-bromo-2-chloro-1,1,1-trifluoroethane ($C_2HBrClF_3$)
HEPA	high efficiency air particulate [filter]
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPS	hantavirus pulmonary syndrome
hr(s)	hour(s)
HTNV	Hantaan virus
HUVECs	human umbilical vein endothelial cells
Hz	hertz
ICTV	International Committee on the Taxonomy of Viruses
IFA	indirect immunofluorescence assay
IFN	interferon
Ig (A/E/G/M)	immunoglobulin (A/E/G/M)
IHC	immunohistochemistry
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
IRF-3	interferon regulatory factor 3
isoflurane	1-chloro-2,2,2-trifluoroethyl difluoromethyl ether ($C_3H_2ClF_5O$)
IU	infectious unit
kb	kilobase (10^3 bp)
kDa	kilodalton (10^3 daltons)
ketamine	2-(2-chlorophenyl)-2-methylaminocyclohexan-1-one ($C_{13}H_{16}ClNO$)
km	kilometer (10^3 meters)
L	large
LB	Luria Bertani
LD_{50}	lethal dose 50
M	medium
mA	milliamps (10^{-3} Amps)
MEM	minimum essential media
MCS	multiple cloning site
mg	milligram (10^{-3} g)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute(s)
mL	millilitre (10^{-3} litre)

mm	millimetre (10^{-3} meter)
mM	millimolar (10^{-3} molar)
MNA	minimum number alive
MNI	minimum number infected
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
µg	microgram (10^{-6} g)
µl	microlitre (10^{-6} litre)
µm	micrometer (10^{-6} meter)
µM	micromolar (10^{-6} molar)
N	nucleocapsid [protein]
NaCl	sodium chloride
NHP	non-human primates
nm	nanometers (10^{-9} meter)
NML	National Microbiology Laboratory
NSs	non-structural S [protein]
NY-1	New York-1 virus
OD	optical density
OPF	oropharyngeal fluids
ORF	open reading frame
Penicillin	(2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylate, ($C_{16}H_{17}N_2O_4S$-)
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PCR	polymerase chain reaction
PFU	plaque forming units
PHV	Prospect Hill virus
PUUV	Puumala virus
RAI	relative avidity index
recAd	recombinant Adenovirus vector
RdRp	RNA dependant RNA polymerase
Ribavrin	1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide ($C_8H_{12}N_4O_5$)
RNA	ribose nucleic acid
RNP	ribonucleocapsid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
S	small
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second(s)
SEM	standard errors of the means
SEOV	Seoul virus
SNV	Sin Nombre virus

Streptomycin	5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)- 4-[4,5-dihydroxy-6-(hydroxymethyl)-3-methylamino-tetrahydropyran-2-yl] oxy-3-hydroxy-2-methyl-tetrahydrofuran-3-carbaldehyde, ($C_{21}H_{39}N_7O_{12}$)
T-705	6-fluoro-3-hydroxy-2-pyrazinecarboxamide
TAMRA	tetramethylrhodamine
TBS	tris buffered saline
TBST	tris buffered saline with Tween 20
TNF	tumor necrosis factor
TPMV	Thottapalayam virus
TULV	Tula virus
U	unit(s)
V	volt(s)
VSV	Vesicular stomatitis virus
VSVΔG*G	replication deficient VSV pseudotyped with VSV G protein
VSVΔG-AND-GPC	VSVΔG*G pseudotyped with ANDV glycoproteins
VSVΔG-C/E1/E2	VSVΔG*G pseudotyped with hepatitis C virus core and envelope proteins
VSVΔG-HTN-GPC	VSVΔG*G pseudotyped with HTNV glycoproteins
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside ($C_{14}H_{15}BrClNO_6$)
xylazine	2-(2,6-Dimethylphenylamino)-5,6-dihydro-4H-thiazine hydrochloride ($C_{12}H_{16}N_2S{\cdot}HCL$)

CHAPTER I:

Literature Review.

1. Introduction

1.1 Brief history. Over the past decades several emerging and re-emerging pathogens have caused outbreaks in human populations. The majority of these episodes are classified as zoonotic diseases because the biological agent responsible is maintained in animal reservoirs and is transmitted, either directly or indirectly through vectors, to humans. Generally, humans are dead-end hosts of zoonotic agents and infections are often serious with life threatening complications associated with disease. Common examples of zoonotic agents include anthrax, Lyme disease, rabies and tularaemia. Among the most important in this category of diseases are those caused by hantaviruses due to the significant levels of morbidity and mortality recorded annually, worldwide. While hantaviral disease (specifically hemorrhagic fever with renal syndrome, HFRS) has probably been recognized for centuries, the association with rodents was only established in the 1970's. In 1976 the agent of Korean hemorrhagic fever (a synonym for HFRS) was identified in a striped field mouse (*Apodemus agrarius*) captured near the Hantan river in South Korea (reviewed in Jonsson & Schmaljohn 2001). The virus, aptly named Hantaan virus (HTNV), was eventually isolated and adapted to cell culture by the early 1980's and became the prototype of a novel group of viruses which was eventually given the distinction of a unique genus within the *Bunyaviridae* (Lee, Lee & Johnson 1978, French et al. 1981, Schmaljohn & Dalrymple 1983, Lee 1989). In 1993, a second disease was attributed to hantaviruses with the outbreak of hantavirus pulmonary syndrome (HPS) in

the Four Corners region of the United States. The etiological agent was isolated from samples of local deer mice (*Peromyscus maniculatus*) and eventually named Sin Nombre (meaning without name) virus (SNV, Nichol et al. 1993, Childs et al. 1994). Since the discovery of HPS, the genus *Hantavirus* has continued to grow with unique viral species and sub-species/genotypes still being discovered throughout the Americas as well as in Europe, Asia and Africa.

1.2 Taxonomy. The *Bunyaviridae* is a large and diverse group of viruses which was established in 1975 and is currently comprised of five genera, *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Hantavirus*, all of which contain viruses of medical importance to humans, and *Tospovirus*, which consists exclusively of plant pathogens (Nichol et al. 2005). The *Hantavirus* genus was conceived in 1983 to account for genetic differences observed between HTNV and previously described bunyaviruses, and by 1985 included four distinct viruses: HTNV, Seoul (SEOV), Puumala (PUUV) and Prospect Hill (PHV, Schmaljohn & Dalrymple 1983, Schmaljohn et al. 1985). Currently, the International Committee on the Taxonomy of Viruses (ICTV) recognizes 22 unique species in the *Hantavirus* genus (Table 1) based on the following criteria: A unique species must 1) demonstrate a minimum 4-fold difference in two-way cross neutralization tests, 2) have at least 7% difference in the glycoprotein precursor (GPC) and nucleocapsid (N) protein sequences, 3) occupy a unique ecological niche (i.e., a distinct primary rodent reservoir species or subspecies), and 4) not form reassortant viruses with other species (Nichol et al. 2005). Since 1993, increased interest in hantavirus research, coupled with the application of modern molecular techniques has lead to the genetic

TABLE 1
Hantaviruses and their associated reservoir hosts

Virus	Disease	Natural host	Distribution
(Order *Rodentia,* family *muridae*, subfamily *Murinae*)			
Hantaan	HFRS	*Apodemus agrarius*	China, Korea, Russia
Amur / Soochong*	HFRS	*Apodemus peninsulae*	China, Korea, Russia
Da Bie Shan	HFRS	*Niviventer confucianus*	China, Korea, Russia
Far East	HFRS	Unknown	Russia
Dobrava-Belgrade	HFRS	*Apodemus flavicollis*	Balkans
Saaremaa	HFRS	*Apodemus agrarius*	Europe
Seoul	HFRS	*Rattus norvegicus / rattus / losea*	Worldwide
Gou*	NR	*Rattus rattus*	
Thailand	HFRS	*Bandicota indica*	Thailand
Sangassou*	NR	*Hylomyscus simus*	Africa
(Order *Rodentia*, family *muridae*, subfamily *Arvicolinae*)			
Puumala	HFRS	*Myodes glareolus*	Europe
Hokkaido	HFRS	*Myodes rufocanus*	Europe
Muju	HFRS	*Myodes regulus*	Europe
Chinese Puumala*	NR	*Myodes rufocanus*	China
Vladivostok*	NR	*Myodes fortis / oceonomus*	Europe
Prospect Hill	NR	*Microtus pennsyvanicus*	Canada, USA
Bloodland Lake	NR	*Microtus ochrogaster*	Canada, USA
Khabarovsk	NR	*Microtus fortis*	Russia
Isla Vista	NR	*Microtus californicus*	South-western USA
Tula	NR	*Microtus arvalis / rossiaemeridionalis*	Europe
Topografov	NR	*Lemmus sibiricus*	Northern Europe
(Order *Rodentia*, family *muridae*, subfamily *Sigmodontinae*)			
Sin Nombre	HPS	*Peromyscus maniculatus*	Canada, USA, Mexico
Blue River	NR	*Peromyscus leucopus*	Central USA
Monongahela	HPS	*Peromyscus maniculatus*	Eastern USA
New York	HPS	*Peromyscus leucopus*	Eastern USA
Cano Delgadito	NR	*Sigmdon alstoni*	Venezuela
El Moro Canyon	NR	*Reithrodontomys megalotis*	South-western USA, Mexico
Rio Segundo	NR	*Reithrodontomys mexicanus*	Costa Rica
Limestone canyon*	NR	*Peromyscus boylii*	USA
Black Creek Canal	HPS	*Sigmdon hispidus*	South-eastern USA
Catacamas*	NR	*Oryzomys couesi*	Honduras
Muleshoe	NR	*Sigmdon hispidus*	Southern USA
Bayou	HPS	*Oryzomys palustris*	South-eastern USA
Playa de Oro*	NR	*Oryzomys couesi*	Mexico
Choclo*	HPS	*Oligoryzomys fulvescens*	Panama
Calabazo*	NR	*Zygodontomys brevicauda*	Panama

Virus	Disease	Natural host	Distribution
Jabora*	NR	*Akodon montensis*	Brazil
Maporal*	NR	*Oligoryzomys fulvescens*	Venezuela
Araucaria / Juquitiba*	HPS	*Oligoryzomys nigripes*	Brazil, Paraguay
Andes	HPS	*Oligoryzomys longicaudatus*	South America
Castelo dos Sonhos*	HPS	*Oligoryzomys moojeni*	Brazil
Pergamino	HPS	*Akodon azarae*	Central Argentina
Maciel	HPS	*Necromys benefactus*	Central Argentina
Araraquara*	HPS	*Bolomys lasiurus*	Brazil
Paranoa*	NR	*Necromys lasiurus*	Brazil
Oran	HPS	*Oligoryzomys longicaudatus*	Argentina, Bolivia
Bermejo	HPS	*Oligoryzomys chacoensis*	Argentina, Bolivia
Neembucu*	NR	*Oligoryzomys chacoensis*	Paraguay
Lechiguanas	HPS	*Oligoryzomys flavescens*	Central Argentina
Hu39694*	HPS	Unknown	Argentina
Laguna Negra	HPS	*Calomys laucha*	Bolivia, Paraguay
Rio Mamore	HPS	*Oligoryzomys microtis*	Bolivia
HTN-007*	NR	*Oligoryzomys microtis*	Peru
Rio Mearim*	NR	*Holochilus sciureus*	Brazil
Anajatuba*	NR	*Oligoryzomys fornesi*	Brazil
Alto Paraguay*	NR	*Holochilus chacoensis*	Paraguay
(Order *Insectivora*, family *Soricidae*)			
Thottapalayam	NR	*Suncus murinus*	India
Cao Bang*	NR	*Anourosorex squamipes*	Vietnam
Seewis*	NR	*Sorex araneus*	Switzerland
Tanganya*	NR	*Crocidura theresae*	Guinea
Camp Ripley*	NR	*Blarina brevicauda*	USA
Ash River*	NR	*Sorex cinereus*	USA
Jemez Springs*	NR	*Sorex monticolus*	USA

The 22 documented species of hantavirus are underlined with identified sub-strains indented below (Nichol et al. 2005). * Indicates probable hantaviruses not yet recognized by the ICTV: Alto Paraguay and Neembucu (Chu et al. 2003); Amur (Yashina et al. 2000); Anajatuba and Rio Mearim (Rosa et al. 2005); Araraquara and Juquitiba (Suzuki et al. 2004); Araucaria (Raboni et al. 2005); Ash river and Jemez Springs (Arai et al. 2008); Chinese Puumala (Zhang et al. 2007); Calabazo and Choclo (Vincent et al. 2000); Camp Ripley (Arai et al. 2007); Cao Bang (Song et al. 2007b); Castelo dos Sonhos (Johnson et al. 1999); Catacamas (Milazzo et al. 2006); Gou (Wang, Kumiko & Zhang 2000); HTN-007 (Powers et al. 1999); Hu39694 (Levis et al. 1998); Jabora (Martinez et al. unpublished data, EF495340); Limestone Canyon (Sanchez, Abbott & Nichol 2001); Maporal (Fulhorst et al. 2004); Paranoa (Melo Silva et al. unpublished data, EF57661); Playa do Oro (Chu et al. 2008); Sangassou (Klempa et al. 2006); Soochong (Jiang et al. 2007); Seewis (Song et al. 2007a); Tanganya (Klempa et al. 2007); Vladivostok (Kariwa et al. 1999). HPS, hantavirus pulmonary syndrome; HFRS, hemorrhagic fever with renal syndrome; NR, none reported.

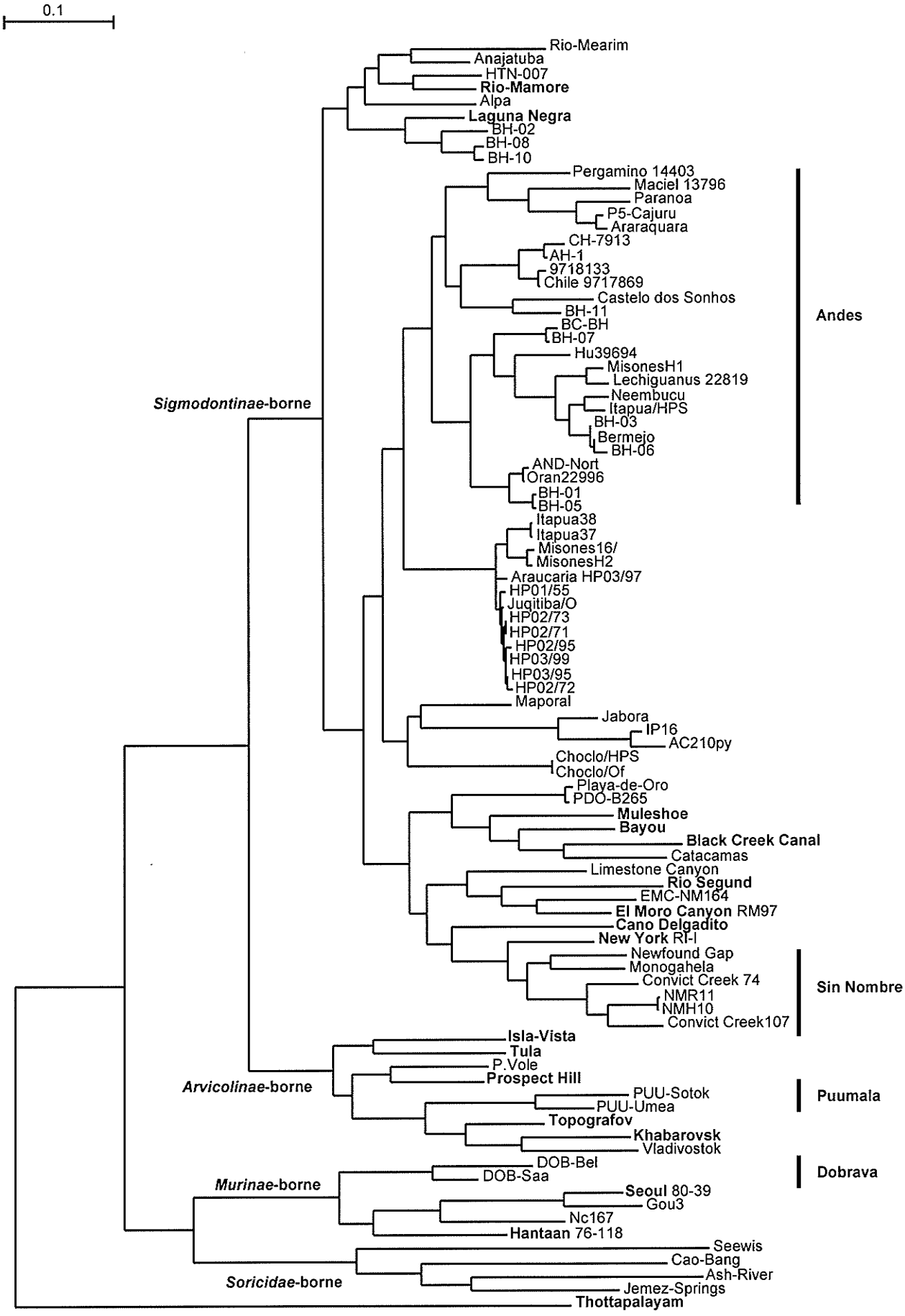
identification and/or isolation of several probable hantaviruses (Table 1). Although these tentative members are not currently classified as novel species or strains of hantaviruses by the ICTV, many of them will undoubtedly be included as the genus continues to expand.

1.3 Distribution. Hantaviruses have been documented essentially worldwide wherever climate and geography allow rodents to thrive. They are serologically, phylogenetically, geographically and clinically divided into two general categories, Old and New World hantaviruses (Figure 1.1). Old World hantaviruses are associated with rodents of the *Arvicolinae* and *Murinae* subfamilies and are commonly found in Europe and Asia. Recently, novel hantavirus sequences (designated Sangassou virus) were documented in tissue samples from an African wood mouse (*Hylomyscus simus*), providing the first evidence that Old World hantaviruses are present in Africa (Klempa et al. 2006). New World hantaviruses are associated with *Sigmodontinae* subfamily rodents and are found throughout the Americas. *Arvicolinae*-borne hantaviruses also exist in North America and include PHV and Isla Vista viruses. Generally, hantaviruses occupy a specific and limited geographical niche based on the distribution of their rodent host. The exception is SEOV, which is found essentially worldwide due to the distribution of rats (*Rattus norvegicus*, *R. rattus*), the virus' natural host.

2. Hantavirus Biology

2.1 Genomic organization. Hantaviruses are single, negative strand RNA viruses with a tripartite genome (Schmaljohn & Hooper 2001). The 3' and 5' termini of each genomic

Figure 1.1. Phylogram of Old and New World hantaviruses. Partial S segment sequences (nucleotides 57-694) were analyzed using PHYLIP software and Maximum Likelihood methods. Species of hantavirus recognized by the ICTV are highlighted in bold (Thailand virus not shown). Modified with permission from Dr. Hideki Ebihara (Institute of Medical Science, University of Tokyo, Tokyo, Japan, unpublished data).


0.1
Rio-Mearim
Anajatuba
HTN-007
Rio-Mamore
Alpa
Laguna Negra
BH-02
BH-08
BH-10
Pergamino 14403
Maciel 13796
Paranoa
P5-Cajuru
Araraquara
CH-7913
AH-1
9718133
Chile 9717869
Castelo dos Sonhos
BH-11
BC-BH
BH-07
Hu39694
MisonesH1
Lechiguanus 22819
Neembucu
Itapua/HPS
BH-03
Bermejo
BH-06
AND-Nort
Oran22996
BH-01
BH-05
Itapua38
Itapua37
Misones16/
MisonesH2
Araucaria HP03/97
HP01/55
Jugitiba/O
HP02/73
HP02/71
HP02/95
HP03/99
HP03/95
HP02/72
Maporal
Jabora
IP16
AC210py
Choclo/HPS
Choclo/Of
Playa-de-Oro
PDO-B265
Muleshoe
Bayou
Black Creek Canal
Catacamas
Limestone Canyon
Rio Segund
EMC-NM164
El Moro Canyon RM97
Cano Delgadito
New York RI-I
Newfound Gap
Monogahela
Convict Creek 74
NMR11
NMH10
Convict Creek107
Isla-Vista
Tula
P.Vole
Prospect Hill
PUU-Sotok
PUU-Umea
Topografov
Khabarovsk
Vladivostok
DOB-Bel
DOB-Saa
Seoul 80-39
Gou3
Nc167
Hantaan 76-118
Seewis
Cao-Bang
Ash-River
Jemez-Springs
Thottapalayam
Sigmodontinae-borne
Arvicolinae-borne
Murinae-borne
Soricidae-borne
Andes
Sin Nombre
Puumala
Dobrava

segment contain non-translated regions, parts of which are composed of conserved and partially complimentary inverted repeats. The consensus termini sequences of the three segments (5' -AUCAUCAUCUG... and 3' -UAGUAGUAUGC...) are unique to hantaviruses and help identify them from other bunyaviruses (Schmaljohn & Dalrymple 1983). While the biological function is unknown, these repeats are predicted to form panhandle-like structures resulting in the genomic segments forming non-covalently closed circles, and as such may play a role in viral replication and/or morphogenesis (Flick et al. 2003, Mir & Panganiban 2005).

In comparison with most other viruses, hantaviruses have the coding capacity for a minimal amount of proteins (Figure 1.2). The large (L) segment is between 6530 and 6562 nucleotides in length and contains a single open reading frame (ORF) which encodes the RNA-dependent RNA polymerase (RdRp). The RdRp is composed of between 2151 and 2156 amino acids (aa) and is estimated to be 250 kDa in size (Kukkonen, Vaheri & Plyusnin 2005). It is responsible for transcription and replication of the genomic segments and, although the processes are not completely understood for hantaviruses, it is assumed the RdRp provides endonuclease, transcriptase, replicase and possibly RNA-helicase functions (Khaiboullina, Morzunov & St Jeor 2005). Based on the mutation rate recorded with some hantaviruses, the RdRp does not appear to have proof reading capabilities (Kukkonen, Vaheri & Plyusnin 2005).

The medium (M) segment ranges in size from 3616 to 3696 nucleotides and encodes the two glycoproteins, G_N and G_C (formerly G1 and G2 respectively), which are 68-76 kDa and 52-58 kDa respectively. The glycoprotein ORF is transcribed and translated as a single GPC (approximately 1135 aa) which is co-translationally cleaved

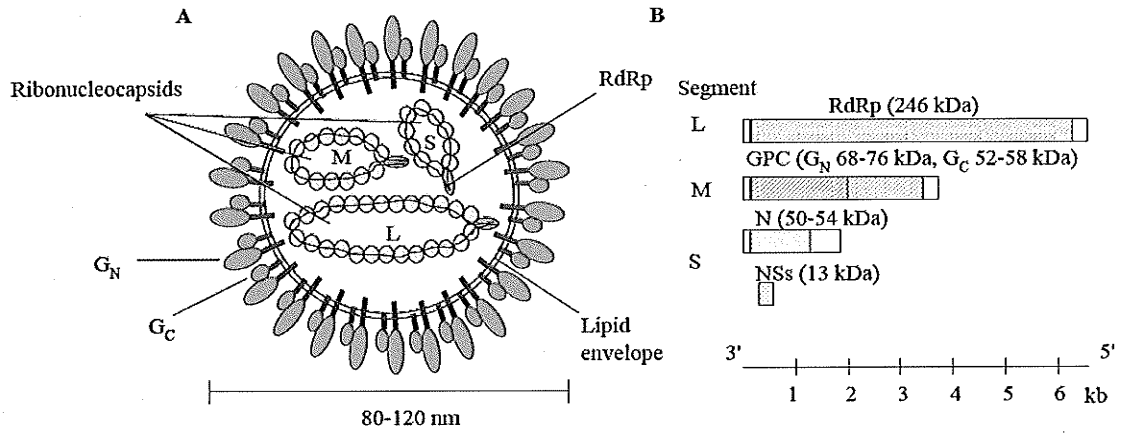


FIGURE 1.2. Schematic illustration of the hantavirus virion and genome. (A) Cross section of a hantavirus particle. Small, medium and large (S, M and L) genomic RNA segments are encapsulated in nucleoprotein (N) to form ribonucleocapsids (RNP). The RNP structures associate with an RNA dependant RNA polymerase (RdRp) and are packaged within a lipid envelope containing the hantavirus glycoproteins (G_N and G_C).
(B) Diagram of the hantavirus genome. The tri-segmented, negative-stranded RNA genome of hantavirus encodes for a minimal amount of proteins. The L and M segments contain one open reading frame each and code for the RdRp and the glycoprotein precursor (GPC) respectively. The GPC is post-translationally cleaved into G_N and G_C. The S segment codes for the N protein and, in some hantavirus species, a non-structural protein (NSs).

into G_N, approximately 665 aa, and G_C, approximately 470 aa (Shi & Elliott 2002). Both glycoproteins are type I integral transmembrane proteins, meaning the amino-termini face the endoplasmic reticulum (ER) lumen and the carboxy-termini are in the cytoplasm. The G_N and G_C proteins are responsible for the early stages of infection and, although attachment and fusion activities have not been assigned to either glycoprotein, a recent study has suggested the fusion domain may reside in G_C (Tischler et al. 2005b).

The small (S) segment contains the most variability of the three segments with respect to size and coding capacity. It varies between 1696 and 2059 nucleotides in length and codes for the N protein. The N protein contains between 429 to 433 aa residues and is approximately 50-54 kDa in size (Kaukinen, Vaheri & Plyusnin 2005). It is the most abundant viral protein produced and is the principal structural component of the viral capsid. The N protein has many functions including protecting RNA from nuclease degradation, forming ribonucleocapsid (RNP) structures and interacting with the glycoproteins and RdRp during replication and morphogenesis. In addition, the N protein has been shown to interact with cellular proteins (i.e., small ubiquitin-like modifier-1 related molecules), leading to the hypothesis that it also functions to regulate some host cell processes (Lee et al. 2003, Maeda et al. 2003, Kaukinen, Vaheri & Plyusnin 2005).

A recent study by Jääskeläinen and colleagues (2007) suggest that, similar to other members of the *Bunyaviridae*, the hantavirus S segment also encodes a second protein, approximately 13 kDa in size, from an overlapping (+1) ORF between nucleotides 83-355. Currently, the protein, termed non-structural S (NSs) protein, has only been detected in Tula (TULV) and PUUV. Although NSs ORFs are present in most hantaviruses, alignment of S segment sequences of *murinae*-borne hantaviruses reveals

no predicted consensus NSs ORF exist. Further, some viral strains of the same species (i.e., TULV) code for the NSs, while others do not (Jääskeläinen et al. 2008). The function of the NSs requires further investigation, although similar to other bunyavirus NSs proteins, it is assumed that it functions to regulate the host cell antiviral response (Jääskeläinen et al. 2007, 2008).

2.2 Viral lifecycle. Virus entry into target cells is mediated by interaction with $\alpha_v\beta_3$ or $\alpha_v\beta_1$ integrins in an RGD (arginine-glycine-aspartic acid) independent manner for pathogenic and non-pathogenic hantaviruses, respectively (Figure 1.3, Gavrilovskaya et al. 1998, 1999). A recent study by Raymond and colleagues (2005) demonstrated that pathogenic hantaviruses preferentially bind inactive $\alpha_v\beta_3$ integrins through interaction with a plexin-semaphore-integrin domain which is exposed at the apex of the integrin following a conformational change to its bent (inactive) form. While secondary receptors have been suggested, for example a novel 30 kDa surface protein was recently described, these reports have yet to be confirmed (Kim et al. 2002). Regardless, following receptor recognition, virus particles are endocytosed and eventually fuse with the endosomal membrane after acidification of the vesicle. Low pH is believed to create a conformational change in the glycoproteins which initiates fusion and allows the virion contents to gain access to the cellular cytoplasm, the site of viral replication (Ogino et al. 2004).

Messenger RNA (mRNA) synthesis is initiated from all three genomic segments shortly after fusion, with N mRNA detected first and at highest levels, followed by GPC

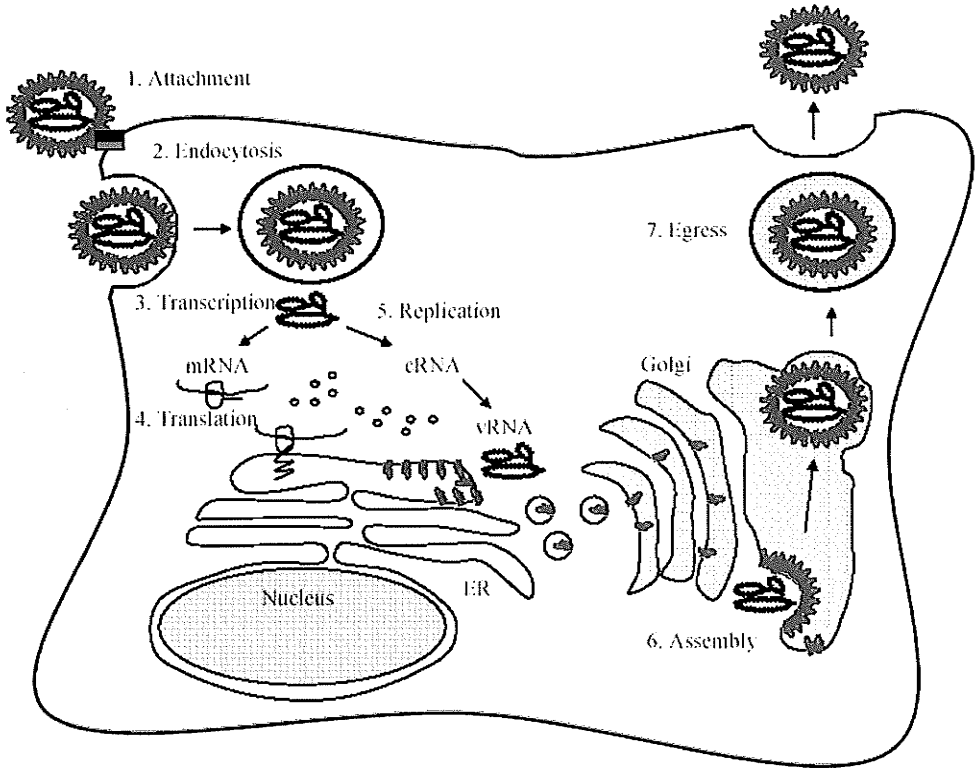


FIGURE 1.3. Replication cycle of hantaviruses. The major events in the hantavirus lifecycle are numbered as follows: 1. attachment to cells via $\alpha_v\beta_3$ or $\alpha_v\beta_1$ integrins (denoted by black and gray rectangles); 2. entry via endocytosis which is followed by pH dependant membrane fusion allowing the virion contents access to the cellular cytoplasm; 3. primary transcription; 4. translation of viral proteins (N protein; small yellow shaded circles, G_N and G_C; blue ovals); 5. replication of the viral genomic segments via a cRNA intermediate, 6. assembly of virions in the Golgi complex (budding at the plasma membrane may also occur for some hantaviruses, though it is not shown in this diagram); 7. egress and exocytosis. ER; endoplasmic reticulum.

mRNA and lastly, and at the lowest levels, L mRNA (Hutchinson, Peters & Nichol 1996). The synthesis of mRNA is initiated by the RdRp using "cap-snatching" mechanisms and is hypothesized to continue following a prime-realign model (Garcin et al. 1995, Jonsson & Schmaljohn 2001, Kukkonen, Vaheri & Plyusnin 2005). The 3' end of the mRNA is not polyadenylated and is truncated by approximately 100 bases compared to genomic RNA, although the transcriptional termination signals remains to be clarified (Schmaljohn, Schmaljohn & Dalrymple 1987, Hutchinson, Peters & Nichol 1996).

Protein translation occurs shortly after mRNA synthesis begins and is carried out by the host cell machinery. The L and S segment mRNAs are translated on membrane bound ribosomes and the proteins have been shown to accumulate in the perinuclear regions of the cell (Kukkonen, Vaheri & Plyusnin 2005). The GPC is translated on ribosomes bound to the ER where the G_N and G_C proteins are co-translationally cleaved at a conserved pentapeptide (WASSA) motif by unknown cellular peptidases (Lober et al. 2001). Intact GPC has not been detected in virus infected cells (Schmaljohn, Schmaljohn & Dalrymple 1987). The glycoproteins are further modified with N-linked oligosaccharides in the ER with five glycosylation sites present in G_N (although only four appear to glycosylated) and one in G_C (Shi & Elliott 2004). During the process of glycosylation, the glycoproteins form heterodimers and are transported to the Golgi compartment, the site of viral maturation. Despite Golgi retention however, the oligosaccharides remain in the high mannose form (Shi & Elliott 2004). Individually expressed G_N and G_C proteins are retained in the ER, demonstrating that G_N/G_C

complexes are required for Golgi localization (Ruusala et al. 1992, Khaiboullina, Morzunov & St Jeor 2005).

At some point after primary transcription the function of the RdRp shifts to complementary RNA (cRNA) synthesis (Kukkonen, Vaheri & Plyusnin 2005). The cRNA functions as a template for viral RNA, which in turn serves as template for additional mRNA as well as viral genome for packaging into progeny virions. The mechanism by which the RdRp function changes from mRNA production to viral replication remains unknown. It has been suggested that the switch may result from a limitation of host mRNA for cap-snatching due to impaired host cell protein synthesis. However, unlike some bunyaviruses (i.e., Rift Valley fever virus), hantaviruses are not known to cause host cell shut-off of protein synthesis (Kukkonen, Vaheri & Plyusnin 2005). It has also been purposed that the accumulation of N protein drives the RdRp to cRNA production possibly by altering the secondary structure of the cRNA, or by masking the transcription termination signals which result in the truncated 3' ends of mRNA. This is a plausible hypothesis since both cRNA and viral RNA are fully encapsidated by N protein, while the mRNA is not (Kukkonen, Vaheri & Plyusnin 2005).

Following transcription and replication the process of viral assembly begins. As viral RNA is produced from cRNA it is encapsidated by N protein creating the RNP. The RNP structures associate with RdRp, and together bud into the Golgi compartment where they interact with the glycoproteins to facilitate budding. Hantaviruses do not code for a matrix protein, meaning the RNP structures directly interact with the glycoproteins. While this process remains unclear, it has been suggested that it may be facilitated through interaction of the RNP with the long cytoplasmic tail of G_N (Pettersson 1991). It

is unknown how individual virions assure each genomic segment is packaged, though experimental evidence for SNV suggests viral particles contain equimolar amounts of each (Hutchinson, Peters & Nichol 1996). Golgi maturation is a hallmark of the *Bunyaviridae*. From the Golgi, vesicles containing virions are transported to the plasma membrane where progeny viruses are released by exocytosis. Some New World hantaviruses (i.e., SNV and Black Creek Canal, BCCV) may also mature from the plasma membrane, however data on this is limited (Goldsmith et al. 1995, Ravkov, Nichol & Compans 1997). The resulting virions are circular, pleomorphic particles approximately 100 nm (80-120 nm) in diameter (Figure 1.2). Virus particles have a distinct pattern which, when viewed under the electron microscope, has a square-grid or checkerboard-like appearance (McCormick et al. 1982).

3. Ecology of Hantaviruses in Nature

3.1 Maintenance. In nature, hantaviruses are maintained in specific rodents with which they are believed to have co-evolved for centuries. It is generally assumed that individual species are predominantly associated with one rodent host; however there are exceptions where one virus may have two closely related rodent hosts (Table 1). Genetic analysis has revealed a high degree of nucleotide variation within individual Old and New World hantavirus species (Plyusnin et al. 1995, Monroe et al. 1999). Within *Peromyscus*-borne hantaviruses, the nucleotide variation of the virus is closely linked to genetic variation within the rodent hosts, further supporting co-speciation of virus and host (Morzunov et al. 1998, Drebot et al. 2001). Occasionally, non-rodent hosts (i.e., cats and dogs) have demonstrated the presence of hantavirus specific antibodies, however these likely

represent non-productive infections due to virus spillover, possibly occurring as a result of predation or environmental contaminate (Leighton et al. 2001).

Evidence of non-rodent-borne hantaviruses have begun to accumulate following the identification of a novel clade of shrew-borne hantaviruses (Table 1, Figure 1.1). Although initially isolated in 1964 from an Asian house or musk shrew (*Suncus murinus*, Order *Insectivora*, family *Soricidae*) captured in India, Thottapalayam virus (TPMV) has only recently been classified as a hantavirus (Carey et al. 1971, Yadav, Vincent & Nichol 2007). Since the characterization of TPMV, other potential insectivore-borne hantaviruses have been genetically described including Camp Ripley virus from Short-tailed shrews (*Blarina brevicauda*), Ash River virus from Masked shrews (*Sorex cinereus*) and Jemez Springs virus from Dusky shrews (*Sorex monticolus*) in the United States (Arai et al. 2007, 2008), Seewis virus from Eurasian shrews (*Sorex araneus*) in Switzerland (Song et al. 2007a), Tanganya virus from Therese shrews (*Crocidura theresae*) in Guinea (Klempa et al. 2007) and Cao Bang virus from Chinese mole shrews (*Anourosorex squamipes*) in Vietnam (Song et al. 2007b). Although the association of some of these viruses with insectivore hosts has not been definitively proven, they are highly divergent from all other known hantavirus species which supports the role of a non-*murinae*, *arvicolinae* or *sigmodontinae* host.

3.2 Hantavirus infections in the natural host. A defining characteristic of hantaviruses is their ability to establish persistent, long-term (probably lifelong) infections in rodent hosts (Lee et al. 1981, Gavrilovskaya et al. 1983, Hutchinson, Rollin & Peters 1998, Bernshtein et al. 1999, Botten et al. 2000, 2003). It is generally accepted that rodents are

asymptomatic carriers with no obvious deleterious effects caused by the virus (Childs et al. 1989, Mills et al. 2007). Although it has been suggested that SNV and New York-1 (NY-1) viruses may cause pulmonary edema and liver pathology in wild-caught rodents, these findings have not been reproduced in experimentally infected deer mice and are contrary to similar studies with other hantaviruses (Lyubsky et al. 1996, Netski, Thran & St Jeor 1999, Botten et al. 2000, 2003). More recently, a negative impact of hantavirus infection on host fitness was proposed based on observations that PUUV infected bank voles (*Myodes glareolus*) had a higher overwinter mortality rate than uninfected voles (Kallio et al. 2007). The significance of these findings remains unclear and warrant further investigation.

Following infection, rodents quickly generate a humoral immune response. Neutralizing antibodies have been detected as early as 7 days post inoculation (dpi) with focus reduction neutralizing titers documented as high as 20,480 in some chronically infected rodents (Botten et al. 2000, 2003). Despite a vigorous antibody response, the virus quickly disseminates throughout the rodent and establishes a persistent infection. In one study, SNV N antigen was detected in heart, lung, liver, spleen and kidney by 7 dpi, as well as pancreas, salivary glands and white and brown fat by 14 dpi in experimentally infected deer mice (Botten et al. 2000). Similar patterns have been documented with other hantaviruses in experimentally infected hosts including, BCCV in cotton rats (*Sigmodon hispidus*, Hutchinson, Rollin & Peters 1998), Cano Delgadito virus in Alstoni cotton rats (*Sigmodon alstoni*, Fulhorst et al. 2002) and PUUV in bank voles (Yanagihara, Amyx & Gajdusek 1985) as well as in naturally infected deer mice (Netski, Thran & St Jeor 1999).

Data on the cytotoxic T-lymphocyte (CTL) response in rodent hosts are scarce due to limited availability of reagents. Nevertheless, studies by Schountz and colleagues (2007) suggest the CTL response in deer mice infected with SNV is predominantly regulatory and anti-inflammatory which limits the immunopathology in the rodent and contribute to the establishment of persistent infection. Similarly, regulatory T cells were shown to mediate persistent Seoul virus infection in rats (Easterbrook, Zink & Klein 2007).

Seroprevalence in rodents is positively associated with age (generally inferred from weight) with increased prevalence often observed in adults (Cantoni et al. 2001, Calisher et al. 2007, Mills et al. 2007). This observation may simply be based on probability, in that older rodents are more likely to come in contact with other rodents harbouring the virus or with behavioural traits (i.e., increased aggression or breeding) associated with age (Childs et al. 1989, Hinson et al. 2004). Alternatively, physiological maturity may influence the susceptibility of rodent hosts through hormonal changes associated with puberty, leading to increased infection rates in these demographics (Klein, Bird & Glass 2000). Field studies conducted on several hantavirus/host relationships have also documented sex related differences, with males more often infected than females (Mills et al. 1997, 1998, 2007, Bernshtein et al. 1999, Cantoni et al. 2001, Calisher et al. 2007). The significance of the sex related difference is unclear, although these findings may also be partially explained by the habits of male and female rodents. For example, male rodents tend to have a larger home range; therefore are more likely to encounter other rodents and thus have a higher probability of becoming infected (Banfield 1974). Alternatively, males and females may not be equally susceptible to

infection. Intracage transmission studies with Andes virus (ANDV) showed that male *O. longicaudatus* were more susceptible than females (Padula et al. 2004). However, experimental studies with SEOV and BCCV have demonstrated male and female animals are equally susceptible (Hutchinson et al. 2000, Klein, Bird & Glass 2000). In addition, some field studies have documented a similar incidence of SNV infection in male and female deer mice, suggesting in at least some populations, they are equally susceptible to hantavirus infection (Calisher et al. 1999).

3.3 Virus transmission between rodents. Unlike other zoonotic agents, hantaviruses have a relatively simple enzootic cycle which is thought to only involve the rodent host. There are no known secondary hosts implicated in transmission cycles, meaning specific rodent species serve both as reservoir and vector for individual hantaviruses. Arthropods and ectoparasites have been proposed to play a role in viral transmission, though this remains to be conclusively demonstrated (Tsai 1987, Houck, Qin & Roberts 2001). The role of secondary vectors should not be ruled out, especially considering all other bunyaviruses are transmitted by arthropod vectors, however, current data indicates hantavirus transmission principally occurs via rodents. Studies on the environmental stability of hantaviruses have shown under some conditions these viruses retain infectivity over prolonged periods of time (up to 14 days) and can result in indirect transmission of virus to naïve rodents. While such mechanisms of transmission requires further study, it does suggest a potential role for fomites, such as communal bedding or food sources, as natural sinks of virus (Kallio et al. 2006).

In nature, the spread and maintenance of hantaviruses may be influenced by several factors including rodent social behaviour, population density and structure, diet, and environmental conditions. Despite numerous field and laboratory-based studies, surprisingly little is known regarding the mechanisms involved in virus persistence and transmission (Hjelle & Yates 2001). Hantaviruses are horizontally transmitted between rodents, most likely through virus contaminated oral secretions, urine or feces from infected rodents (Lee et al. 1981, Yanagihara, Amyx & Gajdusek 1985, Bernshtein et al. 1999, Kariwa et al. 1998, Hutchinson et al. 2000). Vertical transmission of hantaviruses in nature has not been observed and it is thought that pups borne of infected dams are protected by maternal antibodies transferred both *in utero* and through breast milk (Dohmae, Koshimizu & Nishimune 1993, Borucki et al. 2000). The exception to this may be BCCV infections in cotton rats, with one study demonstrating pups borne from infected dams, were themselves infected in spite of the transfer of maternal antibodies (Hutchinson et al. 2000).

The dynamics of virus shedding from individual infected rodents are unknown; however it seems unlikely that every infected host is continuously infectious and able to transmit virus. Few studies have focused on the detection of virus in rodent excreta and secreta. It appears some HFRS associated viruses (i.e., PUUV) are readily detected in urine and oral secretions from infected rodents while HPS viruses (i.e., ANDV) are present primarily in host oral fluids, suggesting possible differences in routes and frequency of transmission between Old and New World hantaviruses (Yanagihara, Amyx & Gajdusek 1985, Padula et al. 2004). Furthermore, it is unknown if outside stimuli (i.e.,

food source, climate) enhance viral shedding, or if recrudescence is mainly associated with certain host biological functions (i.e., hormones, breeding).

Sex related differences in the ability to shed virus have been suggested, with one study indicating that SEOV infected male rats shed virus longer than infected females. While more research is necessary, these differences may be due to differing immune responses between the sexes, with females capable of mounting an efficient immune response which controlls the virus, while the immune response in males is somewhat deficient or impaired (Klein et al. 2004). Interestingly, experimental studies with ANDV demonstrated viral transmission was most efficient when both the donor and recipient rodent were of the same gender (Padula et al. 2004).

Several zoonotic pathogens (e.g., rabies) are known to cause changes in the social behaviour of host animals. Aggressive encounters between rodents are hypothesized to be an important mechanism of hantavirus dissemination between rodents, as suggested by some studies which have observed an increased proportion of infected rodents with obvious scars and wounds (Mills et al. 1997, Hinson et al. 2004, Calisher et al. 2007). It remains unclear whether aggression in rodents increases the probability of infection, or if infection increases aggressive tendencies in rodents. Studies addressing this question are limited, however, there is some experimental evidence that suggest SEOV infection increases aggression in Norway rats and aggression was positively associated with viral titers in lung, kidney and testis implying the possibility of increased viral transmission from these animals (Klein et al. 2004). Hantaviruses are not known to cross the blood-brain barrier, meaning if they were to modulate host behaviour it may be through

regulation of hormone (i.e., androgens) levels (Klein & Calisher 2007). Further study on this topic is required.

Not all studies implicate aggressive encounters in hantavirus transmission. For example, studies with ANDV found that intracage transmission occurred efficiently without evidence of fighting. In these studies the authors suggest wounding is not the primary mechanism of viral transmission and implicate non-aggressive behaviours (i.e., grooming and mutual exploration) in transfer of virus to naive hosts (Padula et al. 2004). Additionally, a review of recently infected deer mice (as determined by documented seroconversion in consecutive trapping sessions) found no relationship between the presence of scars and seroconversion (Douglass et al. 2007).

Regardless of the type of encounters (i.e., aggressive or not) it seems logical that hantavirus prevalence is associated with population density. For example, with an increase in encounters between deer mice (potentially harbouring SNV), the more likely a rodent will become infected. However the association between density and prevalence is not well understood with some field studies documenting a positive relationship (Olsson et al. 2002), while others see no density related affect (Boone et al. 1998, Cantoni et al. 2001), or an inverse association (Abbott, Ksiazek & Mills 1999, Douglass et al. 2001, Pearce-Duvet et al. 2006). Recently two groups reviewed findings of 10 years of field data collected in Montana (Madhav et al. 2007) and Arizona, Montana, Colorado and New Mexico (Calisher et al. 2007) with both studies describing inter- and intra-annual patterns of prevalence. Between successive years both studies documented a delayed density dependant relationship of SNV infection, where the prevalence of SNV correlated best with population and infection dynamics recorded the previous fall (Calisher et al.

2007, Madhav et al. 2007). In addition, both studies found intra-annual or seasonal patterns of infection, where seroprevalence was highest in spring when rodent numbers were lowest with a sharp decline in prevalence occurring with the recruitment (i.e., breeding or immigration) of new individuals into a given habitat. Similar seasonal patterns of infection were observed in shorter field studies which may explain the inconsistencies regarding the affect of density on prevalence (Abbott, Ksiazek & Mills 1999, Douglass et al. 2001, Kuenzi et al. 2005, Mills et al. 2007). Biodiversity may also have an effect on prevalence, but unlike density it is hypothesized to have an inverse association (Mills 2005). For example, the higher the rodent diversity in a given ecosystem, the less likely a specific rodent will encounter infected rodents of its own kind, resulting in decreased transmission of specific viruses.

Environmental variables like temperature, precipitation, and habitat and food availability directly affect rodent populations and therefore have been examined as predictors of prevalence (Glass et al. 2002, Mills 2005). The ultimate goal of many long-term field studies has been to use these variables in geographical modelling to predict HPS outbreaks. The success of these models has been limited thus far, which highlights the complex ecology of these viruses in nature. More recently, it has been speculated that these factors may influence hantavirus transmission in more indirect ways. For example, specific compounds (i.e., 6-methoxy-2-benzoxazolinone and phytoestrogens) present in certain vegetation types can affect rodent reproduction rates and immune functions, possibly leading to viral recrudescence and transmission (Klein & Calisher 2007). The influence of such compounds in the diet of hantavirus infected rodents warrants further investigation, however, this hypothesis may be partially supported by some ecological

studies which have documented a positive relationship between certain vegetation types and hantavirus prevalence (Madhav et al. 2007).

4. Human Disease

4.1 Epidemiology. The geographical distribution of rodents dictates which viruses are indigenous in specific regions and thus disease patterns in humans. Generally, HPS occurs in the Americas, while HFRS is predominantly a disease of Europe and Eastern Asia, although imported cases of each have occurred on other continents (Murgue et al. 2002, Reynolds et al. 2007). In Europe, Sweden, Finland and Russia account for the majority of documented HFRS cases. While other members of European Union are responsible for a fraction of cases, considerable outbreaks have been recorded in countries like Belgium, France and Germany (Mailles et al. 2005). In Eastern Asia, HFRS is more common with over 100,000 cases reported annually in China, Korea and Far East Russia (Lee 1996). Although disease has been observed year round, cases of HFRS are most common in spring and fall with the majority of cases occurring synchronously with peak rodent populations (Sauvage et al. 2002). The majority of HFRS cases occur in rural settings and are epidemiologically linked to agriculture activities, which may explain why disease is more common in males with a ratio of approximately 4 to 1 (Niklasson et al. 1993).

Compared with HFRS, cases of HPS are infrequent, although over the last 15 years greater than 2000 cases have been recorded in the Americas. Since 1993, cases of HPS have been recorded annually in the United States and Canada, with retrospective cases confirmed in both countries dating back to as early as 1959 (Khan et al. 1996,

Drebot, Artsob & Werker 2000, Rooney et al. 2004). With the exception of the initial outbreak of SNV in 1993, HPS tends to occur as sporadic and isolated events, with only a few instances of multiple cases occurring at one time in the same location of North America. In Canada, only one cluster of cases has been documented (Webster et al. 2007). In North America, HPS disease in children is rare and tends to be milder, sometimes not meeting the case definition (Armstrong et al. 1995, Khan et al. 1996, Webster et al. 2007).

The majority of HPS cases occur in South American countries including Argentina, Brazil, Chile, Bolivia, Paraguay, Uruguay and Central American countries (i.e., Panama). Argentina and Brazil account for the bulk of the South American cases of HPS, although the true incidence of disease may be under reported in some of these counties since HPS typically occurs in rural settings where medical diagnostic capabilities tend to be the lowest. Multiple cases of HPS occurring at once are more common in South America, possibly due to larger rural populations in conjunction with increased exposure to a variety of different viral species. Infection in children also appears to be more prevalent in South America when compared to North America, with disease characteristics in children similar to that of adults (Enria et al. 2001)

Like HFRS, cases of HPS have been linked to agriculture, however a large proportion of disease is associated with peridomestic activities reflecting the different behavioural patterns associated with rodent hosts (Drebot, Artsob & Werker 2000, Hjelle & Glass 2000, Douglass et al. 2003). For example, cases of HPS are often linked to spring clean up of outlying or seasonal buildings (i.e. barns, garages, cabins) without adequate personal protection. Rodents such as deer mice typically invade these areas,

while voles tend not to enter human dwellings. In addition, HPS has been epidemiologically linked to other activities which bring humans in close proximity to rodents or their excreta/secreta including, but not limited to, handling rodents, rodent infestation, disturbing rodent nests, and sleeping on the ground (Mills 2005).

4.1.2 Hantavirus disease in Canada. As of 1 May 2008, a total of 70 laboratory confirmed cases of HPS have been documented in Canada. Since 1994, when diagnostic testing was initiated in Canada, an average of 4-5 cases have been diagnosed annually (Figure 1.4A) with yearly numbers fluctuating between two (1999 and 2001) and eight cases (1994). Cases of HPS have been diagnosed in every month, although there is an obvious spring peak of infections, with approximately half of cases documented in Canada occurring between April and June (37/70, 52.8%, Figure 1.4B). The average age of cases has been 40 years old (range 7–76, Figure 1.4C) and the majority of cases have been male (46/70, 65.7%). The current documented case fatality rate in Canada is 30.3%, with higher mortality rates observed in females at 37.5% (9/24), compared with 26.2% (11/42, the outcome of four male patients is unknown) for males. Although genetic identification of the infecting virus is not always possible, when appropriate samples (i.e., acute whole blood) were available for analysis, SNV was always identified as the etiological agent. Epidemiological studies conducted following cases of HPS revealed the trends of exposure to virus outlined above appear to hold true for Canada (Figure 1.4D). Despite the detection of SNV infected mice from across Canada, the overwhelming majority of cases (69/70, 98.6%) have occurred in the western provinces (British Columbia, Alberta, Saskatchewan and Manitoba) with a single case in eastern Canada

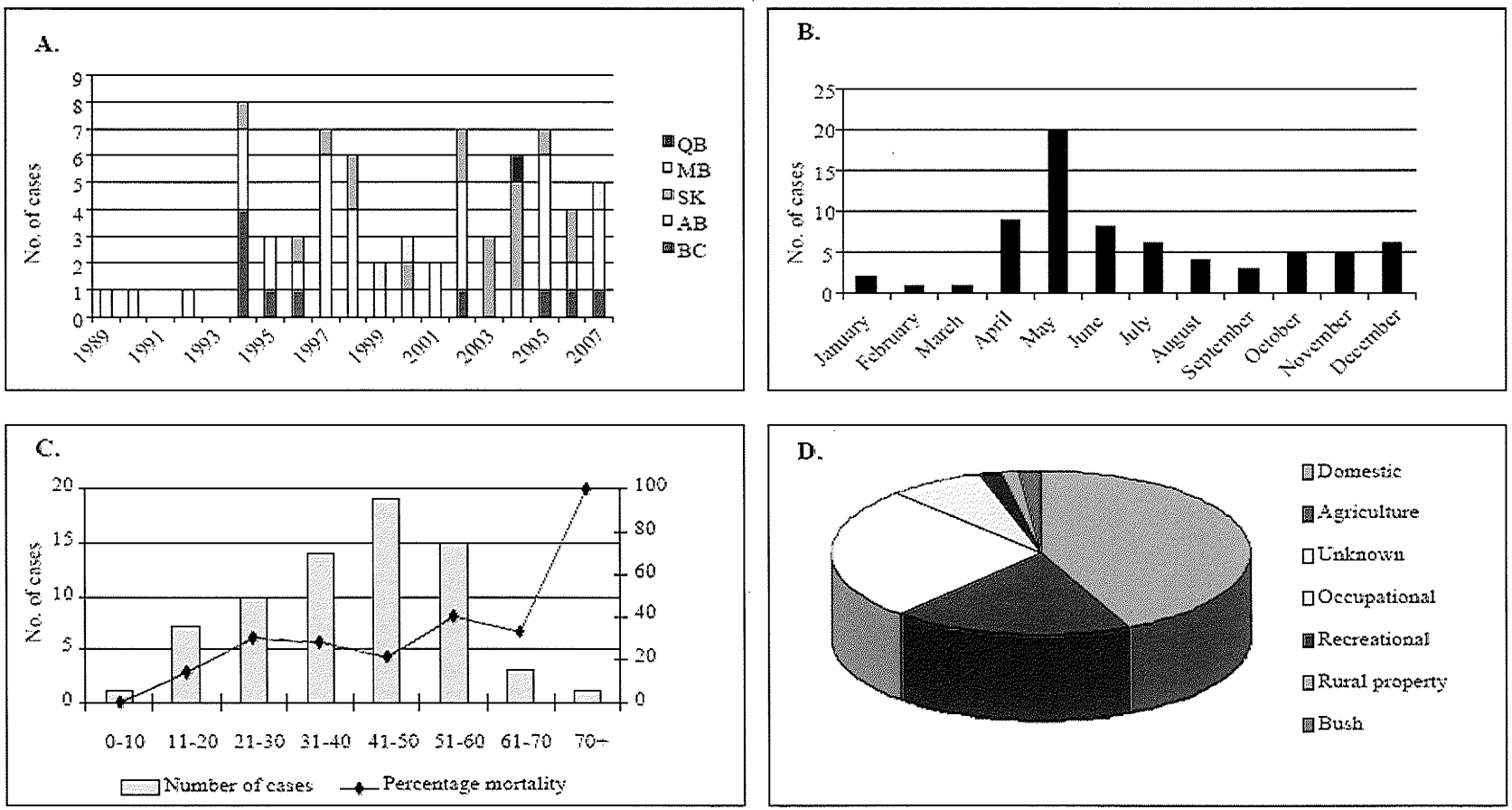


Figure 1.4. Epidemiology of hantavirus pulmonary syndrome in Canada. (A) Number of confirmed cases of HPS per year by province. (B) Predicted month of exposure. (C) Age distribution and fatality rate of cases. (D) Presumed activity/location of exposure.

(Quebec). A similar bias towards western cases of HPS is also observed in the United States (Douglass, Calisher & Bradley 2005). Nucleotide sequence analysis of SNV M and S segment amplicons from infected deer mice collected in Canada has demonstrated polymorphisms which correlate with the geographic location of collection (Drebot et al. 2001). However, it is uncertain if Western strains of SNV are more virulent than Eastern strains, or if other, as of yet undetermined factors, are responsible for the disproportionate number of cases of HPS occurring in Western Canada.

4.2 Transmission to humans. The factors involved in the transmission of hantaviruses to humans remain vague, however they are clearly linked to rodent populations and epizootic transmission of virus. The best example of this is the periodicity of PUUV infections in humans in relation to vole density and seroprevalence. Bank vole density is cyclical and peaks at 3-4 year intervals in accordance with mast (i.e., acorn) years. The incidence of PUUV infections in humans is highest concurrent with peak bank vole population and seroprevalence (Niklasson et al. 1995). Similar patterns of human infections with New World hantaviruses (i.e., SNV) are not observed probably because, unlike voles, deer mice do not undergo exceptional population explosions on a regular basis, although annual population levels are variable due to factors like extended breeding seasons, or food availability (Banfield 1974). This difference may partially explain why annual incidence of HPS in North America is reasonably static compared with PUUV-related HFRS.

Humans are most commonly infected with hantaviruses by inhalation of contaminated rodent excreta and/or secreta, although direct transmission via rodent bites

also occurs. The exception to this is ANDV which, in addition to the classical routes of transmission, has been associated with human to human transmission in Argentina and Chile (Martinez et al. 2005, Ferres et al. 2007).

4.3 Disease. Over half of the species of hantavirus currently recognized by the ICTV are associated with human disease. Seven species, along with associated subspecies, have been documented as etiological agents of HPS, and five have been shown to cause HFRS in humans (Table 1). Although the clinical presentation of the two diseases is quite different, pulmonary manifestations (similar to those of mild HPS) involved with HFRS are becoming more commonly recognized, particularly in cases caused by PUUV and HTNV (Linderholm & Elgh 2001). Likewise, cases of HPS caused by Bayou and BCCV have been reported to have renal involvement (Khan et al. 1995, Hjelle et al. 1996). The actual number of hantaviruses of documented medical importance to humans will probably increase as diagnostics continue to improve allowing for more precise identification of disease causing viruses in areas of high rodent/viral diversity (i.e., South America). Currently, only rodent-borne hantaviruses are known to cause human disease, as the pathogenic potential of shrew-borne hantaviruses in humans remains to be determined.

4.3.1 Hemorrhagic fever with renal syndrome. The earliest documentation of HFRS cases are recorded in Russian medical reports from Siberia dating to 1913 (Casals et al. 1970), although Chinese records describing a similar illnesses have been described from as early as 960 A.D. (Lee, Baek & Johnson 1982). The medical importance of HFRS was

realized through armed conflict within the 20th century when it was referred to as field nephritis and Korean or epidemic hemorrhagic fever. Today, there is an estimated 200,000 cases of HFRS which require hospitalization each year (Lee 1996). The severity of the disease can range from mild to severe depending on the infecting virus. For example, HFRS caused by PUUV (referred to as nephropathia epidemica) or SEOV is generally mild with mortality rates of less than 0.1%, while disease associated with HTNV or Dobrava is more severe with mortality rates reaching between 5 and 10% (Lee 1996). Serological studies conducted on individuals living in endemic regions have found seroprevalence rates ranging from 1% to greater than 20% (Niklasson et al. 1993, Khaiboullina, Morzunov & St Jeor 2005).

Clinically, HFRS is characterized by fever, vascular leakage, which results in hemorrhagic manifestations, and kidney failure. Typically, HFRS presents with five distinguishing phases (febrile, hypotensive, oliguric, diuretic and convalescent) of illness which are typically more pronounced in the severe forms of the disease (Linderholm & Elgh 2001). Symptom onset follows a two to three week incubation period and the febrile phase abruptly commences with non-specific indicators which can include headache, myalgia, fever, nausea and vomiting. Hemorrhagic manifestations occur during the febrile phase and usually present as flushing of the face, injection of the conjunctiva or even a petechial rash. Three to seven days later, the hypotensive phase begins and is characterized by signs of shock. Up to a third of HFRS related deaths occur during this phase, which can last for a few days. The third (oliguric) phase is characterized by hypertension and severe bleeding, which can last for up to a week. Half of all HFRS mortalities occur during this phase and it is not uncommon for severe cases to have

pulmonary edema. Clinical recovery begins with the forth (diuretic) phase, and although kidney function is improved, death can still occur during this period due to pulmonary complications or shock. Fluid and electrolyte imbalance are also common due to excessive urination. The final phase of HFRS is the convalescent stage which can last for months and although long term sequelae are rarely associated with HFRS few studies have addressed this question (Niklasson, Hellsten & LeDuc 1994).

4.3.2 Hantavirus pulmonary syndrome. The original description of HPS occurred in 1993 during an outbreak in the Four Corners region of the United States. Unlike the initial descriptions of HFRS, HPS was noted due to a cluster of fatal cases of pulmonary illness of unknown etiology in previously healthy, young adults. Between May and December 1993, 48 people developed HPS, 27 (56%) of which succumbed to the infection. Since 1993, cases of HPS have been documented throughout the America's however, the severity of the initial outbreak has never been matched in North America with respect to case numbers and fatality rate.

In North America, the proportion of SNV infections to disease is believed to be nearly 100%. Several serological surveys have demonstrated a low seroprevalence to SNV in individuals with a high risk of exposure to SNV (Gonzalez et al. 2001, Fritz et al. 2002, Gardner et al. 2005). It should be noted that the populations examined in these North American serosurveys often exclude, or under represent younger individuals and since HPS in children is often milder, it is possible that asymptomatic infections may occur in this demographic. South America appears to have a higher proportion of asymptomatic cases since serosurveys have documented hantavirus prevalence between

2.2% in Chile and 14.3% in Argentina and Brazil (Castillo et al. 2002, Campos et al. 2003, Pini et al. 2003). Considering Chilean populations have a low documented seroprevalence along with low virus diversity due to isolation by the Andes mountain range, the differences in asymptomatic cases observed between countries in North and South America is likely associated with the genetically diverse viruses (some of which may be less virulent to humans) circulating in countries like Argentina.

Clinically, HPS presents as a febrile disease characterized by bilateral interstitial pulmonary infiltrates and compromised respiratory function which requires supplemental oxygen (CDC 2004). Typically, HPS is characterized by four phases of disease: febrile, cardiopulmonary, diuretic, and convalescent (Enria et al. 2001). The incubation period of HPS was determined to be between 9 and 33 days with a median time of symptom onset of 14-17 days post-exposure, although incubations periods between 46-51 days have been reported (Young et al. 2000, Jonsson, Hooper & Mertz 2008). During the early stages of disease (i.e., around the time of symptom onset) viral RNA is readily detectable in whole blood samples from patients, allowing for genotypic identification of the virus. Generally HPS begins with the febrile phase which consists of similar non-specific symptoms as HFRS, including progressively worsening thrombocytopenia. After three to six days of non-descript symptoms patients enter the cardiopulmonary phase which rapidly progresses from coughing and shortness of breath to shock and severe pulmonary edema requiring intubation and mechanical ventilation. This phase is characterized by vascular leakage, which occurs primarily in the lungs, hypoxemia, and cardiac complications. Death can occur within 48 hrs and in addition to respiratory failure, is due to shock and myocardial dysfunction which has lead some to refer to it as hantavirus cardiopulmonary

syndrome. In total, HPS is fatal in approximately 30% of cases caused by SNV and 40% of cases due to ANDV. The prognosis of patients who proceed to the third (diuretic) phase rapidly improves with resolution of symptoms. The final, convalescent, phase can last for months and long term sequelae have not been characterized, although similar to HFRS few studies have attempted to address this aspect of HPS.

4.4 Post-infection immunity. Following infection, humans quickly generate a humoral immune response with the detection of hantavirus specific immunoglobulin M, G, A and E (IgM, IgG, IgA, IgE) in serum and IgA in saliva (Padula et al. 2000). The N protein is the immunodominant antigen and anti-N antibodies are almost always present at symptom onset and can persist for decades (Lundkvist, Horling & Niklasson 1993, Verity et al. 2000, Kallio-Kokko et al. 2001). The majority of B cell epitopes defined on the N protein are cross-reactive between hantavirus species, though serotype specific epitopes have been defined as well (Tischler, Rosemblatt & Valenzuela 2008). Based on considerable cross-reactivity, in conjunction with the early appearance, serological confirmation of HPS and HFRS disease is often accomplished using N based immunoassays.

Anti-G_N and G_C antibodies appear shortly after those targeting the N protein, and are the major determinant of a protective humoral immune response (Kallio-Kokko et al. 2001, Vapalahti, Lundkvist & Vaheri 2001). The specificity of the neutralizing response may be different for Old and New World hantaviruses. A recent study by Valdivieso and colleagues (2006) compared the neutralizing activity of convalescent patient sera and found for SNV and ANDV related HPS, neutralizing activity was limited to homotypic

viruses. Conversely, vaccine studies with select Old World hantaviruses have shown, in animal models, a cross-reactive neutralizing response can be generated following vaccination with M segment based DNA vaccines (Hooper et al. 2001a). Nevertheless, a strong neutralizing antibody response in HPS patients upon admission appears to correlate with milder disease and thus a favourable prognosis (Bharadwaj et al. 2000). Neutralizing epitopes have been documented in G_N and G_C using specific mAbs, (Arikawa et al. 1989, Wang et al. 1993, Kikuchi et al. 1998), however, the location of neutralizing epitopes within the glycoproteins remains unclear and it is presumed at least some are composed of discontinuous or conformational epitopes, which may be modified by glycosylation (Vapalahti, Lundkvist & Vaheri 2001). A study by Kikuchi and others (1998) described a HTNV escape mutant selected with a G_C specific neutralizing monoclonal antibody with a deduced aa change occurring in G_N, suggesting that at least some neutralizing epitopes are not only discontinuous but occur across G_N and G_C. Similar findings have been observed in protective immunity experiments with neutralizing antibodies only detected in animals immunized with constructs expressing both G_N and G_C (Schmaljohn et al. 1990).

The CTL response to hantavirus in humans is also thought to be vital for viral clearance although details of cellular based immunity remain somewhat unclear. The CTL response is believed to be mainly associated with the N protein (Ennis et al. 1997, Van Epps, Schmaljohn & Ennis 1999, de Carvalho Nicacio et al. 2001), though epitopes within G_N and G_C have been described (Van Epps, Schmaljohn & Ennis 1999, Vapalahti, Lundkvist & Vaheri 2001, Kilpatrick et al. 2004).

5. Pathogenesis. Hantaviruses primarily infect and replicate in endothelial cells, monocytes and macrophages (Duchin et al. 1994, Mackow & Gavrilovskaya 2001, Raftery et al. 2002). Although pulmonary endothelial cells are thought to be the preferential target for HPS-causing viruses, immunohistochemistry (IHC) has demonstrated the presence of viral antigen in endothelial cells of capillaries and small vessels of several organs including heart, kidney, spleen, bladder, pancreas, lymph node, skeletal muscle, intestine, adrenal, and adipose tissue (Zaki et al. 1995, Borges et al. 2006). Dendritic cells have also been shown to support hantavirus infection both *in vivo* and *in vitro* (Zaki et al. 1995, Raftery et al. 2002).

Increased vascular permeability and leakage is a trait of both HPS and HFRS; however, the mechanisms responsible are unclear. Efforts to explain this finding have produced three main hypotheses: direct effects on endothelial cell functions due to viral infection; CTL directed destruction of infected endothelial cells; or increased production of cytokines (Terajima et al. 2007). It has been suggested that at least some hantaviruses may have direct effects on infected cells which include apoptosis (Kang et al. 1999, Li et al. 2005) and cytopathic effect (CPE, Markotic et al. 2003). However, other studies have found that neither SNV nor HTNV cause any apparent CPE in infected endothelial cells and infection alone causes no disruption in the vascular endothelium, suggesting the pathology associated with HPS and HFRS is not due to viral cytotoxicity (Zaki et al. 1995, Sundstrom et al. 2001, Hardestam et al. 2005). There is increasing evidence that the pathology is immune-mediated and involves the CTL response, although the underlying mechanisms for both HPS and HFRS are unclear. Increased levels of activated CD8+ T

cells have been documented in the acute stages of HPS and HFRS and higher frequencies of circulating SNV specific CD8+ T cells have been correlated with severe HPS (Huang et al. 1994, Nolte et al. 1995, Zaki et al. 1995, Kilpatrick et al. 2004). Recent laboratory studies have demonstrated hantavirus specific CTL increased the permeability of infected endothelial cells following antigen recognition (Hayasaka et al. 2007). While the mechanisms responsible for the increase permeability were not addressed, a direct role of cellular immunity in the vascular leakage associated with both HPS and HFRS is suggested. Elevated cytokines levels (including TNF α, IL-6 and IL-10 as well as IFN γ) have also been observed in HFRS and HPS patients and may relate to symptoms (Krakauer et al. 1994, Linderholm et al. 1996). Further, increased cytokine producing cells have been documented in kidney biopsies from HFRS patients, and lung and spleen sections from HPS patients (Temonen et al. 1996, Mori et al. 1999).

Further support for the hypothesis of immune related pathology is the observations of a genetic predisposition and severity of disease. Severe PUUV infection has been associated with HLA-B8 and DRB1*0301 alleles, while the HLA-B27 allele was associated with mild disease (Mustonen et al. 1996). Similarly, the HLA-B35 allele has been associated with a more severe course of HPS (Kilpatrick et al. 2004).

5.1 Immune avoidance. No single virulence factor has been identified to explain the differences between pathogenic and non-pathogenic hantaviruses, or why some cause HFRS while others cause HPS. However, much like other pathogenic RNA viruses, the regulation of the host cell immune response is of paramount importance for hantaviruses. Hantaviruses are sensitive to type I interferon (i.e., IFN α, IFN β) which is activated by

double stranded RNA replicative intermediates. In endothelial cells the addition of exogenous interferon up to 12 hrs post infection is sufficient to block hantavirus replication, however addition of interferon between 15 and 24 hrs post-infection does not impair hantavirus replication and seems to coincide with increased replication, suggesting pathogenic hantaviruses regulate the innate immune response early after infection in order to replicate (Alff et al. 2006). Additionally, hantaviruses are inhibited by interferon inducible genes including MxA, a GTPase active against negative strand RNA viruses and an important component of interferon induced protection (Frese et al. 1996).

To-date, comparative studies on pathogenic and non-pathogenic hantaviruses have produced contradictory results. For example, one microarray study found that non-pathogenic (PHV) viruses differentially regulated up to 67 genes, including 24 interferon specific genes and MxA during early (24 hr) infection of human umbilical macrovascular endothelial cells (HUVECs). In contrast, pathogenic hantaviruses (HTNV, NY-1) induced or repressed three or less genes, and resulted in less than a 4-fold increase in MxA (Geimonen et al. 2002). A second study confirmed the finding that pathogenic (HTNV) viruses delay the expression of MxA protein in early infection of HUVECs as compared to non-pathogenic (TULV) viruses, however this study also found that HTNV (and not TULV) stimulated an interferon response early (24 hrs) in infection, as measured by the induction of IFN- β (Kraus et al. 2004). Recent studies have suggested that during early (24 hrs) infection of human primary lung endothelial cells with PHV but not ANDV resulted in a vigorous IFN-β response, and this correlated with strong activation of interferon regulatory factor 3 (IRF-3), a vital intermediate for interferon stimulated gene expression (Spiropoulou et al. 2007).

In contrast to the above studies, Khaiboullina and others (2004), using microarray analysis, found that pathogenic (SNV) viruses up-regulated as many as 175 genes early (12 hrs) in infection compared with only 35 for non-pathogenic (PHV) viruses, with no difference in the interferon response detected between the two viruses. Additionally, in a second study they found ANDV is a strong inducer MxA mRNA synthesis, and although it was cell type dependant, HUVECs demonstrated the highest level of MxA mRNA early (24 hrs) after infection (Khaiboullina et al. 2005). Although the induction of MxA mRNA was inversely correlated to viral titers, in A549 cells (a human alveolar epithelial cell line) there was no increase in the detection of MxA protein over uninfected cells. Increased MxA and interferon stimulated gene mRNA synthesis has also been observed in endothelial cells treated with infectious or inactivated SNV, suggesting that this induction may be independent of viral replication (Prescott et al. 2005).

The apparent discrepancies in these studies could be due to the experimental approach (i.e., cell lines and the multiplicity of infection, MOI, utilized) or methods employed (i.e., variability in microarrays and real-time RT-PCR), or differences in the viruses themselves. To better understand the regulation of the innate immune response by hantaviruses, other groups have attempted to assign inhibitory functions to N, G_N and G_C proteins. Alff and colleagues (2006) found that PHV RNA and protein synthesis is impaired between 2 and 4 dpi in endothelial cells, compared to NY-1 and HTNV and this correlated with interferon induction in PHV infected cells. Co-infection of PHV and NY-1 reduced interferon induction, suggesting an element of pathogenic viruses was able to regulate the interferon response. Using recombinant proteins it was found that expression of NY-1 G_N cytoplasmic tail, but not NY-1 N protein, or PHV G_N tail, drastically reduced

the interferon response (Alff et al. 2006). It has also been shown that the G_N tails of pathogenic (NY-1, ANDV, HTNV) but not non-pathogenic (PHV) hantaviruses are proteasomally degraded (Sen, Sen & Mackow 2007). The biological significance of this remains unknown however, it has been suggested as a possible virulence factor since proteasomal degradation may enhance antigen presentation. The recently described NSs protein produced by TULV and PUUV may function as a type I interferon antagonist with experimental evidence suggesting it inhibits the promoter activity of IFN β, NK-κB, and IRF-3 (Jääskeläinen et al. 2007). This is an important finding which warrants further study since the majority of RNA viruses studied to-date interfere with the innate immune response through non-structural proteins, including other bunyaviruses such as Bunyamwera virus (Bridgen et al. 2001).

6. Treatment and Prevention. Antiviral therapy for the treatment of HFRS is limited and currently no specific treatment exists for HPS. Lactoferrin and ribavirin have shown the potential to inhibit hantaviruses *in vivo* and *in vitro*, (Huggins et al. 1986, Murphy et al. 2001, Medina et al. 2007) and although clinical studies demonstrated the efficacy of ribavirin for treating HFRS, similar studies conducted with HPS patients are inconclusive, partially due to low enrolment (Huggins et al. 1991, Mertz et al. 2004). Early studies suggest ribavirin's efficacy against HPS may rely on administration early in the course of infection, prior to the onset of severe pulmonary manifestations, suggesting its usefulness is limited since cases of HPS are often only recognized following onset on breathing deficiency (Jonsson, Hooper & Mertz 2008). Several other compounds and chemical entities have shown promise as inhibitory agents for other bunyaviruses,

including Rift valley fever and Crimean-Congo hemorrhagic fever viruses, some of which may also be effective against hantaviruses (Sidwell & Smee 2003). Among these agents is T-705, a pyrazine derivative that is hypothesized to act specifically on the RdRp and was initially described as a potent inhibitor of influenza virus (Gowen et al. 2007). T-705 has now been shown to have broad-spectrum activity against several highly pathogenic RNA viruses, including inhibitory effects on arenaviruses and bunyaviruses (Gowen et al. 2007). T-705, along with other novel chemical entities, may also be effective against hantaviruses, though this assumption requires testing with both *in vivo* and *in vitro* experiments. It should also be noted that since hantavirus disease in humans is believed to be immune-mediated, direct effects of any of these agents in animal models may not result in successful treatment in humans, due to differing immune responses.

Immunotherapy has shown promise in infection models and lethal disease models as a potential therapeutic approach for hantavirus disease. Administration of neutralizing monoclonal antibodies as well as passive transfer of immune sera up to a few days post-challenge can prevent infection and death in animals from homologous challenge (Custer et al. 2003, Medina et al. 2007). While it has been demonstrated that high neutralizing antibody titers upon hospital admission correlated with a good prognosis for HPS patients, treatment of HPS or HFRS via immunotherapy has not been reported (Jonsson, Hooper & Mertz 2008). At this time, treatment of HPS is limited to supportive care, which may include extracorporeal membrane oxygenation for the treatment of severe HPS (Dietl et al. 2008).

Currently, no licensed vaccine against HPS is available, meaning disease prevention relies on educating the general public on how to reduce their contact with

rodents and/or their excreta. The situation for HFRS is essentially the same, although in 1990, the Republic of Korea approved the use of an inactivated HTNV vaccine (termed Hantavax). The efficacy of this vaccine is under debate with at least one study finding the level of protection afforded by the vaccine was not statistically significant (Park, Kim & Moon 2004). Due to safety concerns, inactivated hantavirus vaccines are not likely to be approved for use in North America, especially for indigenous pathogenic (i.e., HPS causing) viral species.

The development of safe and effective countermeasures for hantavirus illnesses is of paramount importance and remains a topic of intense research. As such, several experimental platforms have been developed as potential candidate hantavirus vaccines and tested in small animal infection models (outlined in Chapter V). Among the most successful of these has been plasmid (DNA) based vaccines which encode for the M segment of specific hantavirus species (Hooper et al. 1999, 2001a, 2006, 2008, Kamrud et al. 1999) and currently, a DNA based HFRS vaccine is begining clinical trials in the United States.

Although the role(s) that individual hantavirus proteins play in eliciting a protective response remain the subject of research, typically, candidate hantavirus vaccines are based on the M segment gene products, G_N and G_C, since they are present on the surface of the virion and are targets of the neutralizing antibody response (Schmaljohn et al. 1990). Nevertheless, some studies have demonstrated protective immunity conferred by immunization with N based vaccines in the absence of neutralizing antibodies (Schmaljohn et al. 1990, Lundkvist et al. 1996a). These seemingly contradictory results suggest that the protective immune response against hantaviruses

can be based on either cell mediated or humoral responses or a combination of both. Additionally, at least one study has found that passive transfer of immune sera following immunization with an HTNV N based vaccine was sufficient to protect a minority of suckling mice against lethal HTNV infection, suggesting that non-neutralizing antibodies may also be involved in hantavirus immunity (Yoshimatsu et al. 1993). Currently there is no evidence that the RdRp is involved in generating an immune response, although few, if any, studies have addressed this possibility.

A further topic of interest is that of cross-protection of hantavirus antigens. Serologically, hantavirus species are classified based on minimal cross-neutralizing antibody responses, suggesting that vaccines based on G_N and G_C may not provide good cross protection against heterotypic viruses. However, experimental evidence with both HTNV and SEOV M segment based DNA vaccines suggests that cross protection against closely related HFRS causing viruses is achievable (Kamrud et al. 1999, Hooper et al. 2001a). Similar studies with New World hantaviruses have not been conducted, though based on the findings with HFRS causing viruses, it seems possible that with the appropriate immunogen, a single vaccine could protect against multiple pathogenic hantaviruses circulating in specific geographical areas (i.e., South America). Although few studies have addressed the cross protection associated with the hantavirus N protein, it should not be ruled out for future multivalent vaccines.

CHAPTER II:

Serological differentiation of deer mice recently and chronically infected with SNV.

INTRODUCTION

Since the initial description in 1993, field investigations have been conducted surrounding cases of HPS in attempt to understand the factors involved in SNV spillover to humans (Childs et al. 1994, Cantoni et al. 2001, Lindsay et al. 2001). While these studies have identified several epidemiological factors associated with contracting HPS, they rarely address infection status within rodent populations beyond serological determination of prevalence. While useful, without pre-existing data for infection rates in local rodents, the majority of these studies cannot determine recent changes in hantavirus prevalence. To better predict and monitor hantavirus infection in rodents it would be of value to have a simple test, preferably serological, which could readily identify recently infected mice. These mice would represent a means to assess epizootic intensity, even in small removal studies surrounding case investigations.

To help determine the duration of SNV infection in deer mice and to differentiate between recently and remotely infected rodents, an IgG avidity assay was developed. Similar avidity assays have been utilized to diagnosis acute infections in humans for a variety of pathogens, including hantaviruses, as well as for estimating PUUV infection onset in wild bank voles (Hedman, Vaheri & Brummer-Korvenkontio 1991, Gavrilovskaya et al. 1993, Kallio-Kokko et al. 1993). These assays are based on the observation that the strength of antibody binding to its specific antigen (i.e., antibody avidity) increases with time after exposure to an immunogen (Eisen & Siskind 1964) and

rely on a protein denaturant which can disrupt the antibody-antigen complex affecting low, but not high avidity antibodies. Based on this principle, the presence of low avidity antibodies in a serum sample is indicative of a recent infection, while the detection of high avidity antibodies indicates the infection occurred in the distant past.

In May 2005, four laboratory-confirmed cases of HPS occurred within a one-week period in Hobbema, a small prairie community in central Alberta, representing the first cluster of HPS cases documented in Canada (Webster et al. 2007). At the time of the cases, deer mice collected from this community had an unusually high seroprevalence of SNV. Four months after the cluster of cases, the seroprevalence in deer mice from the same areas had declined substantially. The aim of this study was to evaluate the use of antibody avidity in differentiating recently and chronically infected deer mice and to determine whether the cluster of HPS cases occurred concomitantly with an increased proportion of recently infected deer mice.

HYPOTHESIS

1. Deer mice recently infected with SNV can be differentiated from those infected in the distant past based on the avidity profile of SNV specific IgG antibodies.
2. The occurrence of a cluster of HPS cases in Alberta occurred concurrently with an increased proportion of recently infected deer mice.

MATERIALS AND METHODS

IgG avidity assay. The methodologies employed in the IgG avidity assay are based on the hantavirus enzyme linked immunosorbant assay (ELISA) outlined in Chapter III.

Serum samples were diluted 1:100 in serum diluent and tested in duplicate in 96 well plates previously coated with recombinant SNV N antigen (see SNV ELISA, outlined in Chapter III). Following an initial incubation (60 min, 37°C), serum samples were removed and the wells were washed with phosphate buffered saline (PBS) supplemented with 0.5% Tween 20 (PBST). Sample wells were then treated with either a 35 mM diethylamine (DEA, Sigma-Aldrich) denaturing solution (90 µl DEA in 25 mL PBST) or with PBST alone (3 x 5 min washes). Following the DEA (or PBST) treatments, wells were washed an additional six times with PBST, before incubation with peroxidase labeled goat anti-rodent (a mixture of goat anti-*Peromyscus leucopus* and goat anti-rat, 1:2000 each, Kirkegaard and Perry Laboratories, KPL) secondary antibodies (60 min, 37°C), and substrate (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), peroxidase substrate, KPL) addition (30 min). Color development was quantified by reading the optical density (OD) at the 405 nm wavelength (OD_{405}). The relative avidity index (RAI) was calculated by dividing the OD_{405} of the DEA washed wells by that of the wells washed with PBST and expressed as a percent (RAI = (OD_{405} DEA / OD_{405} PBST) x 100). Samples with an RAI $\leq$ 40% were considered low avidity, while samples with an RAI $\geq$ 60% were considered high avidity. Samples with an RAI between 40 and 60% were deemed to be intermediate avidity.

Assay validation. A total of 60 serum samples from both naturally (n = 29) and experimentally (n = 31) infected deer mice were blind-tested to assess the utility of the avidity assay in identifying recently infected deer mice. Samples from naturally infected deer mice were available from the capture-mark-recapture field studies conducted in

Canada, as described in Chapter III (Safronetz et al. 2008). Samples from experimentally infected deer mice were provided by Dr. Brian Hjelle (Center for Infectious Diseases and Immunity, Department of Pathology, University of New Mexico, Albuquerque, NM, USA). Briefly, four to eight week old deer mice (*P. maniculatus rufinus*) were inoculated with 10-25 infectious dose-50 of SNV (strain SN77734) via intramuscular (i.m.) injections and euthanized between days 14 and 217 post inoculation, all as previously described (Botten et al. 2000, Botten, Ricci & Hjelle 2001). Mice were euthanized with ketamine/xylazine and blood samples collected via cardiac puncture.

Measuring SNV epizootic intensity. In May 2005 a cluster of human cases of HPS were identified in the small prairie community of Hobbema, Alberta, Canada (Webster et al. 2007). As part of a risk-assessment protocol, deer mice were collected from Hobbema (52° 49′ 50″ N - 113° 27′ 25″ W) as well as from areas in a the nearby community of Wetaskiwin (52° 58′ 8″ N - 113° 22′ 36″ W) which had no reported cases of HPS, both at the time of the outbreak (May) as well as in September 2005 as part of a follow-up study. A total of 163 serum samples reactive for hantavirus-specific IgG were used to measure the epizootic intensity of SNV in deer mice collected at these two time points. Where sufficient blood volumes were available, whole blood samples (n = 138) were tested for the presence of hantavirus-specific RNA using the extraction and nested RT-PCR methodologies outlined in Chapter III.

Statistical analysis. Chi-square tests were performed using the statistical software package SAS 9.1 to test for differences in sex and viremia between the high and low avidity groups.

RESULTS

Assay validation. The avidity assay was conducted on coded samples from experimentally infected deer mice and the results were stratified according to the likelihood that samples were collected acutely after experimental infection or during a later phase of infection. The reactivity (binding of deer mouse IgG to SNV N protein) of sera collected from experimentally infected animals within 30 dpi was greatly reduced by the addition of the denaturant (DEA), resulting in an RAI $\leq$ 40% and indicating the presence of low avidity antibodies (Figure 2.1). The average RAI of samples collected between 10 and 20 and 21 and 30 dpi was 9.3 and 19.9% respectively. In contrast, the reactivity of the majority of sera collected at greater than 30 dpi was only modestly affected by the presence of the denaturant, as demonstrated by an RAI $\geq$ 60% (Figure 2.1). The average RAI of samples collected between 31 and 40 dpi was 70% and samples collected at 40 or more dpi had an average RAI of 85.7%. No serum collected from an experimentally infected animal at a time point greater than 35 dpi had an RAI $\leq$ 40% and no sample collected before 34 dpi had an RAI $\geq$ 60%. The avidity assay was 89.5% accurate in distinguishing samples collected during the first 30 dpi.

Similar observations were made with a sub-set of 29 sera collected from naturally infected deer mice which had seroconverted (data not shown). Paired serum samples documenting seroconversion, and collected within 21 days of each other,

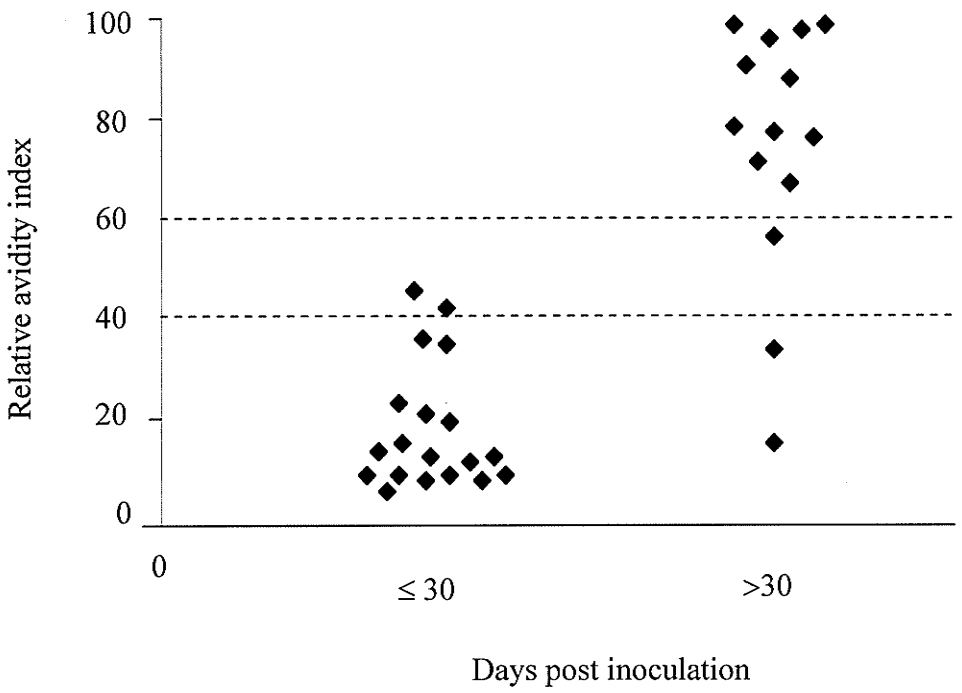


FIGURE 2.1. Comparison of avidity indices of timed serum samples collected from experimentally infected deer mice. Deer mice were inoculated (i.m.) with a uniform dose of SNV strain 77734. Serum samples collected at various time points post infection were screened for the presence of low avidity IgG antibodies. A relative avidity index (RAI) of $\leq 40\%$ indicated low avidity antibodies while an RAI $\geq 60\%$ signified high avidity antibodies. An RAI between 40-60% was intermediate (reproduced, with permission, from Safronetz et al. 2006).

contained low avidity antibodies with an average RAI of 25.3%. However, samples collected from deer mice with seroconversion occurring at greater than 40 days were predominantly high avidity with an average RAI of 80%.

Measuring SNV epizootic intensity. The avidity assay was applied to samples from infected deer mice collected in response to a cluster of HPS cases which occurred in Hobbema Alberta in May 2005, as well as samples collected during a follow-up study in September, 2005. In total, 120 IgG positive serum samples (108 from Hobbema, 12 from Wetaskiwin) from May 2005 and 43 positive samples (35 from Hobbema, 8 from Wetaskiwin) from September 2005 were tested for the presence of low avidity antibodies. A higher proportion of deer mice with low avidity antibodies were collected from both communities during the May sampling period. Eighteen (16.7%) of 108 seropositive mice collected from Hobbema and 1 (8.3%) of 12 mice collected from Wetaskiwin had low avidity antibodies (Table 2). In September, the proportion of mice with low avidity antibodies dropped with 2 (5.7%) of 35 seropositive mice from Hobbema having low avidity antibodies and none (0%) of 8 from Wetaskiwin. Due to the small number of samples from Wetaskiwin, a valid statistical comparison between avidity profiles from deer mice collected in the two communities could not be completed. Additionally, low numbers of deer mice collected from Hobbema in September reduced the power of statistical comparisons between the two time points within that community. However, there was a trend towards a higher proportion of deer mice with low avidity antibodies sampled in May from Hobbema when compared to September ($\chi^2 = 2.9$, df = 1, $p < 0.10$).

TABLE 2
Comparison of avidity profiles from seropositive deer mice collected from Hobbema and Wetaskiwin Alberta in May and September 2005

Month	Community	No. tested	Avidity profiles		
			Low (%)	Intermediate (%)	High (%)
May	Hobbema	108	18 (16.7)	8 (7.4)	82 (75.9)
	Wetaskiwin	12	1 (8.3)	4 (33.3)	7 (58.3)
September	Hobbema	35	2 (5.7)	0 (0)	33 (94.3)
	Wetaskiwin	8	0 (0)	2 (25)	6 (75)

Deer mice were collected from two Alberta communities in response to a cluster of HPS cases reported in Hobbema in May 2005, as well as during a follow-up study four months after the occurrence (reproduced, with permission, from Safronetz et al. 2006).

TABLE 3
Comparison of antibody avidity and the presence of SNV specific RNA in whole blood samples from seropositive deer mice collected from Hobbema Alberta in May and September 2005

	May			September		
Avidity profile	No. Tested	Positive (%)	Negative (%)	No. Tested	Positive (%)	Negative (%)
Low	18	8 (44.4)	10 (55.6)	2	1 (50)	1 (50)
Intermediate	7	0 (0)	7 (100)	0 (0)	0 (0)	0 (0)
High	80	24 (30)	56 (70)	31	19 (61.3)	12 (38.7)
Total	105	32 (30.5)	73 (69.5)	33	20 (60.6)	13 (39.4)

Mice that were RT-PCR positive for SNV specific RNA in whole blood samples were considered viremic. Blood samples from five mice were of insufficient volume to conduct this test (reproduced, with permission, from Safronetz et al. 2006).

To test for an association between the presence of low avidity antibodies and the presence of SNV RNA in blood, nested RT-PCR was conducted on whole blood samples from seropositive mice (n = 138). Over the two sampling periods, there was no significant difference in the proportion of blood samples with detectable SNV RNA between the low and high avidity groups ($\chi^2 = 0.28$, df = 1, $p \leq 1$). In total, SNV RNA was detected in 9 (45%) of 20 mice with low avidity and 43 (38.7%) of 111 mice with high avidity antibodies collected from Hobbema (Table 3). None of the seven deer mice with an intermediate avidity result had detectable SNV RNA in blood.

No significant difference was observed between gender and the presence of low avidity antibodies in seropositive deer mice collected from Hobbema ($\chi^2 = 0$, df = 1, p =1). Over the two sampling periods low avidity antibodies were detected in 8 (14%) of 57 sera samples collected from seropositive female deer mice, and 12 (14%) of 86 samples collected from males.

DISCUSSION

Antibody avidity (otherwise referred to as functional affinity) has been previously used to characterize Old World hantavirus infections in both humans and rodent hosts (Hedman, Vaheri & Brummer-Korvenkontio 1991, Gavrilovskaya et al. 1993, Kallio-Kokko et al. 1993). However, this is the first time these methods have been applied to rodents infected with a New World hantavirus. The time to appearance of high avidity antibodies in deer mice experimentally infected with SNV is similar to those reported for bank voles experimentally infected with PUUV (Gavrilovskaya et al. 1993). Low avidity antibodies (as defined by a RAI $\leq$ 40%) were predominantly documented in sera

collected at time points less than 30 dpi. By 40 dpi, the majority of experimentally infected deer mice tested had high avidity antibodies (as defined by RAI $\geq$ 60%), with the remainder having intermediate avidity (Figure 2.1). Based on the transition of low to high avidity antibodies, the IgG avidity assay reliably identifies deer mice that have been infected within approximately 1 month (30 days).

Previous attempts to define stages of infection (i.e. acute or chronic) in deer mice were based on patterns of serology and the detection of SNV RNA in blood. It has been suggested that a positive serological result coupled with the detection of hantavirus specific RNA in blood samples from rodents indicated a recent infection, however this hypothesis was never properly tested (Netski, Thran & St Jeor 1999). The findings presented here do not support this diagnostic algorithm as a reliable method for identifying recently infected deer mice. The detection of SNV RNA in blood samples did not predict the presence of low avidity antibodies, and the proportion of deer mice with detectable SNV RNA in blood was similar between the low and high avidity groups (Table 3). In the Alberta outbreak study, if one considers all the deer mice collected from Hobbema with low avidity antibodies as recently infected, the positive predictive value for the detection of SNV RNA in blood samples from seropositive mice as an indication of a recent infection is only 17%. This finding is not surprising in light of the recent observations of Kuenzi and colleagues (2005) who demonstrated the detection of SNV RNA in blood samples collected over time from individual, naturally infected deer mice was variable and not consistently positive or negative.

The avidity assay provides the opportunity to indirectly monitor SNV transmission. The identification of acutely infected rodents demonstrates epizootic

transmission of SNV was occurring to some extent in the recent past. As previously mentioned most field investigations use a positive serological result as a surrogate for infection in deer mice, and in conjunction with enumeration of rodent population size, estimate the potential risk of humans acquiring HPS (Mills 2005). Although valuable, seroprevalence as determined by the presence of hantavirus specific IgG antibodies, does not provide an accurate assessment of viral transmission and overall risk of contracting HPS. The utilization of IgG avidity as a means to monitor the epizootic intensity (i.e. indirectly monitoring virus transmission rates) of SNV in a given population of deer mice could provide a more accurate measure of risk than seroprevalence alone. To demonstrate this, the avidity profiles of mice collected in response to a cluster of HPS cases in Hobbema Alberta were determined. Although, far fewer numbers of deer mice were collected in the follow-up study, there was a trend towards a relatively greater prevalence of recent infections in deer mice (as determined by a higher proportion of deer mice with low avidity antibodies) collected at the time of the outbreak. One can speculate that the cause of increased transmission amongst rodents was a function of population size and infection (based on seroprevalence); however, the possibility of yet unidentified factors such as viral, host or environmental stresses or influences should not be ruled out (Mills 2005). If applied to future studies the differentiation of recently infected mice from those infected in the distant past may help clarify some of these factors (e.g., seasonal differences in transmission). Although this study did not address the basis of heightened transmission between rodents, and to humans, it does demonstrate the utility of using an avidity assay to indirectly monitor viral transmission rates (by determining the proportion of recently infected mice), which can help measure the risk of humans contracting HPS.

CHAPTER III:

Assessing SNV prevalence and shedding patterns in naturally infected deer mice collected in Manitoba Canada.

INTRODUCTION

In North America several hantaviruses have been associated with human infection however, SNV remains the principal cause of HPS (Monroe et al. 1999). Since the initial outbreak of HPS in 1993 several field studies have been conducted in the United States to elucidate geographical and temporal patterns of SNV infection in deer mice (Boone et al. 1998, Calisher et al. 1999, 2005, Douglass et al. 2001). The majority of these studies have focused on serology as the sole means to determine patterns of infection in rodent populations. Few studies have looked at patterns of SNV RNA in blood samples from infected mice (Boone et al. 1998, Kuenzi et al. 2005) and none have attempted to correlate infection with possible modes of SNV transmission. While it is assumed transmission of SNV between rodents, and to humans, occurs through contaminated secreta and excreta, it is still unclear at what stages of infection mice shed virus.

In Canada, SNV remains the only etiological agent of HPS identified with a discontinuous distribution of infected deer mice documented across the country (Drebot, Artsob & Werker 2000). To-date, the study of SNV in deer mice in Canada has been limited to passive surveillance (Drebot, Artsob & Werker 2000) and field investigations surrounding HPS cases (Lindsay et al. 2001, Webster et al. 2007). The aim of the field studies described here was to conduct a more extensive ecological survey on the patterns of SNV infection and transmission in deer mice in Manitoba, Canada, using serological and molecular methodologies. To assist in the characterization of infected deer mice, an

IgG avidity assay was employed to help differentiate between recently and remotely infected rodents.

HYPOTHESIS

1. It is possible to elucidate patterns of virus transmission in naturally infected deer mice by differentiating recently from chronically infected individuals and comparing RT-PCR positivity rates in excreta and secreta.
2. An increased proportion of mice with low avidity antibodies (i.e., recently infected) will have detectable SNV RNA in urine and/or oral fluids

MATERIALS AND METHODS

Ethical approval. This research was carried out in accordance with the Canadian Council of Animal Care guidelines and under the Canadian Science Centre for Human and Animal Health Animal Use documents number H99-005 and H04-004 and Manitoba Conservation Wildlife Scientific Permit number WB01963.

Safety. The following field studies were conducted in accordance with previously established guidelines for handling rodents potentially infected with hantaviruses or other zoonotic agents (Mills et al. 2002). In the field, traps containing animals were placed in a sealed plastic container for transport from the site of capture to a nearby field station for processing. While handling animals and collecting samples, personnel wore protective clothing including disposal gowns or coveralls, nitrile gloves and N100 or HEPA filtered positive pressure respirators. In addition, leather gloves were worn when anaesthetizing

animals. The field station was completed disinfected with a 10% bleach solution followed by 70% ethanol at the completion of each day as well as periodically throughout the sampling day. Traps containing rodents were also disinfected prior to being reset in the field.

Field investigations:

Pilot study. From 11-15 August 2003, rodents were captured and sampled from areas near St. Jean Baptiste, Manitoba (49° 16′ 35″ N - 97° 19′ 50″ W) where seropositive deer mice had previously been documented (R. Lindsay, unpublished data). Rodents were trapped using Sherman live-traps (H.B. Sherman Traps, Inc.) baited with a mixture of peanut butter, rolled oats and/or moistened sunflower seeds. Polyester fibre bedding was also placed in each trap to limit hypothermia of captured animals. Up to 300 traps were set between 16:00-20:00 hr and were checked each morning between 07:00-09:00 hr.

Captured animals were anaesthetized by inhalation of isoflurane using an anaesthetic chamber. The weight, sex, relative age, and location of capture were recorded for each rodent. Relative age was inferred from body weight and coat color. Deer mice weighting $\geq$ 16 g with rufous coat above and white below were considered adult, while those weighting < 16 g with grey to light brown coat were considered sub-adult. Blood (approximately 250 µl) was collected via the infra-orbital sinus using heparinized blood collection tubes (i.e., Natelson blood collection capillaries) and placed into Microtainer serum separator or EDTA tubes (all from Fisher Scientific). Oropharyngeal fluids (OPF) were collected by swabbing the oral cavity of each rodent with a Dacron tipped applicator (VWR) which was immediately placed into 400 µl of lysis buffer RLT (Qiagen) for

subsequent RNA extraction. When available, urine was also collected and placed in 400 µl of RLT buffer. Following sample collection, animals were euthanized with an overdose of isoflurane and/or cervical dislocation.

In the field, individually wrapped carcasses, as well as OPF and urine samples, were immediately frozen on dry ice. Blood samples were stored and transported to the National Microbiology Laboratory (NML) on wet ice, where they were immediately separated and serum removed for serology.

In a biosafety level (BSL) 3 suite at the NML, necropsies were performed on 15 mice with serological evidence of SNV infection, and pieces (approximately 15 mg) of salivary gland, skeletal (masseter) muscle, bladder, spleen, kidney, liver, heart, lung, and fat samples were collected. Whenever present, urine (approximately 20-60 µl) was collected from the bladder with a sterile syringe and needle and mixed with 200 µl of lysis buffer RLT. Necropsies were also conducted on 15 antibody negative mice and spleen, kidney, heart, and lung samples were collected. Tissue samples were placed in 1 mL of sterile BA-1 diluent (Hanks' M-199 salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 U/mL penicillin, 100 mg/L streptomycin, 1 mg/L Fungizone in 0.05 M Tris, pH 7.6, Nasci et al. 2002) and mechanically homogenized with a sterile 3-5 mm tungsten bead using a Qiagen Mixer Mill for 5 min at a frequency of 30 Hz (equivalent to 1800 oscillations/min). Homogenates were clarified via centrifugation (≥ 15,000 x g, 10 min) and 200 µl were mixed with 400 µl lysis buffer RLT and removed from BSL 3 following standard operating procedures. The remaining homogenates were stored at -80°C.

Capture-mark-recapture study. A capture-mark-recapture (CMR) study was established in an area of southern Manitoba where SNV infected deer mice had been previously documented (Safronetz et al. 2005). Six sites were selected near the community of Letellier (49° 08′ 09″ N - 97° 18′ 11″ W) and consisted of habitat types typical of the eastern prairie ecozone present in southern Manitoba (e.g., cultivated agricultural land interspersed with oak and ash forests boarding rivers or creeks). All six sites were within an area with an approximate 20 km radius (Figure 3.1).

Trapping was conducted approximately every 21 days beginning in early May and continuing until late September in 2004 and 2005. The duration of the trapping season was determined by weather such that trapping was discontinued once overnight temperatures were consistently below 5°C. Traps were placed approximately 10 meters apart along marked transects. Rodents were live-trapped using methodologies outlined above at each of the six sites for three consecutive nights with 50 Sherman traps per site, for a total of 900 trap nights per session with two exceptions. The final trapping session of both 2004 and 2005 was reduced to two consecutive nights (totaling 600 trap nights each) due to inclement weather. A total of eight trapping sessions were conducted in 2004, however due to severe flooding in and around the study sites in mid-July 2005, only seven sessions were conducted in 2005.

In addition to the live-trapping program, wooden nest boxes were evaluated as a possible mechanism for sampling mice during the winter months. Beginning in August, ten custom-built nest boxes (similar to those described at http://www.wildbirds.org/info/houses.htm) were installed throughout the study area for rodents to inhabit. Nest boxes were elevated approximately 1 meter above the ground by

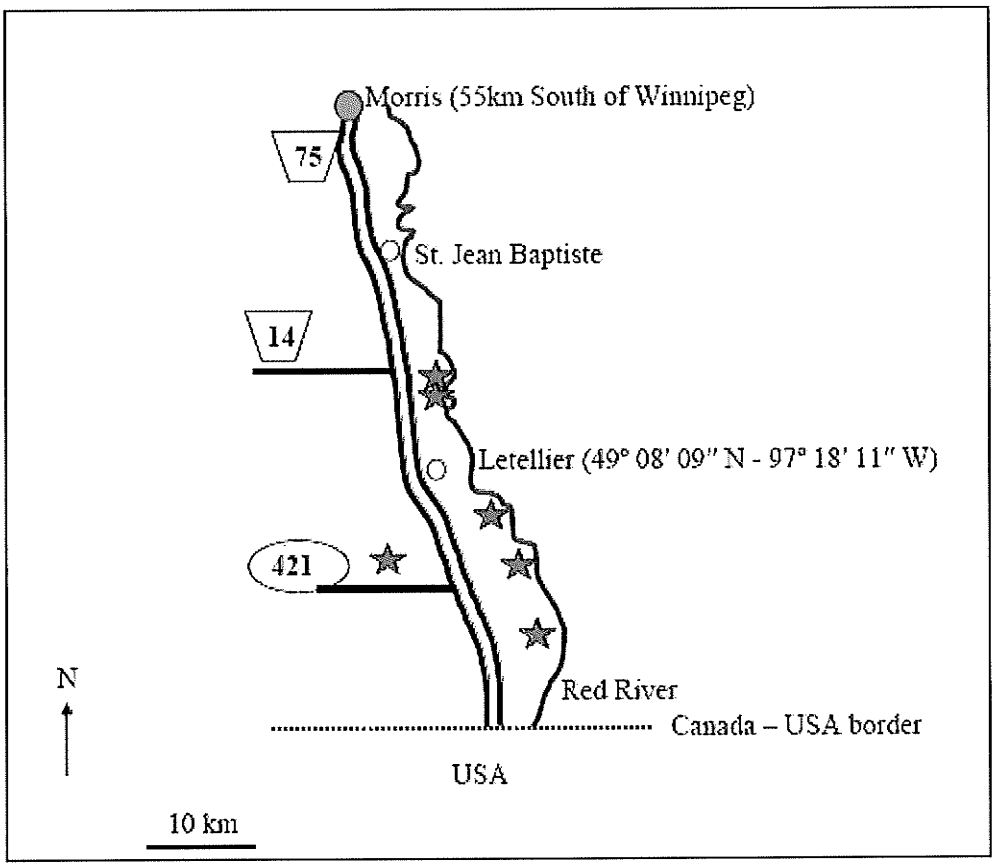


FIGURE 3.1. Map of CMR study trapping sites. Six locations (denoted by red stars) near the town of Letellier and consisting of habitat types indicative of Southern Manitoba were selected as trapping locations for the CMR study.

attaching them to wooden posts or trees and were oriented with the 2.5 cm entry hole facing east or southeast to minimize exposure to winter winds. Resident mouse populations were given two months to colonize the nest boxes. During this time signs of rodent activity and the extent of use of each nest box were monitored; however, samples were not collected from any resident animals.

In January 2004 and 2005, the nest boxes were checked for inhabiting rodents. The entrance to nest boxes populated with rodents were corked and boxes removed from their location and transported to an on-site, heated, field station where inhabiting rodents were sampled and marked.

Throughout the course of the CMR study, captured animals were transported to an onsite field station and processed essentially as described above, with few exceptions. Rodents were initially anaesthetized by inhalation of isoflurane using an anesthetic chamber. Thereafter, rodents were kept inactive using a nose cone attached to anaesthetic machine (T3ISO, Benson Medical Industries) which delivered a precise, low dose of isoflurane mixed with medical oxygen. While inactive, rodents were placed in an individual, sterile Petri dish to catch any expelled urine (collected from dishes with a sterile needle and syringe and placed into 400 µl RLT). Calcium alginate applicators were used to collected OPF samples and were immediately placed into 400 µl RLT. Blood samples were collected as above with the exception that the total volume obtained was adjusted based on body weight with approximately 7-8 µl of blood collected / g (maximum volume collected was approximately 125 µl). Samples were stored and transported as above. Animals were sampled only once per trapping session and recaptures within the same session were immediately released at the site of capture.

For easy identification upon recapture, rodents were marked with a unique identification number by either injecting a permanent dye into specific digits using the Aramis micro-tattooing system (Ketchum Manufacturing Inc) or by toe clipping. Animals were monitored briefly to ensure recovery from the anesthetic and all live rodents were released at the exact site of capture. Any animal that was inadvertently injured during trapping or sampling procedures was humanely killed and the carcass transported to the NML for proper disposal.

Deer mice abundance was estimated using minimum number alive (MNA) enumeration methods (Chitty & Phipps 1966). Using MNA, an individual is known to be alive during a particular trapping session if it is captured during that session, or if it is captured in both a previous and subsequent trapping session. Population dynamics of deer mice captured per trapping session at each site as well as total captures per session were also compared based on trap success (e.g., number of individual deer mice captured per 100 trap nights). The minimum number of infected (MNI) mice (determined by a positive serological result) per session was estimated in a similar manner as MNA, and used to calculate the estimated standing prevalence (ESP = (MNI/MNA) x100) per trapping session (Calisher et al. 1999). Deer mice captured over multiple sessions were included in the recapture rate, however multiple captures of the same rodent, within the same session were not.

Statistical analysis. Chi-square tests were performed to test for relationships between population demographics, seroprevalence and molecular detection of viral genome.

Sampling processing:

Serum separation. Whole blood samples were separated via centrifugation (10 min, ≥ 15000 x g) following which serum was transferred to separate 0.5 mL screw-cap tubes. Both the serum and resultant clot were stored at -80°C for serological and molecular testing, respectively.

Nucleic acid extractions. Total RNA was extracted from blood clots, OPF, urine, and tissue homogenates using spin columns (RNeasy, Qiagen) according to the manufacturer's protocols. Subsequent extracts were either immediately tested for the presence of hantavirus genomic segments or stored at -80°C. For the pilot study, all samples collected were extracted and tested by RT-PCR. However, based on the low amount of positive samples from animals lacking serological evidence of infection (0 of 94 blood or OPF samples tested and only 1 of 15 lung samples), during the CMR study only samples from seropositive animals were extracted and tested. The remaining samples were stored at -80°C and processed if subsequent samples from the individual rodent demonstrated seroconversion.

Hantavirus testing:

Antibody detection. Serum samples from rodents were screened for the presence of IgG reactive to hantavirus using an indirect ELISA as previously described (Feldmann et al. 1993, Lindsay et al. 2001).

Recombinant, *Escherichia coli* (E. *coli*) expressed, SNV N antigen and a negative, non-hantavirus, control antigen (originally prepared by the CDC) were diluted to 1:2000

in PBS and used to coat 96 well "U" bottom microtiter plates (Thermolabsystems, VWR). Each well was coated with 100 µl of the appropriate antigen with the top half of the plate receiving positive antigen (i.e., SNV N) while the bottom half was coated with negative antigen. Plates were placed in a sealed plastic container and stored overnight at 4°C. The following morning, plates were washed three times with PBST on a mechanical plate washer, after which excess fluid in the wells was tapped out onto absorbent paper.

Serum samples were diluted 1:100 in serum diluent (PBST containing 5% skim milk) and 100 µl of each was added to a positive and corresponding negative control well. Plates were covered and incubated at 37°C for 60 min in a sealed plastic container lined with a moistened paper towel for humidity. Serum samples were then removed, and wells washed three times before incubation at 37°C for 60 min with 100 µl of a mixture of goat anti-*Peromyscus leucopus* and goat anti-rat peroxidase labeled secondary antibodies (1:2000 each, KPL) diluted in master plate diluent (PBS supplemented with 0.1% Tween 20 and 5% skim milk). Sample wells were washed again prior to the addition of the peroxidase substrate (ABTS peroxidase substrate kit, KPL) prepared according to the manufacturer's instruction. Color was allowed to develop for 30 min in the dark and quantified by reading the OD_{405}. The net OD_{405} was calculated by subtracting the OD_{405} of the negative control well from that of the positive antigen well for each sample. Samples were considered positive if the net OD_{405} was ≥ 0.95.

All serum samples were screened at a dilution of 1:100 and positive samples were titrated to a maximum dilution of 1:6400. Samples were considered positive if they were reactive at a dilution of 1:400 or greater, and equivocal if positive only at a 1:100 screening dilution. For the CMR study, seroconversion was defined by a serologically

negative sample followed by a subsequent positive sample, or a four-fold increase in titer between two samples.

IgG avidity assay. Recently infected deer mice were differentiated from those infected in the distant past using the IgG avidity assay (outlined in Chapter II). All serum samples with an IgG titer of $\geq$ 100 were tested.

Real-time RT-PCR. The RNA extracts were tested for hantavirus-specific genomic sequences using a one-step real-time RT-PCR assay on an ABI 7700 sequence detector essentially as previously described (Botten et al. 2000, 2002). A 66 bp fragment of the SNV S segment was amplified using primers SNV S-179f and SNV S-245r and detected with a dual labeled (5' fluorescein aminohexyl, FAM, 3' tetramethylrhodamine, TAMRA) fluorescent probe, SNV TM (binding coordinate 198, all three synthesized by TIB Molbiol, oligo sequences available in appendix 1). Reactions were prepared using TaqMan One Step RT-PCR Master Mix Reagent kits (Applied Biosystems) as described by the manufacturer with 0.2 µM of each oligo. Each reaction consisted of 45 µl of master mix and 5 µl of extracted RNA (representing 12.5% of the total RNA). Samples were amplified in three stages: reverse transcription (50°C for 30 min); initial denaturation (95°C for 10 min) and three-step amplification (40 cycles of 95°C for 10 sec, 50°C for 10 sec and 72°C for 30 sec). Data acquisition occurred at the end of the annealing stage (50°C for 10 sec) of each amplification cycle.

Nested RT-PCR. Nested RT-PCR was conducted on RNA extracts with previously described M-segment primers (Johnson et al. 1997, primer sequences in appendix 1). Stage one was carried out using a one-step RT-PCR kit (Qiagen) according to the manufacturer's instruction with 0.5 µM of oligos SM 1687C and SM 2255R. Each reaction contained 45 µl of master mix and 5 µl of extracted RNA (representing 12.5% of the total RNA extracted). Following complimentary DNA (cDNA) synthesis (50°C for 30 min) and Taq activation (95°C for 15 min) steps, a 568 bp fragment was amplified with 40 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec, with a final elongation step of 72°C for 10 min. In the second stage PCR, a 293 bp fragment was amplified in 100 µl reactions with 2 µl of the first stage reaction serving as template. Reactions contained 0.5 µM of primers SM 1723C and ASM 2016R with 0.2 mM dNTPs (Invitrogen), 1 U AmpliTaq (ABI), 2 mM $MgCl_2$ and 1x PCR buffer. Second round cycling parameters were the same as round one without the initial cDNA synthesis and Taq activation steps. Post-PCR, 20 µl of the second stage reaction was electrophoretically separated in a 1.5% w/v agarose gel stained with ethidium bromide (EtBr) and visualized with ultraviolet light.

For the pilot study, a subset (n = 50, including all positive blood, oral swabs, and urine samples along with one or more representative tissue samples from each SNV infected mouse dissected) of real-time RT-PCR positive samples were confirmed using the nested RT-PCR assay. However, due to problems adapting the SNV real-time assay to a new sequence detector, genetic detection for the CMR study was done using only the nested RT-PCR procedures.

Nucleotide sequencing. To verify the presence of amplified SNV genome, selected M-segment amplicons were purified using QIAquick columns (Qiagen) according to the post-PCR centrifuge clean-up protocols outlined in the product insert. Amplicons were submitted to the DNA Core facilities at the NML and sequenced on an ABI 3100 or 3730xl DNA analyzer using the second round amplification primers and BigDye Terminator version 3.1 cycle sequencing kits.

RESULTS

Pilot Study:

From 11-15 August 2003, a total of 122 deer mice were collected over 1200 trap nights, representing a trap success of 12.2 captures per 100 trap nights. The majority of deer mice collected were male, 81 (66.4%) of 122, and adult, 78 (63.9%) of 122.

Antibodies (IgG) to hantavirus were detected in 28 (23%) of 122 deer mice, with the majority (21) of these animals having titers ranging from 400 to $\geq$ 6400. Serum samples from the remaining seven mice were reactive only at the 1:100 screening dilution and as such were considered equivocal titers. Seropositive mice were predominantly adult with 18 (23.1%) of 78 adults and 3 (6.8%) of 44 sub-adult considered positive ($\chi^2 = 4.34$, $df = 1$, $p < 0.05$). A significant sex related difference in seropositive deer mice was not observed ($\chi^2 = 0.23$, $df = 1$, $p \leq 1$). In total, 15 (18.5%) of 81 males and 6 (14.6%) of 41 females were serologically positive.

Samples (including 28 blood, 28 OPF, 11 urine and tissue homogenates from 15 dissected mice) from seroreactive (i.e., mice with equivocal or positive serological results) mice were extracted for RNA and tested for the presence of hantavirus specific

sequences using real-time and nested RT-PCR methodologies. Positive RT-PCR results were obtained from 6 (21.4%) of 28 OPF samples, 2 (18.2%) of 11 urine samples, and 9 (32.1%) of 28 blood samples from these mice. Viral genome was also amplified from 9 (81.8%) of 11 fat samples; 12 (80%) of 15 salivary gland, masseter muscle, spleen, kidney, heart and lung samples; 10 (66.7%) of 15 liver samples; and 6 (40%) of 15 bladder samples (Table 4). Sin Nombre virus RNA was detected in mice with antibody titers ranging from equivocal (100) to $\geq$ 6400. Only two seropositive mice (Table 4, DM53 and 78) lacked viral genome in any sample, while another seropositive mouse (Table 4, DM 45) had detectable viral RNA in only the blood sample. Two serologically equivocal mice (DM 37 and DM 57) had SNV RNA in a variety of tissues. A subset of samples (n = 50) tested by nested M-segment RT-PCR demonstrated 100% correlation with the real-time RT-PCR results. Analysis of the nucleotide sequence of M-segment amplicons from the lung samples of five seropositive deer mice showed significant identity with SNV genotypes previously reported in Manitoba (Drebot et al. 2001), and confirmed the presence of the virus within these mice.

Samples collected from seronegative deer mice (94 OPF and blood samples, and spleen, kidney, heart and lung samples from 15 dissected animals) were also extracted for RNA and tested for the presence of SNV. Results from RT-PCR conducted on these samples were uniformly negative, with the exception of lung samples from one mouse.

Capture-mark-recapture study:

Between May 2004 and October 2005, a total of 12,900 trap nights were conducted over 15 trapping sessions. Deer mice were the predominant rodent species captured with a

TABLE 4

Summary of serology and RT-PCR results from 15 deer mice collected in August 2003 from Southern Manitoba

Rodent I.D.#	Sex (Weight; g)	Antibody Titer	Whole Blood	OPF [b]	Salivary Gland	Masseter Muscle	Urine	Bladder	Fat	Spleen	Kidney	Liver	Heart	Lung
DM-2	F (22.5)	≥6400	+	+	+	+	n.a.[a]	+	+	+	+	+	+	+
DM-4	M (24.5)	≥6400	-	-	+	+	-	-	n.a.	+	+	+	+	+
DM-41	M (21.5)	≥6400	+	-	+	+	-	-	+	+	+	+	+	+
DM-51	M (28)	≥6400	+	+	+	+	-	+	+	+	+	+	+	+
DM-84	M (23)	≥6400	-	+	+	+	-	-	+	+	+	+	+	+
DM-87	M (23.5)	≥6400	-	-	+	+	+	+	+	+	+	-	+	+
DM-91	F (17)	≥6400	+	+	+	+	-	-	n.a.	+	+	+	+	+
DM-110	M (20)	≥6400	-	-	+	+	-	-	+	+	+	-	+	+
DM-26	M (22)	1600	+	-	+	+	-	-	+	+	+	+	+	+
DM-53	M (18.5)	1600	-	-	-	-	-	-	-	-	-	-	-	-
DM-54	M (21)	1600	+	+	+	+	+	+	+	+	+	+	+	+
DM-78	M (14)	400	-	-	-	-	n.a.	-	n.a.	-	-	-	-	-
DM-45	M (14)	400	+	-	-	-	-	-	-	-	-	-	-	-
DM-37	M (18)	100[b]	+	-	+	+	n.a.	+	+	+	+	+	+	+
DM-57	M (16)	100	+	+	+	+	n.a.	+	n.a.	+	+	+	+	+

Necropsies were performed on 15 deer mice with serological (anti-N IgG) evidence of hantavirus infection collected in during a pilot study conducted in August 2003. Tissue homogenates were tested for the presence of SNV specific genomic sequences using real-time and nested RT-PCR methodologies. [a] not available; [b] a titer of 100 was considered equivocal; [c] oropharyngeal fluids (modified, with permission, from Safronetz et al 2005).

total of 388 individuals. Male and female mice were captured at roughly equal frequencies with 201 (51.8%) male and 187 (48.2%) female mice collected from the six study sites. Eighty-six mice were captured and sampled over multiple sessions (the most for any rodent was four) resulting in a total of 502 samples from deer mice. Only six deer mice were captured in both 2004 and 2005. The overall trap success for deer mice was 3.9 captures per 100 trap nights (range among sites: 1.7 to 6.1) and the average recapture rate was 23.1%. The trap mortality rate for deer mice was 5.4% (21 of 388) over the two years of study with the greatest proportion (7 deaths) occurring in the final session of 2005. Relatively few sympatric rodent species were captured, the most prominent of which were red-backed voles (*Myodes gapperi*) with 168 individuals captured. Other infrequently encountered species included meadow jumping mice (*Zapus hudsonius*) with 13 individuals captured and 2 meadow voles (*Microtus pennsylvanicus*). Samples were collected from the majority (125) of these animals.

Over the two years a total of 108 serum samples were reactive for the presence of hantavirus specific anti-N antibodies, representing samples collected from 86 (22.2%) of 388 deer mice. A total of 102 serum samples were titrated, the majority of which (75) had antibody titers $\geq$ 6400. Seropositive deer mice were predominantly male with 53 (26.3%) of 201 males and 33 (17.6%) of 187 of females having serological evidence of hantavirus infection ($\chi^2 = 4.16$, df = 1, $p < 0.05$). Additionally, seropositive mice were more likely to be adult with 75 (25.6%) of 301 adults and 9 (10.2%) of 87 sub-adults having serological evidence of hantavirus infection upon first capture ($\chi^2 = 9.40$, df = 1, $p < 0.01$). Over the two years seroconversion was observed in 12 (3.8%) of 388 deer mice.

Seropositive deer mice were collected during all 15 trapping sessions with the ESP per session ranging from 7.9 to 60% (Figure 3.2). In 2004, seropositive mice were captured at five of six sites with an annual ESP of 28.1% (individual sites ranging from 0 to 58.1%). In 2005, seropositive mice were captured at all six sites with an annual ESP of 17% (individual sites ranging from 5.9 to 30.4%). In both 2004 and 2005, the highest ESP was documented in May or June.

Recently infected deer mice were differentiated from chronically infected rodents based on the detection of low avidity IgG antibodies. The avidity assay was performed on 99 seropositive serum samples (nine serum samples were of insufficient volumes to perform the avidity assay). High avidity IgG antibodies were detected in 73 (73.7%) samples, while low avidity IgG antibodies were detected in 19 (19.2%) samples. Seven (7.1%) samples had intermediate avidities. Deer mice with low avidity antibodies were collected in 9 of 15 trapping sessions with the proportion of recently infected mice varying from 0 to 9.4% of captured mice (Figure 3.3). The proportion of seropositive deer mice with low avidity antibodies varied from 0 to 33.3% per session, with the highest rates occurring in May or June each year (Figure 3.3). Low avidity antibodies were predominantly detected in samples with low (≤ 1600) antibody titers with 12 (46.2%) of 26 of samples with low titers having low avidity antibodies compared with 7 (9.6%) of 73 samples with high titers ($\chi^2 = 16.5$, df = 1, $p \leq 0.001$).

Evidence of SNV infection was not detected in any of the other (i.e., non-deer mice) rodents tested (n = 125). However, PHV genomic sequences were amplified from two meadow voles with serological evidence of hantavirus infection (data not shown). Both voles were adult and male and were captured from the same trap site, one per year.

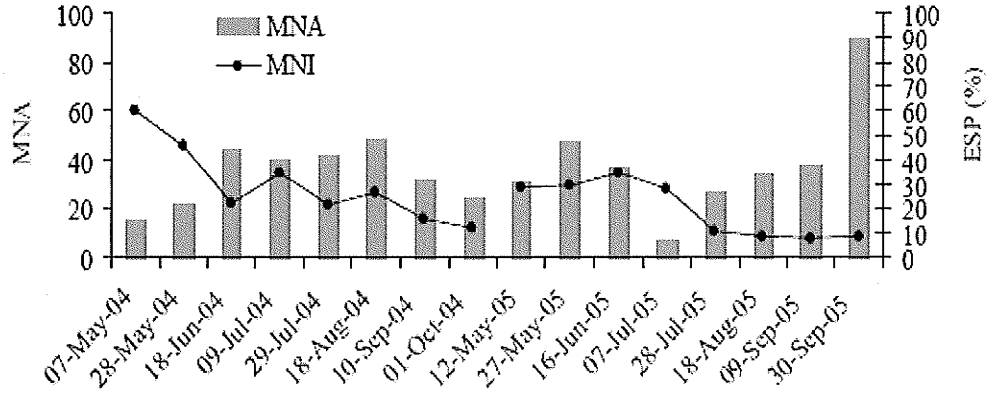


FIGURE 3.2. The minimum number of deer mice known to be alive (MNA) and estimated standing prevalence (ESP = (minimum number known to be infected per interval / MNA) x100). Reproduced with permission from Safronetz et al. 2008.

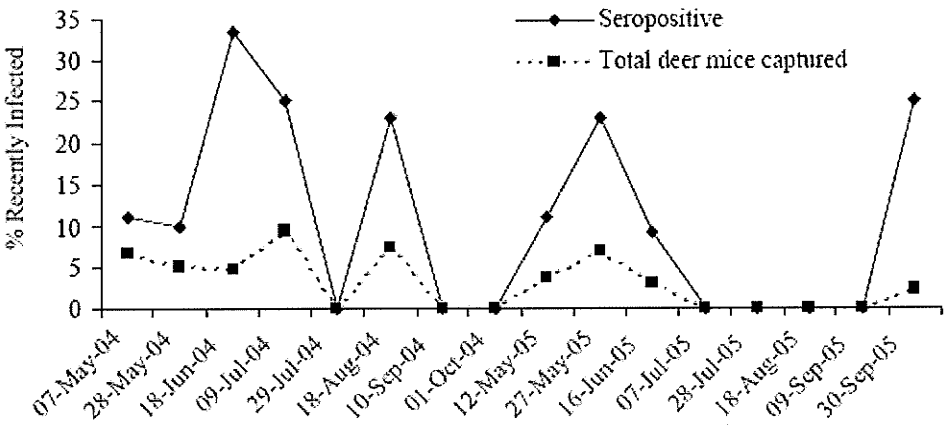


FIGURE 3.3. Proportion of recently infected deer mice in relation to seropositive and total deer mice captured per interval. Deer mice were considered recently infected based on the detection of low avidity IgG antibodies.

In total, RNA was extracted from 29 urine and 108 OPF and blood samples collected from seropositive rodents. Sin Nombre virus RNA was detected in 51 (47.2%) of 108 blood samples from seropositive deer mice. Forty-four (51.2%) of 86 seropositive deer mice had at least one RT-PCR positive blood sample over the course of the study. Positive blood samples were detected in mice collected in 14 of 15 trapping sessions with the proportion of seropositive deer mice with detectable SNV RNA in blood per session ranging from 18.2 to 90% when more than one seropositive mouse was sampled. The prevalence of RNA in blood in 2004 and 2005 was 53.8% and 36.4%, respectively (Figure 3.4). Similar to ESP, the detection of SNV RNA in blood samples from seropositive mice peaked in May of both 2004 and 2005, although in 2004 rates remained high throughout the year (Figure 3.4).

A positive association between high titer antibody ($\geq$ 6400) and RT-PCR positive blood samples was observed ($\chi^2 = 5.5$, df = 1, $p \leq 0.025$). Forty-two (56%) of the 75 blood samples with antibody titers $\geq$ 6400 were RT-PCR positive, compared with eight (29.6%) of 27 samples with titers of $\leq$ 1600. However, the proportion of blood samples with detectable SNV RNA was similar for the low and high avidity groups ($\chi^2 = 0.21$, df = 1, $p \leq 1$). In total, 11 (57.8%) of 19 samples with low avidity and 38 (52.1%) of 73 samples with high avidity were RT-PCR positive (Figure 3.5). Also, there was no significant difference between seropositive males or female with detectable SNV RNA in blood samples. In total 29 (54.7%) of 53 males had at least one RT-PCR positive blood sample, compared with 15 (45.5%) of 33 females ($\chi^2 = 0.70$, df = 1, $p \leq 1$). Four (12.1%) female and six (11.3%) males had multiple blood samples with demonstrable SNV RNA.

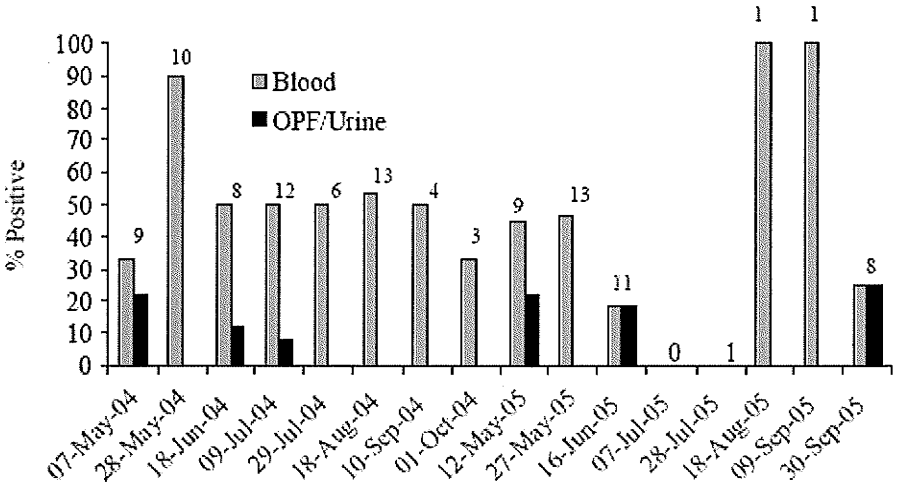


FIGURE 3.4. Proportion of seropositive deer mice with detectable SNV RNA in blood samples and OPF/urine samples. Total number of seropositive animals captured per interval is indicated above. Reproduced with permission from Safronetz et al. 2008.

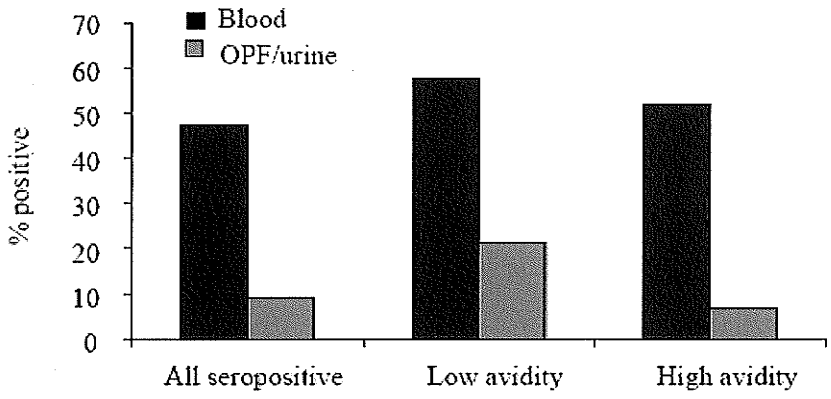


FIGURE 3.5. Comparison of RT-PCR positive blood and OPF/urine samples collected from seropositive deer mice with low and high avidity antibodies.

The overall detection rate of SNV RNA in OPF and urine collected from seropositive mice was similar with 8 (7.4%) of 108 OPF and 2 (6.9%) of 29 urine samples testing positive. In total, 10 (11.6%) of 86 individual, seropositive deer mice had detectable SNV RNA in OPF or urine. Seropositive animals did not have more than one positive OPF or urine sample collected throughout the course of the study. Two of the positive OPF samples and both positive urine samples were collected from mice with low avidity antibodies. Of the remaining positive OPF samples, five were collected from mice with high avidity antibody, and one from a mouse with intermediate avidity. The small number of deer mice collected with low avidity antibodies reduces the power of statistical comparisons between the detection of SNV RNA in urine or OPF samples and avidity groups. However, there was a trend towards a greater proportion of mice with low avidity antibody and RT-PCR positive OPF or urine samples when compared to mice with high avidity antibodies ($\chi^2 = 3.45$, df = 1, $p < 0.10$). In total, four (21%) of 19 mice with low avidity antibodies had detectable SNV RNA in OPF/urine compared with five (6.8%) of 73 samples from mice with high avidity antibodies (Figure 3.5).

Nucleotide sequence of M segment PCR amplicons from a subset of seropositive mice confirmed the presence of SNV and demonstrated a high identity (> 95%) with SNV genotypes previously reported on the prairies (Figure 3.6, Drebot et al. 2001).

Blood samples (12), OPF (12), and urine (4) were tested from 11 deer mice prior to antibody acquisition (i.e., pre-seroconversion). Six (50%) of the 12 blood samples were positive for SNV RNA, including two blood samples from the same mouse collected in consecutive intervals immediately prior to the detection of SNV specific antibodies. Urine and OPF samples collected from these mice were uniformly negative.

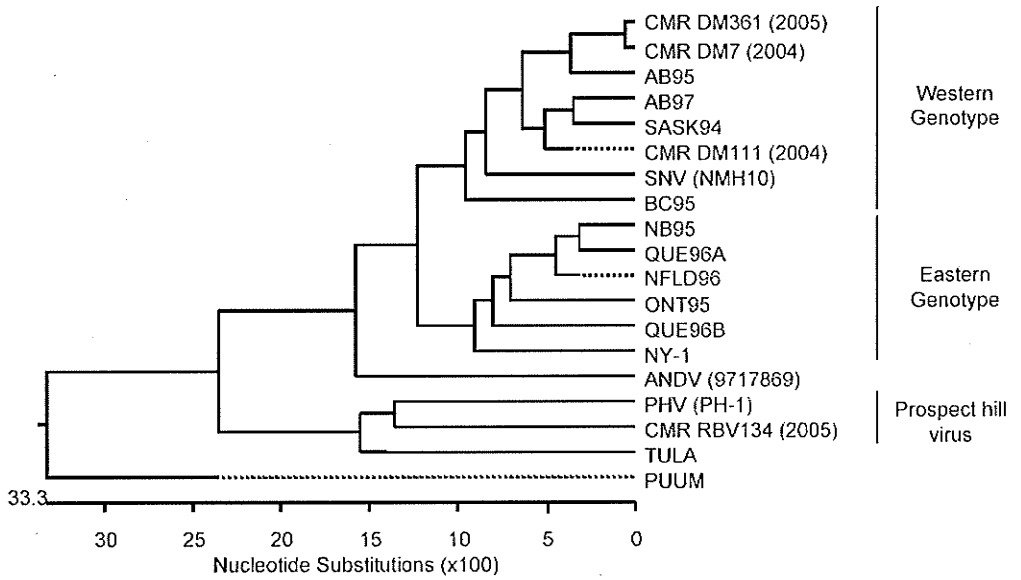


FIGURE 3.6. Phylogenetic analysis of SNV sequences from deer mice collected during the CMR study. The nucleotide sequence of a 200 bp fragment of the G1 coding region was analyzed with Lasergene DNAstar software (version 7.2) using the MegAlign applications. Sequences identified in the CMR study (denoted by CMR with the appropriate deer mouse, DM, identifier number and the year of collection in brackets) were compared with those described by Drebot et al. (2001) from Newfoundland (NFLD), New Brunswick (NB), Quebec (QUE), Ontario (ONT), Saskatchewan (Sask), Alberta (AB) and British Columbia (BC) as well as reference sequences including SNV (NMH10, Spiropoulou et al. 1994, accession # NC005215), NY-1 (Hjelle et al. 1995, accession # U36802), ANDV (9717869, Meissner et al. 2002, accession # AF291703), PHV (Plyusnin, Vapalahti & Vaheri 1996, accession # AZ49098), Tula (Vapalahti et al. 1996, accession # NC_05228) and PUUV (Vapalahti et al. 1992, accession # NC_005223).

DISCUSSION

The overall patterns of SNV infection in deer mice collected in southern Manitoba are similar to those from studies conducted elsewhere (Boone et al. 1998, Calisher et al. 1999, Douglass et al. 2001). In the CMR study, adult and male deer mice were disproportionately infected, confirming the existence of an age and sex bias of SNV infection. Studies with both naturally and experimentally infected deer mice have demonstrated the presence of SNV (viral antigen and/or RNA) in a variety of tissues (Netski, Thran & St Jeor 1999, Botten et al. 2003). Necropsies performed on 15 seropositive mice collected during the pilot study confirmed a similar systemic infection in naturally infected mice from Manitoba. Most of the seropositive deer mice examined had detectable levels of SNV RNA in a number of tissue types, with seropositive adult mice having the highest frequency of RT-PCR positive tissues. Although a few of the mice considered to be serologically equivocal (i.e., positive only at the 1:100 screening dilution) likely represented carry over of maternal antibodies, many of these mice were adult and those examined yielded RT-PCR positive tissues suggesting that some were true infections.

During the two year CMR study, the annual peak in seroprevalence was documented in spring or early summer (May or June) with the individual trapping session prevalence peaking in May 2004 at nearly 60%. As in other studies, prevalence decreased with the appearance of juvenile deer mice (i.e., during breeding season) and fluctuated considerably for the remainder of the season (Douglass et al. 2001). However, the average seroprevalence rate of 22.2% in this study is higher than those reported in similar studies from Montana (13%, Douglass et al. 2001), Colorado (between 2.6% and 9.6%

Calisher et al. 1999, 2005) and various national parks in eastern and central United States (7%, Mills et al. 1998). It has been suggested that seroprevalence of SNV in areas with low rodent diversity might be elevated due to increased interaction of infected rodents with susceptible individuals (Mills 2005). In the above mentioned studies, significant numbers of sympatric rodent species were also captured, some of which harboured other hantavirus species. The effect of low biodiversity might partially explain the infection rates observed here since deer mice dominated the study locations in Manitoba with only one other rodent species (red-backed voles) consistently captured every trapping session. It should, however, be noted that the overall seroprevalence documented here may also be biased by the length of the study as well as the lack of winter data.

Efforts to evaluate overwinter prevalence of SNV were largely ineffective. In total, two deer mice were found inhabiting the nest boxes overwinter (January 2004); however one was found dead, presumably due to freezing temperatures, while the other was sampled, but was not seen again (data not shown). Studies conducted in Montana have also experienced difficulties in attempting to address overwinter prevalence of SNV, and have concluded that overwinter activity of SNV in sylvan populations of deer mice remains a "black box" (Douglass et al. 2001). In any case, six deer mice were captured in both early fall 2004 and spring 2005, three of which seroconverted between these sampling points. Furthermore, in both years the highest annual prevalence was observed in adult mice collected in spring which supports overwinter transmission of virus. Deer mice remain active all winter long, albeit at a reduced capacity, and are known to cohabitate in communal dwellings with as many as 13 individuals observed per nest (Banfield 1974). It is conceivable if one of these deer mice were infected, a large majority

(if not all) would emerge the following spring infected with SNV. Although unable to collect overwinter data, this suggests that SNV transmission does occur during the winter months.

Few studies have monitored the presence of SNV RNA in blood samples during a multi-year study. Previously it has been suggested that peak viral RNA detection in blood corresponds with the early breeding season (Kuenzi et al. 2005). The CMR study findings support this hypothesis. Additionally, the peak detection of SNV RNA in blood also corresponded with peak seroprevalence levels though the detection of RNA in blood was less variable than seroprevalence rates. In the CMR study, the overall detection rate (47.2%), as well as the proportion of infected mice with detectable SNV RNA in blood (51.2%) were similar to those documented in Nevada and California (51% of seropositive deer mice, Boone et al. 1998), but considerably higher than those documented in sylvan deer mouse populations in Montana (19% of blood samples from seropositive mice, Kuenzi et al. 2005). Although difficult to ascertain, the differences observed between the Montana and Manitoba studies are likely due to many naturally occurring variables which have been previously shown to influence seroprevalence (i.e., food availability and population size and structure). It is interesting to note that both the CMR study presented here and the study by Boone and others (1998) in Nevada/California both documented high rates of detectable SNV RNA in blood in populations of deer mice with a high seroprevalence, implying that one may drive the other.

It has been suggested that chronically infected deer mice undergo viral recrudescence and this hypothesis has been used to explain findings of seropositive rodents with alternating patterns of RT-PCR positive and negative results from blood

samples collected over a period of time (Kuenzi et al. 2005). Although similar (alternating) patterns were not observed in the CMR study, this is most likely because individual deer mice were not recaptured a sufficient number of times to discern them. Nevertheless, it seems plausible that viral reactivity could be involved in the high rate of detection of SNV RNA in blood reported here, especially in light of its association with high titer antibodies. Similar to the studies conducted on deer mice collected from Hobbema, Alberta (outlined in Chapter II) there was no significant difference in the detection of SNV RNA in blood samples from mice with high and low avidity antibodies. Interestingly, over the course of the CMR and Hobbema studies, only 1 (7.1%) of 14 mice with intermediate avidity had an RT-PCR positive result from blood. Although the number of mice with intermediate avidities were low, the data suggest as the antibody response matures, SNV is cleared (or reduced to levels below the detection of nested RT-PCR) from blood, only to reactivate later during infection despite a robust humoral immune response. As in other studies (Boone et al. 1998, Kuenzi et al. 2005), a brief period of viremia was documented in five of 11 infected deer mice prior to antibody acquisition, with one deer mouse having detectable SNV RNA in blood samples collected in the two consecutive sampling sessions prior to the detection of SNV specific IgG antibodies.

Hantavirus transmission is believed to occur primarily through virus contaminated excreta and secreta. Studies with PUUV have described infectious virus in OPF, urine and/or feces from experimentally infected bank voles by inoculating samples directly into weaning voles. The same study also demonstrated intracage transmission of virus from infected to naïve rodents (Yanagihara, Amyx & Gajdusek 1985). More recently, Fulhorst

and colleagues (2002) used cell culture to detect infectious Caño Delgadito virus in oral swabs and urine samples collected from experimentally infected Alstoni cotton rats, however they found the sensitivity of virus recovery using these methods was low. While it is widely assumed that SNV transmission occurs through the same routes, infectious SNV in OPF, urine or feces has not been well documented. Transmission studies with SNV using experimentally infected deer mice have yielded limited results with only a single documented case of intracage transmission and no infectious virus detected by passaging pooled excreta or secreta samples in cell culture or naïve rodents (Botten et al. 2002). Despite failed attempts at transmission, this remains the only study to demonstrate the presence of SNV RNA in OPF and, to-date, no studies have demonstrated the presence of SNV RNA in urine samples (Botten et al. 2002). As such, the transmission dynamics of SNV remain poorly understood and, due to the low sensitivity and reliability of infectious virus assays for SNV, few field studies have attempted to collect and test secreta and/or excreta to address the subject. In the two field studies presented here SNV RNA was documented in OPF and urine from a small proportion of seropositive mice. Although viral RNA does not necessarily predict the presence of infectious virus, it is suggestive of the potential for transmission via these samples.

One of the enduring mysteries about SNV is why it appears that only a very small fraction of persons who appear to be exposed to SNV infected mice actually contract infection and develop HPS. It is reasonable to question whether seropositive deer mice differ markedly in their infectiousness to man. Thus it is of potential interest that individual deer mouse specimens differ substantially in the degree to which viral RNA can be detected in their excreta/secreta. Laboratory based studies on PUUV infections in

Myodes glareolus (Yanagihara, Amyx & Gajdusek 1985) and Caño Delgadito virus infections in *Sigmodon alstoni* (Fulhorst et al. 2002) have suggested that these hantaviruses are shed at higher rates early after virus acquisition (in the acute stages of infection) with periodic shedding occurring in the persistent stages of infection. Additional studies with BCCV in cotton rats have also suggested that transmission may be highest during the early stages of infection, based on increased detection of viral antigen and/or virus in numerous tissues and urine and high rates of intracage transmission within the first few weeks post inoculation (Hutchinson et al. 1998, 2000). The findings of these laboratory based studies are supported by observations of PUUV dynamics in naturally infected bank voles. Bernshtein et al (1999), found intracage transmission of PUUV between naturally infected donors and naïve voles occurred most frequently during the first month of infection and this corresponded to peak accumulation of viral antigen in tissues. To determine if similar transmission patterns exist in naturally infected deer mice, the avidity assay was used to differentiate between acutely and chronically infected rodents. Using this assay low avidity antibodies were detected in 19 serum samples (representing 18.6% of samples reactive for hantavirus specific antibodies collected during the CMR study). Although the numbers of recently infected mice (as defined here by the presence of low avidity antibody) in this study were low, there was a trend towards an increased proportion of recently infected deer mice with detectable SNV RNA in urine or OPF (Figure 3.5, 19% of samples from low avidity mice versus 6.8% of samples from high avidity mice). Retrospective analysis on the samples collected as a part of the pilot study revealed similar patterns with 3 (37.5%) of 8 mice with low avidity having detectable SNV RNA in OPF and/or urine, compared with 3 (18.75%) of 16 mice

with high avidity (data not shown). The remaining positive OPF sample was collected from a mouse with intermediate avidity. Combined the two field studies presented here provide the first evidence supporting this hypothesis in a population of naturally infected deer mice.

Despite high rates of seroprevalence and detection of SNV RNA in blood samples from seropositive deer mice, only a small proportion of infected deer mice collected in southern Manitoba appear to be actively shedding SNV at any one time (as suggested by the presence of SNV RNA in urine or OPF). This may partially explain why HPS remains a rare disease with only 70 laboratory confirmed cases in Canada from 1992 to present (H. Artsob, unpublished data). However, the apparently low proportion of mice actively shedding SNV may not sufficiently explain the high exposure rates of deer mice to the virus (as predicted by the seroprevalence). Aggressive encounters between infected and naïve rodents have been hypothesized as an important route of viral dissemination (Glass et al. 1988). Although viral transmission between aggressive rodents could occur via SNV contaminated OPF, urine or blood, based on the data presented here it is reasonable to speculate that direct blood-borne transmission (i.e., through wound to wound contact) of virus may contribute significantly to the enzootic cycle of SNV. This hypothesis may also be supported by other field studies which have found high seroprevalence rates occurring in concert with high rates of detection of SNV RNA in blood samples from seropositive mice (Boone et al. 1998). Botten et al (2003), described two discrete patterns (disseminated and restricted) of persistent SNV infection in experimentally infected deer mice, based on the detectability of viral RNA in blood, replicative (positive strand) RNA in the heart, lungs or brown fat, and the number of antigen positive tissues. The study also

found that SNV was more readily recovered from mice in the disseminated stage of persistent infection, although the authors only focused on solid tissue samples and not blood, excreta or secreta. Similar patterns of persistent infection may also exist in naturally infected deer mice as suggested by the detection of SNV RNA in blood from chronically infected rodents. These presumably viremic mice may represent the fundamental reservoir for the enzootic cycle of SNV.

CHAPTER IV:

Establishing the Syrian hamster animal model for the study of ANDV HPS.

INTRODUCTION

The study of hantaviruses and their associated diseases has been hampered by lack of a suitable animal model which reflects disease progression observed in humans. Experimental infections with several hantaviruses in their natural hosts have been utilized with limited success to glean information on chronic infection, transmission, and maintenance in nature (Yanagihara, Amyx & Gajdusek 1985, Hutchinson, Rollin & Peters 1998, Botten et al. 2000, Fulhorst et al. 2002). Natural host models have also been utilized to evaluate potential therapies and vaccines (Lundkvist et al. 1996a, Medina et al. 2007), however reservoir hosts do not make an ideal animal model for the study of hantaviruses since they do not respond to infection in the same way humans do. Several small animal species have been evaluated as potential models for the study of HFRS and HPS, with little success. Numerous laboratory rodents (mice, rats, guinea pigs, gerbils, and hamsters) have been inoculated with HFRS causing hantaviruses, and although they are susceptible to infection, much like the natural rodent hosts, they are generally asymptomatic (Kawamura et al. 1991, Tamura et al. 1991). Non-human primates (NHP) have also been assessed as models for disease, however the majority of Old and New World NHPs infected resulted in asymptomatic infections (Yanagihara et al. 1988, Groen & Osterhsus 1999). The lone exception may be Cynomolgus macagues (*Macaca fascicularis*) which appear to develop mild symptoms (i.e. lethargy, proteinuria, and microhaematuria) suggestive of acute nephropathy following inoculation with lung

homogenates from voles naturally infected with PUUV (Yanagihara et al. 1988, Groen & Osterhsus 1999). Similar studies conducted with ANDV concluded that Cynomolgus monkeys are susceptible to infection but do not display symptoms of HPS (McElroy et al. 2002). With the exception of HTNV in suckling (McKee et al. 1985) and adult (Wichmann et al. 2002) mice, lethal models for HFRS viruses have not been reported and currently no animal models exist which reflects the symptomology of HFRS.

In 2001, the first lethal disease model for the study of hantaviruses was described (Hooper et al. 2001b). Hooper and colleagues reported that, unlike HFRS causing viruses, ANDV infection in Syrian hamsters (*Mesocricetus auratus*) results in a lethal illness clinically similar to HPS. Beginning at approximately 10 dpi, hamsters develop respiratory symptoms presenting as progressively worsening breathing deficiency which leads to death, generally within 36 hrs of onset. In addition to these symptoms, ANDV infected hamsters become hypotensive in the days immediately preceding death and exhibit signs of cardiogenic shock as suggested by a rapidly increasing heart rate and heart rate variability (Campen et al. 2006). Although hamsters are highly susceptible to ANDV infection with the 50% lethal dose (LD_{50}) for intramuscular (i.m.) injections calculated at 8 plaque forming units (PFU), other HPS causing viruses, including SNV, do not appear to cause disease in this model.

Several studies, listed above, have demonstrated the utility of the hamster model of HPS in recent years, however, prior to initiating experiments with the model it is important to re-evaluate it in individual laboratories with in-house virus preps. The purpose of the following studies was to establish the hamster model of HPS at the NML and evaluate it for future use in vaccine trials.

HYPOTHESIS

1. Similar to previous descriptions, Syrian Golden hamsters injected with ANDV will develop disease characteristics which both clinically and pathologically resemble HPS in humans.

MATERIALS AND METHODS

Establishing the hamster model of HPS:

Ethical approval. The studies outlined in the following two chapters were carried out in accordance with the Canadian Council of Animal Care guidelines and under the Canadian Science Centre for Human and Animal Health Animal Use document H06-009.

Biosafety. The Public Health Agency of Canada in conjunction with the Office of Laboratory Security is responsible for classifying biological agents in Canada according to their associated disease and transmission characteristics. Hantaviruses are defined as risk group 3 agents meaning in humans they can cause severe disease (which represents a high personal risk), but are not typically transmitted from person-to-person (therefore are of low community risk). Based on this classification, and in accordance with the CDC's recommendations, *in vitro* experiments (i.e., cell culture) conducted with hantaviruses require BSL 3 containment. However, due to the increased risk of aerosolization and/or direct transmission of virus when doing animal experiments in natural or susceptible hosts (i.e., permissive hosts potentially capable of transmitting virus), *in vivo* experiments with hantaviruses requires BSL 4 containment (CDC 1994).

Virus propagation. Vero E6 cells (ATCC # CRL-1586, an African green monkey kidney cell line) were cultured at 37°C with 5% atmospheric CO_2 in culture media consisting of Dulbecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin [100 U/mL] and streptomycin [100 μg/mL]) and L-glutamine (2 mM) (all from Invitrogen).

Andes virus (strain Chile 9717869, gift from Dr. Connie Schmaljohn, US Army Medical Research Institute of Infectious Diseases, Ft. Detrick, MD, USA) was propagated in a BSL 3 laboratory. Monolayers of Vero E6 cells were infected at approximately 80% confluence with ANDV diluted 1:10 in a minimal amount of pre-warmed DMEM. Following adsorption (1-2 hrs at 37°C), the volume of media was increased according to the size of flask with culture media containing reduced (2%) FBS and incubated (37°C, 5% CO_2). After 10-14 days, supernatant was collected and clarified by low speed centrifugation (500 x g, 10 min), after which the FBS concentration was increased to 20% and aliquots stored at -80°C.

Infectious virus titration. A focus-forming assay was used to titer the ANDV stocks. Ten-fold serial dilutions of ANDV were prepared in DMEM supplemented with 5% FBS and added (100 μl per well for 24 well culture plates) to triplicate confluent monolayers of Vero E6 cells. Following adsorption (1 hr, 37°C), cells were washed twice with DMEM, and overlayed with culture media containing 1.4% methylcellulose. Plates were incubated (37°C, 5% CO_2) for 7-10 days, after which time the overlay was removed and cell monolayers were fixed with a 1:1 methanol-acetone mix for 15 min. Cells were air dried in a biosafety cabinet (15-30 min) and removed from BSL 3 following standard

operating procedures. In BSL 2, cells were washed twice with PBS and incubated with rabbit anti-SNV N hyperimmune sera (diluted 1:1000 in PBS, gift from Dr. Brian Hjelle) for 1 hr at room temperature (RT). Cells were washed twice with PBS and incubated with peroxidase conjugated goat anti-rabbit IgG antibodies (diluted 1:500 in PBS, Dako) for 1 hr, RT. Following two more PBS washes, foci were visualized using NovaRED peroxidase substrate kits (Vector Laboratories) according to manufacturer's instructions. Titers were calculated using the highest dilution resulting in at least 20 foci, and were expressed as focus forming units (FFU) per mL.

Animal husbandry. Syrian golden hamsters (Charles River) were housed within a BSL 4 laboratory either individually (females), or in groups of 3-4 (males) in microisolator cages. Water and food were provided *ad libitum* and animals were maintained on 12:12 hr light/dark cycle. Hamsters were allowed to acclimatize for a minimum of one week to BSL 4 conditions prior to commencing the indicated experiments.

Determining the route of inoculation. Ten female hamsters (approximately 10 weeks old) were randomly divided into two groups of five, individually anaesthetized in a sealed chamber by inhalation of halothane and inoculated with ANDV diluted in sterile DMEM. One group was challenged via i.m. injection at two sites in the hind leg musculature, with 0.1 mL inoculum, representing a challenge dose of 4000 FFU. The second group was challenged via intraperitoneal (i.p.) injection at two sites with 0.2 mL inoculum, representing a dose of 8000 FFU. A single control hamster was inoculated with sterile DMEM (i.p.). Inoculations were administered using a 1 mL syringe fitted with a 25-ga

½" needle and delivered with the aid of a volumetric stepper (Tridak) to ensure a precise, uniform dose. Following inoculation, hamsters were carefully monitored for signs of illness and weighted daily.

Lethal dose 50 calculations. Thirty-six female hamsters, approximately 6-8 weeks old, were randomly divided into six groups of six and inoculated (i.p. injections, as above) with 10-fold serial dilutions of ANDV prepared in sterile DMEM representing challenge doses between 80000 and 0.8 FFU. Post-inoculation, hamsters were monitored daily for signs of illness. Using these data the LD_{50} for i.p. injections of ANDV in hamsters was calculated.

Time course pathogenesis study:

Serial sample study. Twenty-five male hamsters, approximately 6-8 weeks old, were randomly divided into two groups and injected (i.p., as above) with either 100 LD_{50} (154 FFU) ANDV diluted in DMEM (n = 18), or DMEM alone (n = 7).

At 2, 4, 6, 8, 10 and 12 dpi, one control and three infected hamsters were anaesthetized, and exsanguinated via cardiac puncture with blood collected into 5 mL EDTA tubes. The oral cavity of each hamster was swabbed with a sterile applicator, pre-wetted in DMEM, which was immediately placed into 1 mL DMEM. Urine was collected by cystocentesis and expelled directly into 1 mL DMEM. Necropsies were performed on each animal and the heart, right and left lung, right and left kidney, liver, spleen, brain, bladder and one testicle were removed, placed in individual sterile petri-dishes and briefly washed in PBS. An approximately 100 mg piece of each organ was removed for

homogenization and the remainders of each organ were placed into 10% phosphate buffered formalin (for histopathology and IHC). The 100 mg sections of each tissue were mechanically homogenized in 1 mL DMEM with a sterile 3 mm tungsten bead using a Qiagen Mixer Mill for 10 min at a frequency of 30 Hz. Homogenates were clarified (15,000 x g, 10 min), and the supernatants transferred to clean tubes. One hundred and forty microlitres of cardiac blood, OPF, urine and tissue homogenates were mixed with 560 µl lysis buffer AVL (Qiagen) and immediately extracted for RNA, while the balance of each sample was stored at -80°C for viral titration.

Real-time quantitative RT-PCR. Viral RNA was extracted from samples using QIAamp viral RNA extraction kits (Qiagen) according to spin protocols outlined in manufacturer's instructions. Quantitative, real-time, one-step RT-PCR was carried out using a Rotor-Gene RG-3000 instrument (Corbett research) with ANDV S segment specific primers (ANDV S129f and ANDV S291r) and dual labeled FAM/TAMRA fluorescent probe (ANDV TM, binding coordinate 183, all from TIB Molbiol, sequences in appendix 1). A 162 bp fragment was amplified in 25 µl reactions using QuantiTect Probe RT-PCR kits (Qiagen) as specified in the product insert with 0.4 µM of each primer and 0.2 µM of probe. Each reaction contained 20 µl of master mix and 5 µl of extracted RNA. Reverse transcription-polymerase chain reaction was carried out in three stages: reverse transcription (50°C for 30 min); Taq activation (95°C for 15 min); and amplification (40 cycles of 94°C for 15 sec, 60°C for 60 sec). Data acquisition occurred at the end of the annealing/extension stage (60°C for 60 sec) of each amplification cycle.

Samples were quantified against a standard curve of ANDV RNA extracted (as above) from 10-fold serial dilutions of titered ANDV stocks.

Assay for infectious virus. Infectious ANDV titers in tissue homogenates were determined using the focus assay as outlined above with few exceptions. The overlay media (DMEM, with 10% FBS and 1.4% methylcellulose) contained twice as much penicillin (200 U/mL) and streptomycin (200 μg/mL). Following the 7-10 day incubation, infected cells were fixed overnight with 10% phosphate buffered formalin. The next day the formalin was changed and the plates were sealed in plastic pouches and removed from BSL 4 according to standard operating procedures. The remaining steps of the focus assay were conducted in BSL 2 as outlined above.

Histopathology. Formalin fixed lung, kidney, heart, spleen, liver, brain, bladder and testis from each hamster were dehydrated and embedded in paraffin according to standard procedures. Thin (4 μm) sections of each tissue were prepared, mounted on slides and air dried. The following day, slides were deparrafinized at 60°C for 60 min, followed by washes in xylene (2 x 5 min) and absolute ethanol (2 x 2 min). Samples were re-hydrated with successive washes in decreasing concentrations of ethanol (90%, 70%; 1 x 2 min each), rinsed in water and stained with hematoxylin and eosin. Briefly, slides were over-stained in hematoxylin (5 min), rinsed in water and slightly de-stained in 1% acid alcohol (approximately 30 sec). Following another rinse in water, slides were immersed in Scott's tap-water substitute (30 sec), rinsed again, and checked by light microscopy to ensure nuclei were stained blue and cytoplasm appeared grey in color. Slides were then

counterstained with 1% eosin to stain cytoplasm (3 min), rinsed in water and dehydrated with 30 sec washes in increasing concentrations of ethanol (70%, 90%, 100% x 2) and two 30 sec washes in xylene. Cover slips were mounted with Permamount (Fisher) and slides were allowed to dry overnight before being analyzed by light microscopy.

Assay for viral antigen. An IHC assay was used to test for ANDV antigen in tissue sections. Thin (4 μm) tissue sections were prepared and deparrafinized as above. Slides were briefly washed in Tris buffered saline (TBS), submerged in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6) and autoclaved at 121°C for 20 min for antigen retrieval. After rinsing with TBS containing 0.01% Tween 20 (TBST), slides were blocked with Casein solution (PowerBlock, Biogenex) for 10 min, followed by another three TBTS washes. Tissue sections were then incubated overnight at 4°C with a monoclonal antibody specific for ANDV N antigen (clone 1A8/F6, Austral Biologicals) diluted at 1:100 in TBST. The next morning, slides were washed six times with TBST. Detection of the primary antibody was accomplished using LSAB2 Streptavidin-Biotin kits (Dako) with a universal secondary antibody and peroxidase-conjugated strepavidin, according to manufacturer's instructions. NovaRED peroxidase substrate kits were used as the chromogen and were supplemented with levamisole (Vector Laboratories) to inhibit endogenous alkaline phosphates activity. After incubation with substrate (10 min), slides were washed with TBST, counterstained with methyl green (5 min at 60°C), rinsed in water and dehydrated and mounted as above.

RESULTS

Establishing the hamster model of HPS:

In the initial experiment, groups of five hamsters were inoculated with ANDV in DMEM by way of i.m. or i.p. injections and monitored (and weighed) daily for signs of illness. A single hamster (i.p. group) was showing signs of illness the morning of day 7 and was found dead later the same day. The remaining 9 hamsters began showing symptoms between 7 and 9 dpi and all had succumbed to infection by day 10 (Figure 4.1A). Symptoms mainly consisted of breathing distress, beginning with rapid, shallow breaths which quickly advanced to severe dyspnea. At symptom onset, hamsters appeared active but as symptoms progressed they quickly became moribund. Death occurred within approximately 12-36 hrs of symptom onset. Post-mortem observations included cyanosis in lips of the majority of hamsters as well as the occasional finding of dried blood around the nares and mouths. The majority of animals gained weight until 6 or 7 dpi and only lost an average of 3.5% of their body weight (i.m. average 2.9%, range 1% to 4.6%; i.p. group average = 4.1%, range 1.2% to 6.2%, Figure 4.1B). The single control hamster showed no signs of illness, and was euthanized two weeks after the last infected hamster died. No significant differences in disease progression were observed between the different sites of injection.

In order to determine the LD_{50} of ANDV for i.p. injections, 36 hamsters were separated into groups of six and challenged with 10-fold dilutions of virus (80,000 to 0.8 FFU, Figure 4.2A). The time to symptoms onset occurred in a dose dependant manner with animals receiving higher doses showing symptoms (and dying) earlier than those receiving lower doses (Figure 4.2B). However, ANDV was not 100% lethal at higher

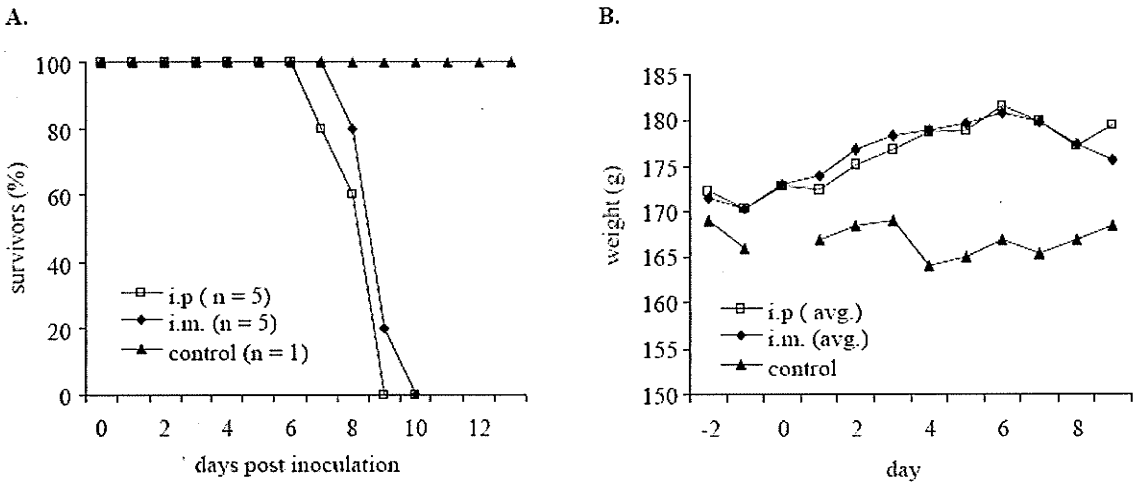


FIGURE 4.1. Evaluation of intramuscular and intraperitoneal injection of ANDV in hamsters. (A) To assess the virulence and influence of route of inoculation of ANDV in hamsters, groups of five animals were injected via i.m. or i.p. routes and monitored daily for signs of illness. A control hamster was injected with DMEM alone (i.p. injection). Survival curves are shown. (B) Weight charts show a steady increase in body weight following ANDV injection with only a slight loss of weight in the terminal stages of illness. Average weights for both i.m. and i.p. groups are shown along with those of the single control hamster.

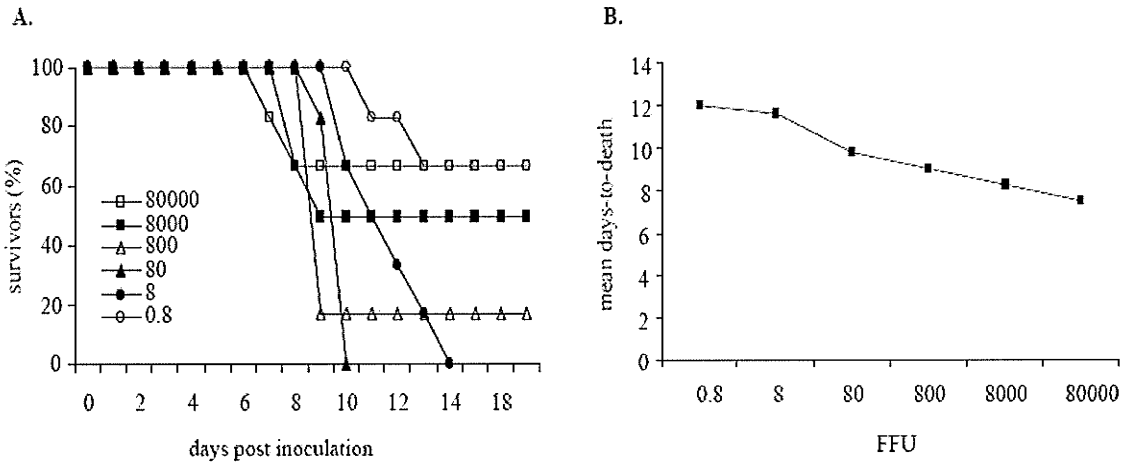


FIGURE 4.2. Determination of the lethal dose required to kill 50% of hamsters inoculated with ANDV. (A) Groups of six hamsters were i.p. injected with serial 10-fold dilutions of ANDV (inoculum ranging from 80000 to 0.8 FFU). Survival curves are shown. Based on the data the LD_{50} for i.p. injections was calculated at 1.54 FFU. (B) Average time to death is inversely related to inoculum dose.

doses with only 2 (33.3%) of 6 hamsters inoculated with 80,000 FFU and 3 (50%) of 6 hamsters inoculated with 8,000 FFU appearing ill and subsequently succumbing to infection. In the 800 FFU group, 5 (83%) of 6 hamsters expired, with all five hamsters dying between 8 and 9 dpi. Challenge doses of 80 and 8 FFU were both 100% lethal, with death occurring between days 8 and 10 and 10 and 14, respectively. Only two hamsters challenged with 0.8 FFU ANDV, showed symptoms of infection and died at 11 and 13 dpi. Hamsters which survived challenge were monitored for an additional two weeks after the last death and did not show any clinical signs of illness throughout the experiment. Based on these data the LD_{50} for i.p. injections of ANDV was calculated to be 1.54 FFU.

Time course pathogenesis study:

To monitor disease progression over time, 18 hamsters were inoculated with 100 LD_{50} of ANDV and at two day intervals, one control and three infected hamsters were euthanized and necropsies performed (in total 20 necropsies were performed throughout the experiment). Hamsters inoculated with ANDV were clinically normal until 9 dpi at which point 5 of 6 remaining hamsters began showing characteristic symptoms of HPS (i.e., breathing distress consisting of rapid, shallow breaths). At 10 dpi, three hamsters were found dead (H22, 23, 24, necropsies not performed on these animals), and the remaining three were moribund and dyspneic. Findings of the study are summarized in Table 5. Gross abnormalities at necropsy were mainly observed following symptom onset and were most notable in the lungs from these animals. Edema was noted as early as 6 dpi in the lungs of the three infected hamsters. In addition, one of the infected hamsters (animal H11) also had an enlarged spleen (approximately 3x regular size). However at 8

TABLE 5

Temporal analysis of the detection of virus and histpathological changes in hamster tissues collected during a serial sample study

Tissue / Sample	RT-PCR	IHC	Infectious virus	Gross abnormalities	Histopathology
2 dpi (no symptoms)					
Blood	0/3	-	n.d.[a]		
Lung	2/3	0/3	n.d.		
Heart	0/3	0/3	n.d.		
Liver	0/3	0/3	n.d.		
Kidney	2/3	0/3	n.d.		
Spleen	2/3	0/3	n.d.		
Bladder	0/3	n.d.	n.d.		
Testis	0/3	n.d.	n.d.		
Brain	0/3	n.d.	n.d.		
OPF	0/3	-	n.d.		
Urine	0/3	-	n.d.		
4 dpi (no symptoms)					
Blood	0/3	-	n.d.		
Lung	3/3	0/3	n.d.		
Heart	3/3	0/3	n.d.		
Liver	2/3	0/3	n.d.		
Kidney	3/3	0/3	n.d.		
Spleen	3/3	0/3	n.d.		
Bladder	0/3	n.d.	n.d.		
Testis	3/3	n.d.	n.d.		
Brain	3/3	n.d.	n.d.		
OPF	0/3	-	n.d.		
Urine	0/3	-	n.d.		
6 dpi (no symptoms)					
Blood	3/3	-	n.d.		
Lung	3/3	1/3	n.d.	3/3 edema	
Heart	3/3	0/3	n.d.		
Liver	3/3	1/3	n.d.		
Kidney	3/3	0/3	n.d.		
Spleen	3/3	0/3	n.d.	1/3 enlarged	
Bladder	3/3	n.d.	n.d.		
Testis	3/3	n.d.	n.d.		
Brain	3/3	n.d.	n.d.		
OPF	0/3	-	n.d.		
Urine	0/3	-	n.d.		
8 dpi (no symptoms)					
Blood	3/3	-	n.d.		
Lung	3/3	3/3	n.d.		
Heart	3/3	0/3	n.d.		

Tissue / Sample	RT-PCR	IHC	Infectious virus	Gross abnormalities	Histopathology
Liver	3/3	1/3	n.d.		
Kidney	3/3	0/3	n.d.		
Spleen	3/3	0/3	n.d.		
Bladder	3/3	n.d.	n.d.		
Testis	3/3	n.d.	n.d.		
Brain	3/3	n.d.	n.d.		
OPF	1/3	-	n.d.		
Urine	1/3	-	n.d.		
10 dpi (3 FD[b], 3 moribund and dyspneic)					
Blood	3/3	-	3/3		
Lung	3/3	3/3	3/3	3/3 edema	Yes [c]
Heart	3/3	2/3	3/3		
Liver	3/3	3/3	3/3		Yes [d]
Kidney	3/3	2/3	3/3		
Spleen	3/3	1/3	3/3		Yes [e]
Bladder	3/3	0/3	n.d.		
Testis	3/3	0/3	n.d.		
Brain	3/3	0/3	n.d.	1/3 meningitis	
OPF	2/3	-	0/3		
Urine	3/3	-	0/3	increased volume	

Symptom onset was 9 dpi in 5/6 remaining hamsters.
[a] n.d., not done; [b] FD, found dead; [c] pneumonitis, lymphohistiocytic infiltrates with thickening of alveolar walls and congestion; [d] mild hepatitis, hepatocyte necrosis; [e] red-pulp congestion, histiocytosis.

dpi, lungs from three infected animals appeared normal, and their spleens were only slightly enlarged. Severe edema was observed in lungs of the three terminally ill hamsters (10 dpi), and appeared to be more prominent in the lower portions of both lungs. Meningitis-like pathology was observed in the brain of one hamster (H20). Increased urine volumes were extracted from the bladders of two infected hamsters (H18 and 19) and unlike the cloudy, concentrated urine collected from all the other hamsters, the urine from these animals was clear and low pH. No gross abnormalities were observed in the liver and significant amounts of pleural effusion were not seen during the course of infection.

A total of 130 tissue sections (including 20 of 20 lung and liver; 18 of 20 kidney, heart and spleen; and 12 of 20 brain, bladder and testis samples collected) were stained with hemotoxylin and eosin and examined by light microscopy. Kidney, heart, brain, bladder, and testis from infected animals collected throughout the study were unremarkable andshowed no signs of pathological changes attributable to ANDV infection. Spleen samples collected from infected hamsters at 10 dpi appeared to have mild to moderate red-pulp congestion with moderate histiocytosis. Liver samples from infected hamsters at 10 dpi showed signs of mild hepatitis which was random and subacute, with multifocal individual hepatocyte necrosis. Lung samples demonstrated the greatest degree of pathological changes which were most pronounced in samples collected at 10 dpi. Microsopic examination of representative sections of lung tissue revealed a mild to moderate, diffuse subacute pneumonitis with mild perivascular lymphohistiocytic infiltrates. Moderate alveolar edema was also observed along with mild to moderate thickening of the alveolar walls (Figure 4.3).

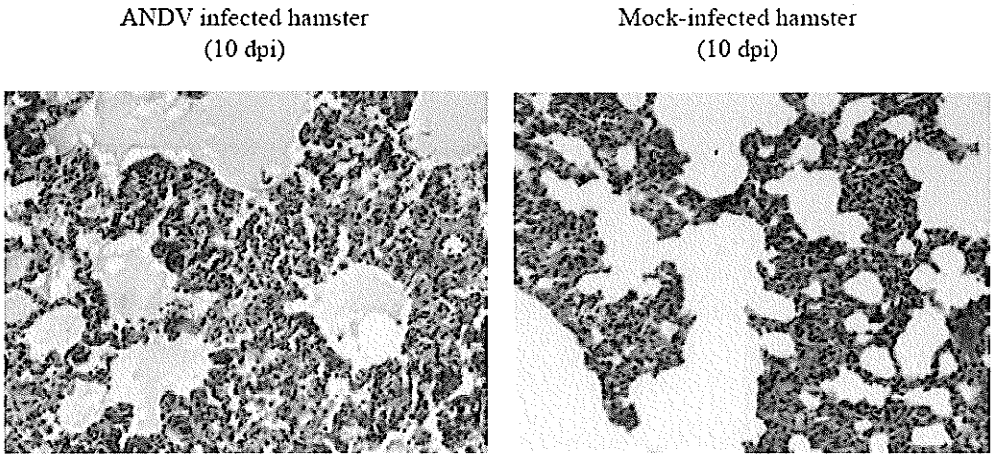


FIGURE 4.3. Hemotoxylin and eosin stained sections of hamster lungs post-inoculation. The most striking histological changes observed in hamsters occurred in the lungs and included congestion and thickened alveolar walls, which was most pronounced after symptom onset. Lung sections from an ANDV and mock infected hamster collected at 10 dpi.

In total, 100 tissue sections were examined for the presence of viral antigen using IHC protocols, including all tissue samples collected from animals at 10 dpi, as well as all remaining lung and liver samples and all heart, kidney and spleens collected after 2 dpi. Lung and liver sections contained the greatest amounts of ANDV N antigen. Beginning at 6 dpi, the lung and liver sections from one hamster had detectable amounts of viral antigen, although immunostaining was infrequent and limited. By 8 dpi, 3 of 3 lung sections from infected animals were positive, however antigen was only detected in 1 of 3 liver samples from these animals. At 10 dpi, 3 of 3 lung and liver samples from infected hamsters were immunoreactive with positive cells easily detectable throughout the sections (Figure 4.4). In contrast, ANDV N antigen was detected less frequently in kidney, heart and spleen sections and only in those collected at the 10 dpi time point. No viral antigen was detected in brain, bladder or testis sections.

A real-time RT-PCR assay was developed to quantify ANDV RNA levels in tissue homogenates against a standard curve derived from RNA extracted from serial dilutions of titrated viral stocks. The RNA profiles from the collected tissues are shown in Figure 4.5. Significant RNA titers were measured beginning at 4 dpi in lung and kidney homogenates and by 6 dpi all tissue samples analyzed were positive for ANDV RNA. Peak RNA levels were observed in liver samples collected at 8 dpi. Blood samples were first positive at 6 dpi with increasing levels of ANDV RNA documented in samples collected up to 10 dpi. The urine and oral swabs collected from one hamster (H15) at 8 dpi and all three infected hamsters at 10 dpi were positive for ANDV RNA, however due to the method of collection (i.e., differing volumes) the RT-PCR analysis was only qualitative.

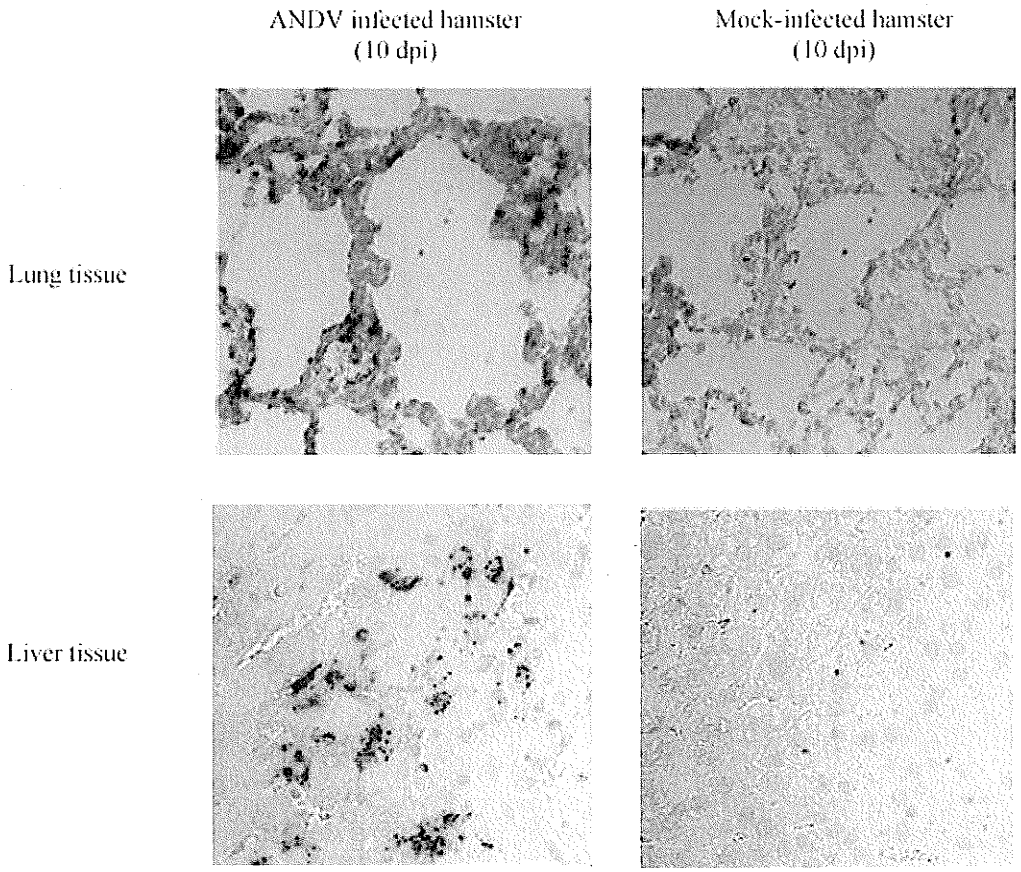


FIGURE 4.4. Detection of ANDV N antigen in hamster lung and liver sections. Immunohistochemistry was performed on tissue samples collected from hamsters at various time points post-inoculation. Lung and liver samples demonstrated the most consist staining with highest levels of ANDV antigen (labelled red) observed in those collected at day 10 (shown above).

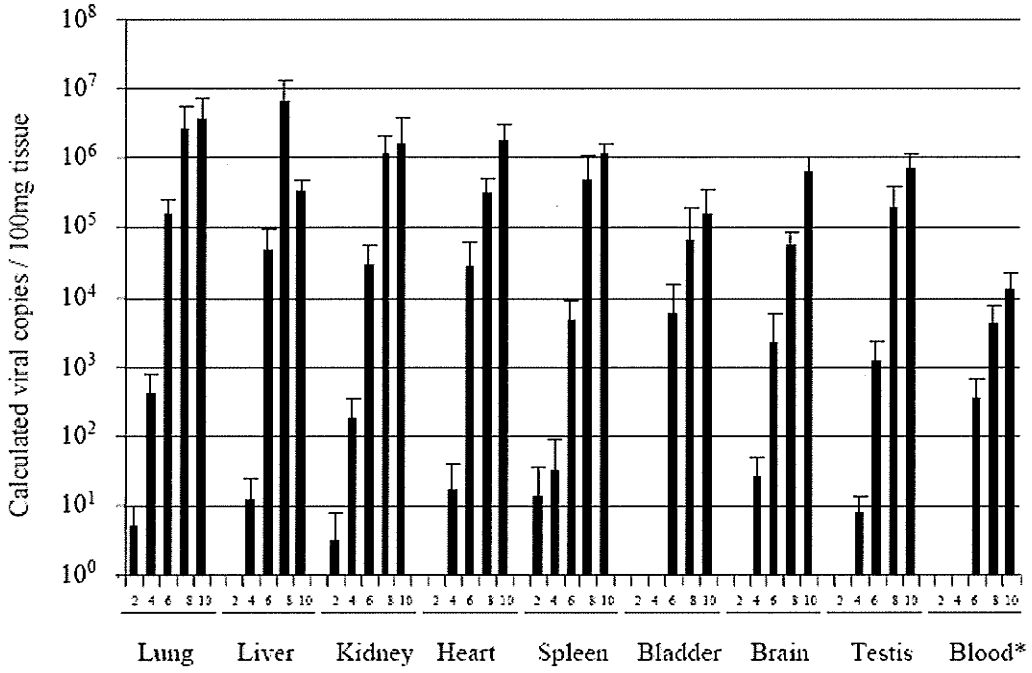


FIGURE 4.5. Detection of ANDV RNA in tissues of infected hamsters. Real-time, quantitative RT-PCR was conducted on RNA extracted from homogenized tissues collected at two day intervals post-ANDV challenge. Data shown represents the average values from tissues collected from three infected hamsters per time point. Error bars represent 2x the calculated standard errors of the means (SEM). * Blood measured in calculated viral copies / 100 µl.

To confirm the presence of infectious virus, tissue homogenates collected at 10 dpi from infected hamsters were titrated using a focus assay (Figure 4.6). The highest viral titers were observed in liver ($4.1x10^5$ FFU/100 mg) and lung ($1.8x10^5$ FFU/100 mg) samples. Slightly lower titers were documented in heart ($3.2x10^4$ FFU/100 mg), spleen ($2.7x10^4$ FFU/100 mg), kidney ($2.2x10^4$ FFU/100 mg) and blood ($3.1x10^3$ FFU/100 µl).

DISCUSSION

The lethal disease observed in hamsters following inoculation with ANDV closely resembles that of HPS in humans, with similarities including incubation period / time to symptom onset, clinical presentation and pathophysiology. Clinically, HPS in humans is characterized by four phases of disease (febrile, cardiopulmonary, diuretic and convalescent). Following a period of incubation, estimated at between 9 and 33 days for SNV, and 11 and 32 days for ANDV (Young et al. 2000, Vial et al. 2006), patients enter the prodrome (febrile phase) which generally lasts 3 to 5 days and is typified by non-descript symptoms (i.e., fever, headache, general malaise and myalgia). In humans, the cardiopulmonary phase is the defining stage of HPS which is distinguished by sudden onset of severe respiratory symptoms. Typically patients rapidly progress from coughing and shortness of breath to shock and severe pulmonary edema requiring intubation and mechanical ventilation. This phase is characterized by vascular leakage (primarily in the lungs), hypoxemia, and cardiac complications. The majority of HPS related fatalities occur during this phase, often within 48 hrs, and mortality is generally due to shock and cardiac failure. Resolution of pulmonary symptoms in nonfatal cases is rapid and patients who progress to the third and forth (diuretic and convalescent) stages of disease have a

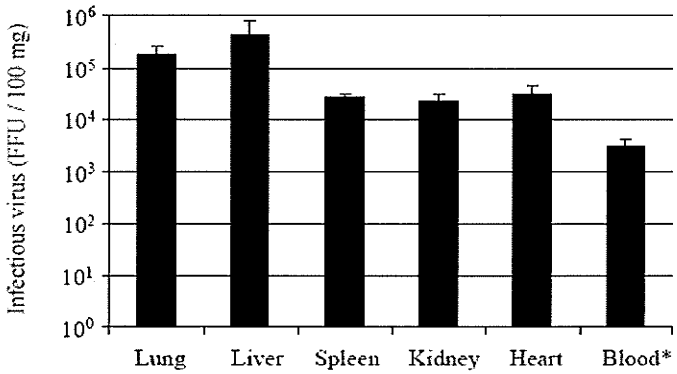


FIGURE 4.6. Detection of infectious ANDV in tissue samples collected at 10 dpi. Tissue samples (100 mg) collected during necropsies were mechanically homogenized and titrated in duplicate using a focus forming assays. All data shown represents the average values for the three hamsters necropsied at 10 dpi. Error bars represent 2x SEM. * Blood measured in FFU / 100 µl.

good prognosis.

At approximately 9 days, the incubation period described here in hamsters is similar to those in previous descriptions of the model, between 9 and 28 days depending on the route of inoculation, as well as the incubation period defined in humans (Hooper et al. 2001b, Campen et al. 2006, Hooper, Ferro & Wahl-Jensen 2008). While the unremarkable symptoms indicative of the febrile phase of HPS in humans are unapparent in hamsters, the transition from the febrile to cardiopulmonary phase is a hallmark of HPS and is strikingly similar in hamsters. The first signs of illness consist of breathing distress which quickly progresses from short, shallow breaths to severe dyspnea. Typically, symptom onset precedes death by approximately 24-36 hrs. While not measured here, classic signs of shock are also present in the terminal stages of disease as suggested by Campen and colleagues (2006). Using radiotelemetry, a rapid decrease in blood pressure and increased heart rate was observed in hamsters immediately preceding death. The lungs are the primary organ involved in the development of HPS in hamsters with vascular leakage associated with ANDV infected endothelial cells leading to pulmonary edema and pleural effusion. Other similarities to HPS in humans include the development of thrombocytopenia and neutrophilia (Wahl-Jensen et al. 2007).

The clinical observations made here in the i.m. and i.p. injected groups were indistinguishable and agreed with the findings of previous reports (Hooper et al. 2001b, Campen et al. 2006). The rapidity of disease onset and death was reflected in the weight charts of both i.p. and i.m. challenged hamsters. On average, inoculated hamsters lost 3.5% of their body weight, with only a single hamster losing more than 5%, demonstrating that such measurements do not provide a means to monitor disease

progression. Consistent with previous descriptions of the model, hamsters in the studies outlined here succumbed to ANDV infection with an average time to death of approximately 9-10 dpi (Hooper et al. 2001b, Campen et al. 2006, Wahl-Jensen et al. 2007). In addition, the route of inoculation did not appear to influence the outcome, with similar kinetics and identical mortality rates (100%) observed in both the i.m. and i.p. challenge groups. As previously reported with i.m. inoculations, ANDV is also highly pathogenic in hamsters when delivered by i.p. injection, as demonstrated by similar LD_{50} calculations of 8 PFU for i.m. (Hooper et al. 2001b) and 1.54 FFU for i.p. (described here). These observations are not surprising in light of recent findings that ANDV is highly virulent and uniformly lethal in hamsters regardless of inoculation route (Hooper, Ferro & Wahl-Jensen 2008).

An interesting observation in the LD_{50} study described here was the survival rate of hamsters which received the highest doses of ANDV (80,000 and 8000 FFU) was greater than those which received lower doses (80 and 8 FFU). Similar observations have been made with animal models for other infectious diseases (e.g., mouse-adapted Ebola) and, although not addressed in these studies, these findings may be due to an innate immune response which recognizes high doses of ANDV and is capable of limiting the infection while the adaptive immune response forms, as suggested by Ebihara and colleagues (2006). Due to the limited inoculum range resulting in 100% mortality, the challenge dose was set at 100 LD_{50} (equivalent to 154 FFU ANDV) for subsequent experiments.

Based on the similar symptomology, it is not surprising the pathophysiology of HPS in hamsters is similar to that described for humans. Autopsies conducted on fatal

cases of HPS have documented high viral loads from endothelial cells with numerous tissues in the body having detectable hantaviral antigen by IHC procedures (Zaki et al. 1995, Toro et al. 1998). In the studies described here, the presence of ANDV in hamster organs was monitored by RT-PCR, IHC and virus isolation. Following i.p. injection, ANDV rapidly disseminated throughout the hamster with viral RNA detected as early as 4 dpi in lungs and kidneys. By 6 dpi ANDV RNA was also detectable in heart, liver, spleen, bladder, brain, testis and blood. The highest RNA levels were detected in lung and liver samples, with peak ANDV RNA observed in 8 dpi liver samples. Infectious virus titers from these organs were approximately one log higher than those from other tissues analyzed at 10 dpi and IHC revealed the largest amounts of N antigen in sections from these tissues between 6 and 10 dpi.

Despite the systemic distribution of viral RNA and antigen, the lungs were the primary organ responsible for symptoms and lethality in hamsters. The brief histological analysis conducted here revealed pathological changes in lungs similar to those observed in both HPS patients and previous descriptions of the model (Zaki et al. 1995, Toro et al. 1998, Hooper et al. 2001b, Wahl-Jensen et al. 2007, Eyzaguirre et al. 2008). These include alveolar edema with thickening of the alveolar walls, diffuse subacute pneumonitis, and perivascular lymphohistiocytic infiltrates in terminally ill hamsters. Although significant amounts of pleural effusion were not observed in the hamsters necropsied over the course of the studies presented here, this may be because necropsies were not performed on any of the hamsters which succumbed to infection.

In humans, the lungs and spleen of patients have the most consistent histiopathological abnormalities including interstitial pneumonitis, mononuclear cell

infiltrates, congestion and alveolar edema in lungs, and red pulp congestion in spleens (Zaki et al. 1995, Toro et al. 1998). Although red-pulp congestion was also noted in the spleens of some of the terminally ill hamsters described here, this finding was not consistent. In contrast to humans, the liver of hamsters appears to play an important role in disease progression, based on the detection of ANDV RNA, antigen and high viral titers. The involvement of the liver as a primary target organ in hamsters remains to be explained since HPS causing viruses are only rarely reported to have liver involvement, although the detection of antigen in hepatic cells may be more prominent in South American cases of HPS (Zaki et al. 1995, Toro et al. 1998, Enria et al. 2001). Liver tropism may be an artifact of the route of inoculation, though further studies are required to illustrate its role in HPS disease progression in hamsters.

The remaining organs analyzed from infected hamsters were essentially normal and lacked any discernable pathological irregularities. Although recently, cardiogenic shock has been demonstrated in ANDV infected hamsters immediately preceding death (Campen et al. 2006), heart tissue collected at 10 dpi appeared normal. This may not be surprising in light of findings by Zaki and others (1995), who found little evidence of cardiac tissue damage in SNV HPS, despite numerous deposits of N antigen in heart muscle. Despite being highly lethal, with hamsters succumbing to infection shortly after the appearance of respiratory symptoms, during the serial sample study increased volumes of urine were observed in the bladders of two of three terminally ill hamsters necropsied at 10 dpi, which may be an indication of the diuretic phase.

The virulence factors responsible for lethal, HPS-like disease caused by ANDV in hamsters remain unknown. While ANDV is lethal in hamsters, SNV is not. Further, all

Old World hantaviruses tested to-date result in asymptomatic infections in hamsters. The only other virus described to cause HPS-like disease in hamsters is Maporal, a South American hantavirus believed to be non-pathogenic in humans. Unlike the mortality associated with ANDV in hamsters, Maporal is far less virulent with milder symptoms and reduced mortality (Milazzo et al. 2002). More recently Choclo virus, a South American hantavirus phylogenetically close to Maporal and ANDV, was reported be non-pathogenic in hamsters, although unlike with SNV infection (where antigen was rarely found in the endothelial cells of lungs), Choclo antigen was detected throughout the endothelial cell lining of the microvasculature in lung tissue (Wahl-Jensen et al. 2007, Eyzaguirre et al. 2008). The presence of antigen without obvious symptoms or histological abnormalities in the lungs suggests in hamsters, endothelial cell leakage is not solely due to viral replication. One of the original studies conducted in hamsters attempted to ascribe pathogenesis to specific ANDV proteins by infecting animals with a reassortant virus containing the S and L segments of SNV and the M segment of ANDV. While the "SAS" virus was highly infectious in hamsters, it was non-lethal, suggesting the glycoproteins alone are not responsible for disease in the model (McElroy et al. 2004). Based on the recent findings with Maporal and Choclo, similar studies conducting in hamsters with reassortant viruses containing specific segments of ANDV in the genetic background of other, more closely related, South American viruses may help elucidate the viral virulence factors involved in lethal disease in hamsters.

Currently there are no vaccines or antiviral agents available for the treatment and prevention of HPS, due in large part to the inability to perform clinical studies in a faithful animal model which reflects HPS disease. The ANDV hamster model of HPS has

been well characterized by at least two independent laboratories (Hooper et al. 2001b, Campen et al. 2006, Wahl-Jensen et al. 2007), however prior to commencing the vaccine experiments outlined in Chapter V, it was imperative to re-evaluate and verify the results of others with our own in-house viral stocks of ANDV. The results of the studies described here confirm the findings of others and demonstrate the ANDV hamster model is a convenient and relevant model with which to study HPS pathogenesis as well as putative vaccines and therapeutics.

CHAPTER V:

Assessing the protective efficacy of two replication deficient viral vectors for ANDV vaccines.

INTRODUCTION

Since the first appearance of HPS in 1993, greater than 2000 cases have been diagnosed from across the Americas, with several indigenous species of virus implicated in human disease. Following a brief incubation period, humans who have become infected undergo a rapid disease progression which involves severe pulmonary manifestations and case-fatality rates between 30-50% depending on the virus species involved. Despite the severity of disease, no methods of medical intervention exist for the treatment or prevention of HPS, with patient management limited to supportive care.

There is an urgent need to develop safe and effective vaccines against HPS which could be incorporated into immunization programs targeting high risk individuals to reduce the morbidity and mortality associated with human disease. Several vaccine platforms have been evaluated for protective efficacy and/or immunogenicity, including inactivated hantaviruses (Yamanishi et al. 1988, Song et al. 1992), recombinant vaccinia virus (Pensiero et al. 1988, Schmaljohn et al. 1990, Xu et al. 1992, Chu, Jennings & Schmaljohn 1995, Maeda et al. 2005), baculovirus derived lysates and proteins (Schmaljohn et al. 1990, Lundkvist et al. 1996b), replication deficient adenovirus (Maeda et al. 2005), *E. coli* or yeast expressed recombinant proteins (Lundkvist et al. 1996a, Dargeviciute et al. 2002), chimeric Hepatitis B virus like particles (Ulrich et al. 1998, 1999), naked (plasmid) DNA (Bharadwaj et al. 1999, 2002, Hooper et al. 1999, Kamrud

et al. 1999, Maeda et al. 2005), transgenic plants (Kehm et al. 2001), Sindbis replicons (Kamrud et al. 1999) and VSV pseudotypes (Lee et al. 2006). The majority of these studies aimed at dissecting the protective immune response associated with hantaviruses were conducted with HFRS causing viruses and were performed in small animal infection models, which do not reflect disease progression in humans.

In 2001, a lethal disease model for the study of ANDV HPS was described in Syrian hamsters (Hooper et al. 2001b). The ANDV hamster model remains the only small animal model for the study of hantavirus disease in which disease progression in animals closely mimics that in humans. Despite this, relatively few studies focusing on vaccine development or antiviral therapies have been conducted in the model and no vaccine platform has been described which invokes a specific immune response in hamsters sufficient to protect from lethal ANDV challenge. To-date, only DNA based vaccines have been tested in the lethal ANDV hamster model, however, despite being highly efficacious against HFRS causing viral infections in hamsters (Hooper et al. 1999, 2001a), and eliciting strong humoral immune responses in NHP (Hooper et al. 2006), DNA vaccines platforms based on the ANDV M segment were neither immunogenic nor protective in hamsters (Custer et al. 2003).

The objective of the studies outlined here was to test the ability of two recombinant replication deficient viral vectors, which either incorporate (VSV pseudotypes) or express (adenovirus) ANDV proteins, to induce a specific immune response in hamsters capable of protecting them against an otherwise lethal dose of ANDV.

HYPOTHESIS

1. Immunization with replication deficient viral vaccines which either incorporate or express the ANDV structural proteins will result in an adaptive immune response in hamsters capable of protecting them from lethal HPS disease.
2. Vaccine candidates based on the M segment gene products (i.e., GPC or G_N/G_C) will provide increased protection in hamsters, compared with those based on the N antigen.

MATERIALS AND METHODS

ANDV glycoprotein expression plasmid construction:

Trizol RNA extraction. Vero E6 cells infected with ANDV (as outlined in Ch IV) were collected at 7-10 dpi and extracted for total RNA using Trizol LS reagent (Invitrogen) essentially as described by the manufacturer. Briefly, approximately $6x10^6$ cells were pelleted, resuspended in 0.25 mL PBS and mixed with 0.75 mL Trizol reagent. Following incubation for 5 min at RT the mixture was removed from BSL 3 according to standard operating procedures. In BSL 2, 0.2 mL of chloroform was added and the mixture was briefly vortexed. Samples were incubated for 15 min at RT and separated into three phases by centrifugation at 12000 x g for 15 min. The upper aqueous phase (containing RNA) was transferred to a clean tube and the RNA precipitated with the addition of an equal volume of isopropyl alcohol and 1 µl of glycogen. Following 10 min incubation at RT, samples were centrifuged at 15000 x g for 10 min. The resultant pellet was washed once with 1 mL 75% ethanol, centrifuged again, and air dried for 5-10 min. The RNA

was dissolved in 30-50 µl nuclease-free water (Qiagen) and immediately used in subsequent steps, or frozen at -80°C.

Plasmid construction. The ANDV GPC coding region was cloned using established primers (SN-Fj and PUUM R, see appendix 1) essentially as previously described (Custer et al. 2003). Trizol extracted ANDV RNA was reverse transcribed using Superscript II (Invitrogen) in 20 µl reactions. Reaction mix (containing 1-5 µg RNA and 0.25 µM primers SN-Fj and PUUM R) was prepared, incubated at 70°C for 10 min and placed on ice. Master mix (1x first strand buffer, 10 mM DTT, 0.5 mM dNTPs, and 1 U RNase inhibitor, all from Invitrogen) was prepared and 7 µl added to 12 µl of reaction mix. The mixture was preheated to 50°C prior to the addition of Superscript II enzyme (5 U). The reactions were then incubated at 50°C for 50 min, followed by 70°C for 10 min to inactivate the enzyme. Following cDNA synthesis, RNA was degraded with RNase H (Invitrogen) at 37°C for 20 min and the reactions were cleaned using Montage PCR clean-up spin columns (Millipore) according to the manufacturer's instructions.

The ANDV GPC was amplified from the cDNA using the SN-Fj and PUUM R primers with Platinum Taq Hi-fidelity and TA cloned into pCR2.1 (both from Invitrogen). Each 50 µl PCR reaction contained 0.5 µM of each primer with 1x Hi-fidelity buffer, 0.2 mM dNTPs, 2 mM $MgSO_4$ and 4 µl cDNA. Cycling parameters were 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec and 68°C for 8 min with a final extension of 68°C for 10 min. Products were analyzed on a 1.5% w/v agarose gel stained with EtBr and reactions producing the appropriate size band (approximately 3.5 kb) were ligated overnight at 14°C into pCR 2.1 according to the manufacturer's specifications.

The next day, chemically competent, Top 10 E. *coli* cells (Invitrogen) were transformed with 1 µl of the ligation reaction according to standard "heat-shock" methodologies and cultured over night at 37°C on LB agar plates containing carbenicillin (40 µg/mL) and X-gal (40 mg/mL, Invitrogen). The next day, individual white colonies (indicating the presence of the insert) were picked and used to inoculate 5 mL of LB broth with 50 µg/mL ampicillin and incubated for 12-16 hrs at 37°C with agitation (220 rpm). Plasmids were extracted from the liquid cultures using mini-prep columns (Qiagen) according to the manufacturer's instructions and the complete insert was sequenced in both directions using 12 ANDV GPC sequencing primers (sequences available in appendix 1, for a brief description of sequencing see nucleotide sequencing section in Ch. III).

The ANDV GPC insert was sub-cloned into the pCAGGs/MCS expression vector. The pCR 2.1 clone was digested with *Eco* RI and the insert was gel purified in a 1.5% w/v low melt agarose gel using QIAquick clean-up columns (Qiagen) with gel purification methodologies described in the product insert. The ANDV insert was used as template and amplified using Platinum Taq Hi-fidelity as described above and oligos with *BsmBI* (*Xho I*) and *Bgl II* restriction sites engineered in. The ANDV insert contains an *Xho I* site, therefore *BsmBI* was used create an *Xho I* site in the forward primer. The ANDV GPC insert and pCAGGs/MCS vector were double digested with the appropriate enzymes and gel purified, ligated, and transformed into E. *coli* all as outlined above. Nucleotide sequencing was performed to select a mutation free clone.

Transfections. Flasks of 293T cells (a human embryonic kidney cell line) were cultured at 37°C with 5% CO_2 in DMEM enhanced with 10% FBS, antibiotics (penicillin [100

U/mL] and streptomycin [100 μg/mL]) and L-glutamine (2 mM). Six-well dishes were pretreated with poly-D-Lysine (Sigma) for 30-60 min, washed twice with PBS and seeded with 293T cells (cultured as outlined above). The following day, 293T monolayers (approximately 70-80% confluence) were transfected with glycoprotein expression plasmids as follows: For each well, 2 μg of plasmid DNA and 4 μl of TransIT-293 transfection reagent (Mirus) were added to separate tubes containing100 μl of Opti-MEM media (Invitrogen). After incubation for 5 min at RT, the DNA/Opti-MEM mix was added to the TransIT-293/Opti-MEM mix and incubated for a further 15-30 min, RT. The culture media on the 293T cells was replaced with 1 mL of fresh culture media and 200 μl of DNA/TransIT-293/Opti-MEM mixture was added to individual wells in a drop wise manner. The next morning, another 1 mL of culture media was added to each well, and the cells were incubated for a further 24-36 hrs.

Western Blots. Transfected cell pellets were mixed with Laemmli sample buffer (Biorad), boiled for 5 min, and resolved by SDS-PAGE on a 12% gel (Invitrogen). Gels were run at a constant 22 mA (44 mA for 2 gels) for approximately 1.5-2 hrs, until the dye front began to run out of the gel. Proteins were electrophoretically transferred to a nitrocellulose membrane using a semi-dry transfer system at 15-20 V for 20-25 min for one gel or 20-35 V for 30-35 min for two gels. Membranes were blocked for 1 hr, RT in TBS containing 5% skim milk (blocking buffer), and probed overnight at 4°C with primary antibodies diluted as indicated in blocking buffer. The following day, membranes were washed three times (≥ 10 min per wash) with TBST and incubated for 1 hr, RT with the appropriate secondary antibody (diluted 1:1000 in blocking buffer). Membranes were

washed five times with TBST and protein bands were visualized on X-ray film using the ECL western blot detection system (Amersham Biosciences). Expression of ANDV G_N and G_C proteins from pCAND-GPC was confirmed using either convalescent patient sera (1:100 dilution, gift from Dr. Delia Enria, National Institute of Human Virology Disease, "Dr. J Maiztegui", Pergamino, Argentina) or monoclonal antibodies (1:1000 dilution, clones 3F3/H2, 3C7/B10, and 2H4/F6, Austral Biologicals) as primary antibodies with peroxidase labeled goat anti-human IgG (KPL) or goat anti-mouse (Dako) secondary antibodies.

Creation of VSV pseudotypes bearing ANDV glycoproteins:

VSVΔG*G. The recombinant VSVΔG*G virus (kindly provided by Dr. Michael Whitt, Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, TN, USA) is replication deficient virus with the gene encoding the envelope protein replaced with that of GFP (Takada et al. 1997). In order to propagate VSVΔG*G, the VSV glycoprotein must be expressed *in trans* (see preparation of VSV pseudotypes, below).

Preparation of VSV pseudotyped viruses. Pseudotype viruses were prepared essentially as outlined by Ogino and colleagues (Figure 5.1, Ogino et al. 2003). Six-well dishes were seeded with 293T cells and transfected with plasmids expressing the glycoproteins of ANDV (pCAND-GPC), HTNV (pCHTN-GPC, provided by Dr. Jiro Arikawa, Graduate School of Medicine, Hokkaido University, Sapporo, Japan), or VSV (pCVSV-G, provided by Dr. Yoshihiro Kawaoka, School of Veterinary Medicine, University of

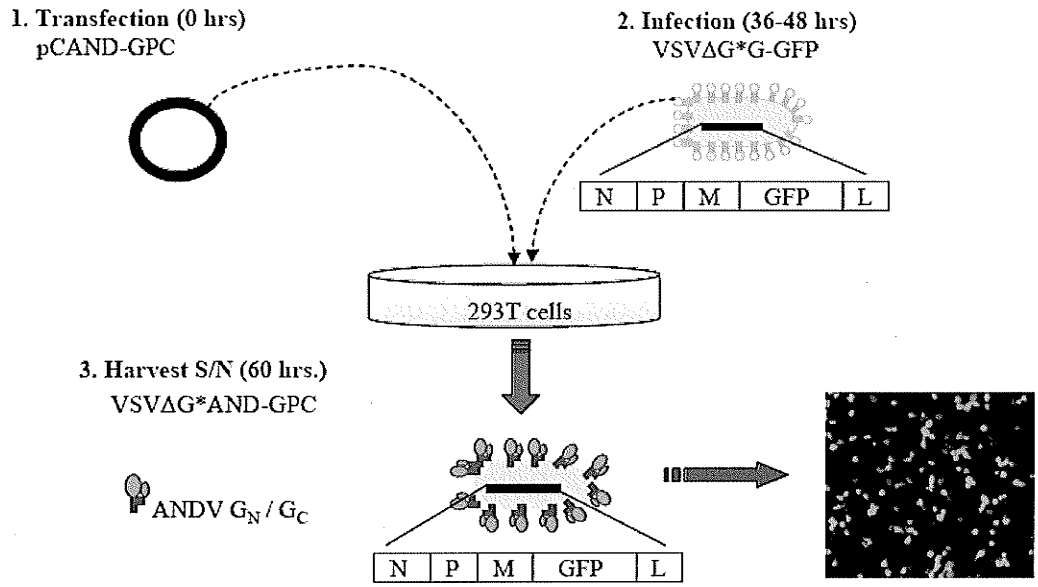


FIGURE 5.1. Generation of VSVΔG*AND-GPC. The ANDV glycoproteins were expressed in 293T cells by a chicken beta-actin promoter driven expression vector. Between 36-48 hrs post-transfection, cells were infected with VSVΔG*G at an MOI of 1, for 1 hr. Cells were washed twice and incubated overnight. Supernatant (S/N) was collected and used to infect Vero E6 cells. Green fluorescent protein (GFP) expression was enumerated the following day.

Wisconsin, Madison, WI, USA) or empty pCAGGs/MCS as outlined above. At 48 hrs post-transfection, cells were infected at an MOI of 1 with VSVΔG*G for 1 hr at 37°C. Cells were washed twice with pre-warmed DMEM, and further incubated at 37°C with culture media. At 16-18 hrs post-infection, supernatant containing pseudotype viruses were collected, clarified by low speed centrifugation and titrated.

VSV pseudotype titration. Ten-fold serial dilutions of VSV pseudotypes were made in DMEM and used to infect Vero E6 cells in 48 well culture dishes. After infection at 37°C for 1 hr, the inoculum was removed, cells washed twice with DMEM, and incubatedovernight at 37°C with culture media. Cells expressing GFP were enumerated on an Olympus IX70 microscope using an X-cite 120PC fluorescence illumination system with the appropriate (FITC) filters and titers calculated and expressed as infectious units (IU)/mL.

Purification and concentration of VSV pseudotypes. Pseudotyped VSV were purified and concentrated by centrifugation through a sucrose cushion. A 20% w/v sucrose solution was prepared in TNE buffer (10 mM Tris, 135 mM NaCl, 2 mM EDTA, Lee et al. 2006) and filter sterilized with a 0.45 μm filter. Culture supernatant (12 mL), containing VSV pseudotypes, was placed in ultraclear centrifuge tubes and 2 mL of the sucrose solution was carefully added to the bottom of the tube, lifting the supernatant. The VSV pseudotypes were pelleted through the cushion by centrifugation in Beckman ultracentrifuge with an SW-40 Ti rotor at 35,000 rpm for 2 hrs, 4°C. Pseudotypes were resuspended in a minimal volume of PBS and titrated as above.

Confirmation of foreign glycoprotein incorporation. To confirm the incorporation of the ANDV glycoproteins in VSV particles, western blots were performed on purified VSVΔG*AND-GPC (both as outlined above).

*Characterization of VSVΔG*AND-GPC:*

VSV pseudotype neutralizations. Neutralizations were performed essentially as previously described (Ogino et al. 2003). Convalescent serum samples were tested for the ability to neutralize VSVΔG*AND-GPC, VSVΔG*HTN-GPC or VSVΔG*G at doubling dilutions between 1:40 and 1:1280. The serum samples were from confirmed cases of HPS from South America (kindly provided by Dr. Delia Enria), and North America as well as from a confirmed case of HFRS (both provided by Daryl Dick, Public Health Agency of Canada, Winnipeg, MB, Canada) and control sera from an individual with no history of hantavirus-like illness. Serum samples (50 µl) were mixed with 200 IU of VSVΔG*AND-GPC, VSVΔG*HTN-GPC, or VSVΔG*G, diluted to 50 µl, and incubated at 37°C for 1 hr. The pseudotype/serum mix was used to infect nearly confluent Vero E6 cells in 48 or 96 well plates (seeded the day before). After 1 hr attachment at 37°C, the inoculum was removed, cells washed twice with pre-warmed media, and incubated overnight with culture medium. Serum samples were considered neutralizing if an 80% reduction in infectivity (as measured by GFP positive cells) was observed in Vero E6 cells at 16-24 hrs post infection.

Cellular receptor blockage studies. The effect of vitronectin, fibronectin (both from Sigma-Aldrich) and an anti-β_3 monoclonal antibody (ReoPro, Eli Lilly and Company) on

pseudotype infectivity was assessed essentially as previously described (Gavrilovskaya et al. 1999, Larson et al. 2005). Vero E6 cells were cultured in 24 well culture plates and pre-treated for 1 hr with vitronectin, fibronectin (5, 25, or 50 μg/mL) or the anti-β_3 antibody (5, 25, 50 or 100 μg/mL) when they neared 100% confluence. Cells were then washed with PBS and infected with 200 IU of VSVΔG*AND-GPC, VSVΔG*HTN-GPC, or VSVΔG*G as outlined above. The effect of each compound was assessed 16-24 hrs post infection by enumerating GFP positive cells.

*Assessing the protective efficacy of VSVΔG*AND-GPC:*

Protection studies. Fifteen male hamsters (approximately 4-6 weeks old) were randomly divided into three groups of five animals. At 0, 3 and 6 weeks, hamsters were anaesthetized and immunized with 10^7 IU of VSVΔG*AND-GPC, VSVΔG*HTN-GPC, or VSVΔG*G (purified and titrated as above) diluted in DMEM. Immunizations consisted of i.m. injections at two sites in the hind leg musculature, 50 μl per site. At 9 weeks hamsters were anaesthetized and challenged (i.p.) with 100 LD_{50} ANDV and monitored daily for signs of illness. Survivors were monitored for 3 times the average time to death (until approximately 40 dpi) before they were anaesthetized and exsanguinated via cardiac puncture.

In a second experiment, two groups of hamsters (male, 4-6 weeks old) received either two (prime-boost, n =12) or three (prime-boost-boost, n =13) immunizations with 10^7 IU of VSVΔG*AND-GPC (8 or 9 per group) or VSVΔG*G (4 per group), as above. Hamsters were challenged at 21 days after the final vaccination with 100 LD_{50} ANDV and monitored for signs of illness.

Concurrent to the challenge experiments, 10 hamsters (age and sex matched) were group housed in a BSL 3 laboratory and immunized according to the same schedule with VSVΔG*AND-GPC. Prior to each immunization, hamsters were anaesthetized and blood samples (approximately 125 μL) collected via the infra-orbital sinus using heparinized glass capillary tubes into Microtainer serum separator tubes. Blood samples were immediately separated and serum removed and stored at -80°C for subsequent serological analysis.

Antibody determinations. Sera from hamsters were individually tested for the presence of hantavirus specific antibodies using ELISA and neutralization assays. Antibodies to the N antigen of ANDV cross react with SNV N antigen; therefore, the recombinant SNV ELISA was used to test for hantavirus specific N antibodies in hamsters. The methodologies used were identical to those described in Chapter III with two exceptions; hamster sera was initially screened at a 1:40 dilution with titers determined using doubling dilutions thereafter, and a peroxidase conjugated goat anti-hamster IgG antibody (diluted 1:1000, KPL) was used as the secondary antibody. Neutralizing antibody titers were determined using VSVΔG*AND-GPC according to the protocols outlined above.

Preparation of recombinant Ad-ANDV vectors:

Cell culture. Flasks of 293IQ cells were cultured at 37°C with 5% CO_2 in minimum essential media (MEM, Invitrogen) supplemented with 10% FBS, antibiotics (penicillin [100 U/mL] and streptomycin [100 μg/mL]) and L-glutamine (2 mM).

Preparation of recombinant Adenovirus vectors. Three recombinant Adenovirus (recAd) vectors expressing the ANDV N, G_N and G_C proteins (Ad-ANDV-N, Ad-ANDV-G_N, Ad-ANDV-G_C) and a control Adenovirus (Ad-EMPTY) were kindly provided by Dr. David Johnson (Dept of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland OR, USA). The recAd viruses lack the E1/E3 coding regions thereby making them replication deficient. Propagation of the recAd vectors was accomplished using 293IQ cells (a packaging cell line derived from the 293 cells, which supplies the necessary transcriptional units *in trans*, Matthews et al. 1999). Briefly, nearly confluent monolayers of 293IQ cells were infected at an MOI of 5 with each of the recAd vectors and cultured as above. At approximately 36-48 hrs, cells and media were collected and centrifuged at low speed (1200 x g, 10 min). Supernatants were discarded, while cell pellets were re-suspended in 10 mM Tris-Cl buffer (pH 8.1, approximately 0.5 mL per T150) and immediately frozen at -80°C for subsequent purification.

Confirmation of protein expression. To confirm the expression of the foreign ANDV proteins, western blots were performed on cells infected with the three recAd vectors. Briefly, Vero E6 cells were infected with the recAd vectors (MOI < 1) and cell pellets were harvested 24-36 hrs later. Western blots were performed using monoclonal antibodies as outlined above for the detection of ANDV G_N and G_C. For the detection of ANDV N, a monoclonal antibody (clone 1A8/F6, Austral Biologicals, 1:1000 dilution) was used as the primary antibody.

Purification of Adenovirus vectors. Infected 293IQ cell pellets were rapidly freeze-thawed three times and the lysates spun at 2000 x g for 10 min at 4°C. Gradients consisting of light (22.39 g CsCl in 77.61 mL 10 mM Tris-Cl, pH 8.1) and heavy (42.23 g CsCl in 57.77 mL 10 mM Tris-Cl, pH 8.1) solutions of CsCl were prepared by slowly adding 10 mL of heavy CsCl below 10 mL of light CsCl in a 35 mL ultra-clear ultracentrifuge tube (Beckman). Lysate supernatant was carefully layered on top of the gradient and tubes were spun at 4°C for 2 hrs at 20,000 rpm (53,000 x g) in a SW-28 rotor using a Beckman ultracentrifuge. The virus, a narrow white band appearing approximately 2/3 down the gradient, was collected by puncturing the tube with a 23-ga needle and aspirated using a 3 mL syringe. The viral band was diluted 1:1 with 10 mM Tris-Cl and layered atop a second gradient prepared as above with 4 mL light and heavy CsCl solutions in a 12 mL ultra-clear ultracentrifuge tube. Gradients were spun at 4°C, overnight (approximately 16 hrs) at 20,000 rpm (50,500 x g) in an SW-40 rotor. Viral bands were collected as above, and dialyzed in 3 mL slide-a-lyzer dialysis cassettes (10,000 molecular weight cut-off, Pierce) according to the manufacturer's instructions. Following dialysis, individual recAds were frozen at -80°C in 0.5 mL aliquots for subsequent experiments.

Titration of Adenovirus vectors. The recAd vectors were quantified in 12 well tissue culture dishes using Adeno-X rapid titer kits (Clonetech) as outlined in the product insert. Briefly, duplicate wells of nearly confluent 293IQ cells were infected with 10-fold serial dilutions of each recAd. After 48hrs, infected cells were fixed by methanol treatment (10

min, -20°C) and stained with a mouse anti-hexon primary antibody using an anti-mouse horseradish peroxidase / 3'3' diaminobenzidine detection system.

Assessing the protective efficacy of Ad-ANDV vectors:

Protection study. Thirty-seven male hamsters (approximately 4-6 weeks old) were randomly divided into six groups and a pre-immunization blood sample was collected via the infra-orbital sinus. Hamsters were individually anesthetized and immunized at two sites in the hind leg musculature via i.m. injections with a total dose of 100 µl containing 10^8 infectious particles of each recAd either alone (4 groups: Ad-ANDV-G_N, Ad-ANDV-G_C, Ad-ANDV-N, or the control Ad-EMPTY) or in combination (2 groups: Ad-ANDV-G_N/G_C; and Ad-ANDV-G_N/G_C/N). At 28 days post immunization a second blood sample was collected prior to challenge with 100 LD_{50} ANDV. Post-challenge, hamsters were examined twice daily for signs of illness. Survivors were monitored for approximately 3x the average time to death (44 days) at which point they were anaesthetized and exsanguinated via cardiac puncture. Serology was performed on sera samples using ELISA and neutralization assays outlined above.

Test for sterile immunity. In a follow-up study four groups of 12 hamsters were immunized as above with Ad-ANDV-G_C or Ad-ANDV-N alone, Ad-ANDV-G_N and Ad-ANDV-G_C together, or Ad-EMPTY and challenged 21 days later with 100 LD_{50} ANDV. At 6 and 9 dpi, three hamsters from each group were anesthetized, exsanguinated via cardiac puncture and necropsies performed. A 100 mg piece of lung and liver tissue was placed into individual tubes containing 600 µl of RNAlater (Qiagen) and stored overnight

at 4°C to stabilize the RNA. The following day the RNAlater was removed and the tissues were mechanically homogenized in 600 µl of lysis buffer RLT as outlined in Ch IV. Homogenates were clarified and 200 µl was further diluted into 400 µl of buffer RLT and removed from BSL 4 according to standard operating procedures. Blood samples were prepared for RNA extraction according to the methodologies outlined in the serial sample study in Ch. IV. Samples were extracted for RNA using RNeasy (solid tissue) or QIAamp viral RNA (blood) extraction kits according to the manufacturer's specifications and tested for the presence of ANDV specific RNA using the quantitative, real-time RT-PCR methodologies outlined in Ch IV.

The remaining 6 hamsters in each group were monitored daily for signs of illness. Survivors were held for approximately 3x the average time to death (40 days) at which point they were anaesthetized and exsanguinated via cardiac puncture. Serology was performed on samples as above.

Statistical analysis. One-way analysis of variance (ANOVA) was conducted on the data using vaccination groups as the treatment factor with Tukey-Kramer multiple comparison tests to determine the pairwise differences amongst the groups. Statistical analysis was carried out on the quantitative RT-PCR data from lung, liver and blood samples collected at 6 and 9 dpi using InStat v3.0 statistical software (GraphPad).

RESULTS

pCAND-GPC construction and protein confirmation:

The ANDV GPC coding region was amplified and cloned into the *Xho* I and *Bgl* II restriction sites of pCAGGs/MCS. The resultant plasmid, designated pCAND-GPC, had 99.7%, 94.5%, and 94.4% homology with ANDV strains Chile-9717869 (AF291703, Meissner et al. 2002), CHI-7913 (AY228238, Tischler et al. 2003), and AH-1 (AF324901, Padula et al. 2002), respectively. Alignment of the nucleotide sequences of pCAND-GPC and Chile-9717869 revealed 11 nucleotide substitutions within pCAND-GPC, 10 transitions and 1 transversion, the majority of which were silent and matched the sequences of the other ANDV isolates. Western blots with monoclonal antibodies and convalescent patient sera were performed on cells transfected with pCAND-GPC and confirmed the presence of two proteins of approximately 55 and 70 kDa.

*Characterization of VSVΔG*AND-GPC:*

The ANDV glycoproteins complemented infectivity of VSVΔG*G at levels similar to those described for the HTNV and SEOV glycoproteins (Ogino et al. 2003). Both VSVΔG*AND-GPC and VSVΔG*HTN-GPC pseudotypes were efficiently rescued with average titers ranging between 10^5 of 10^6 IU/mL. The empty plasmid (pCAGGs/MCS) did not complement VSV infectivity (Figure 5.2A). Western blots performed on purified pseudotype virions confirmed the incorporation of the ANDV glycoproteins (Figure 5.2B)

The functionality of the foreign hantavirus glycoproteins in the VSV particles was assessed through virus neutralization and receptor blockage studies. The infectivity of

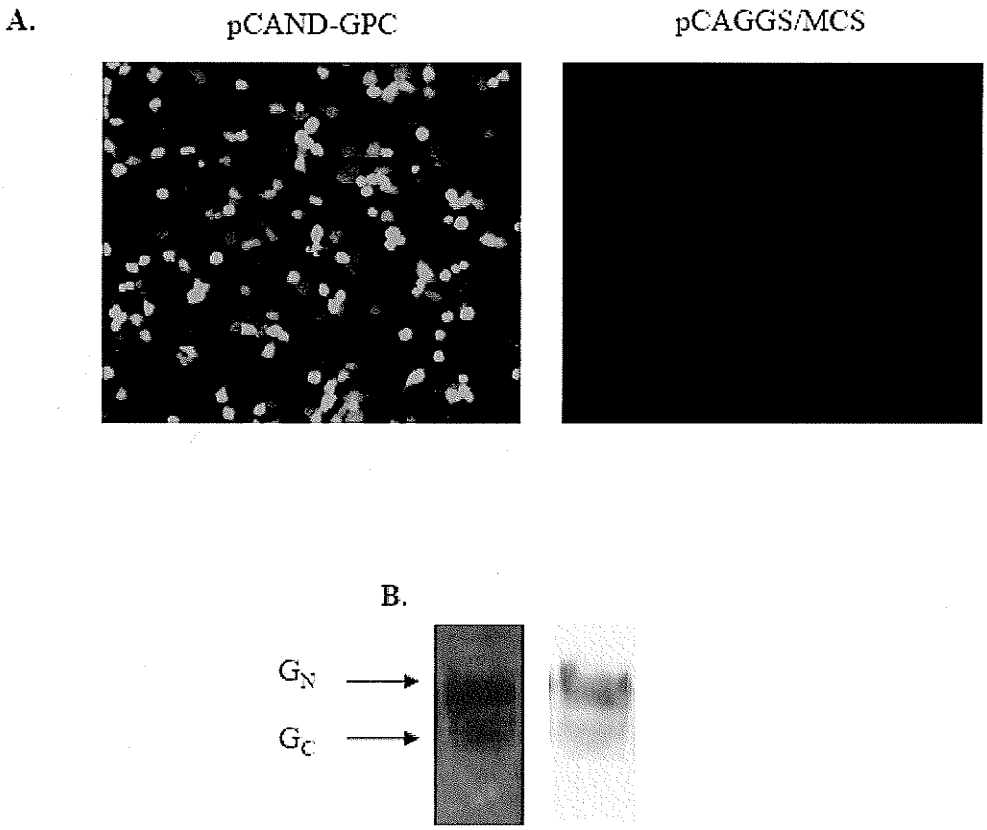


FIGURE 5.2. Rescue of VSV pseudotyped with ANDV glycoproteins. Cells transfected with pCAND-GPC or pCAGGS/MCS were infected with VSVΔG*G at 36-48 hrs post transfection. The following day supernatant was transferred to Vero E6 cells and GFP expression was assessed 16-24 hrs later. (A.) The presence of GFP positive Vero E6 cells indicates the foreign hantavirus glycoproteins were able to complement VSV infectivity (10x magnification). (B.) Psuedotype virions were pelleted through a sucrose cushion and the incorporation of the ANDV glycoproteins was confirmed via western blots with convalescent patient sera (left) and monoclonal antibodies (right).

VSVΔG*AND-GPC was neutralized by convalescent patient sera from a South American case of HPS in a dilution dependant manner, but not by sera from North American HPS or HFRS cases (Figure 5.3). Sera from a South American case of HPS was able to neutralize VSVΔG*AND-GPC to a maximum reciprocal serum dilution of 320, but had no neutralizing effects on VSVΔG*HTN-GPC or VSVΔG*G. Conversely, convalescent patient sera from an HFRS case neutralized VSVΔG*HTN-GPC to a maximum dilution of 160, while having no effect on VSVΔG*AND-GPC. Sera from a North American case of HPS and negative control sera from a volunteer with no history of hantavirus disease had no effect on either VSVΔG*AND-GPC or VSVΔG*HTN-GPC, and none of the sera had any neutralizing effects on VSVΔG*G.

Blocking the hantavirus receptor ($\alpha_v\beta_3$) by pre-treating Vero E6 cells with an anti-β_3 monoclonal antibody or vitronectin reduced the infectivity of VSVΔG*AND-GPC and VSVΔG*HTN-GPC in a dose dependant manner. Fibronectin had no effect on either VSVΔG*AND-GPC and VSVΔG*HTN-GPC, and none of the pretreatments diminished the infectivity of VSVΔG*G (Figure 5.4).

*Assessing the protective efficacy of VSVΔG*AND-GPC:*

Immunization with VSVΔG*AND-GPC, but not VSVΔG*HTN-GPC or VSVΔG*G is partially protective against a lethal dose of ANDV (Figure 5.5A). Groups of 6 hamsters were immunized with VSVΔG*AND-GPC, VSVΔG*HTN-GPC or VSVΔG*G three times, at three week intervals before ANDV challenge. Post-challenge, hamsters immunized with VSVΔG*HTN-GPC or VSVΔG*G began showing symptoms of HPS at day 9 with all animals in both groups succumbing to infection by day 11. In

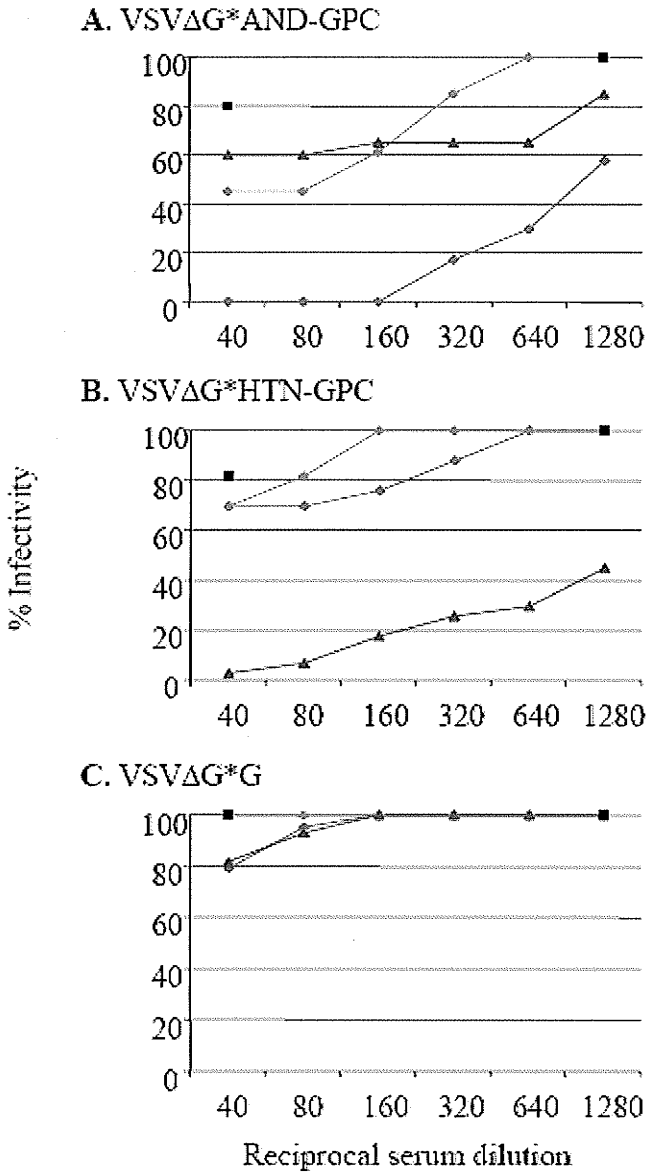


FIGURE 5.3. Neutralizing activity of convalescent patient sera on VSV pseudotype infectivity. Dilutions of patient sera from a North (green) and South (red) American case of HPS, as well as HFRS patient sera (blue) and negative control sera (black) were incubated for 1 hr with 200 IU of VSVΔG*AND-GPC (A), VSVΔG*HTN-GPC (B), or VSVΔG*G (C). Mixtures were then used to infect Vero E6 cells and the neutralizing effects of patient sera were assessed the next day by enumerating GFP positive cells and calculating the percent infectivity.

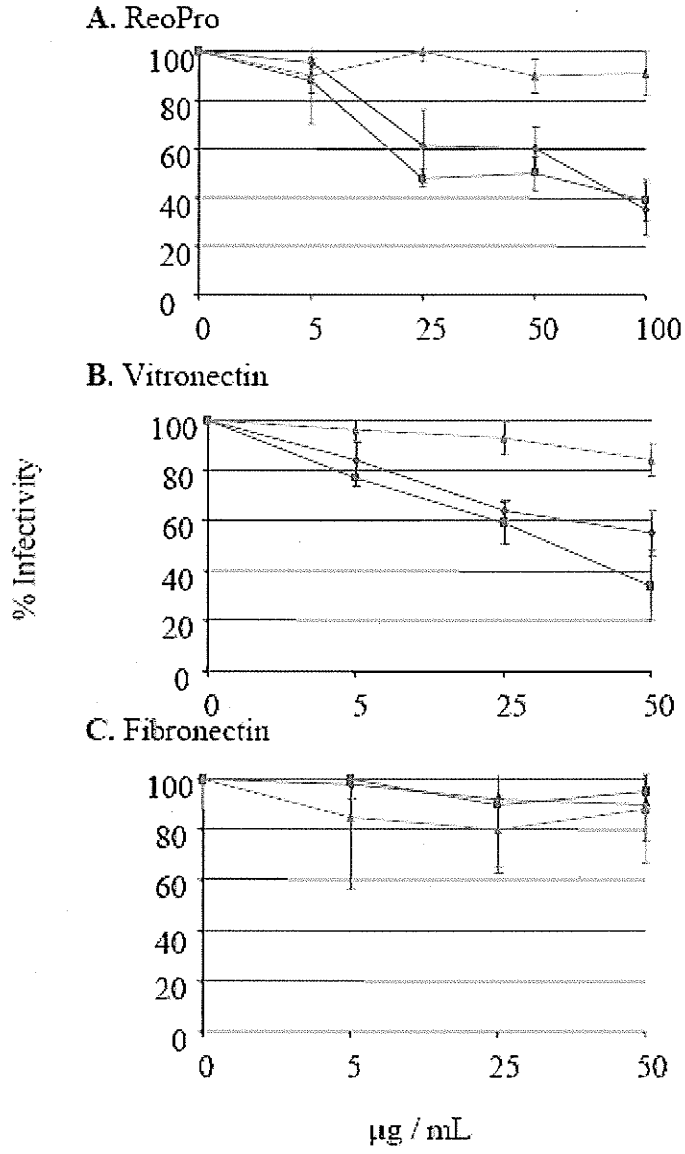


FIGURE 5.4. Dose-response curves showing the effect of a β3-specific monoclonal antibody, vitronectin and fibronectin on pseudotype infectivity. Nearly confluent VeroE6 cells were pre-treated with a anti-β_3 monoclonal antibody (Reopro, A), vitronectin (B), or fibronectin (C) at the indicated concentrations for 1hr, washed, and infected with 200 IU of VSVΔG*AND-GPC (red), VSVΔG*HTN-GPC (blue), or VSVΔG*G (green). The effect of each pretreatment was determined the following day by GFP enumeration and calculation of percent infectivity. Average values of three experiments are plotted with error bars representing twice the standard deviation.

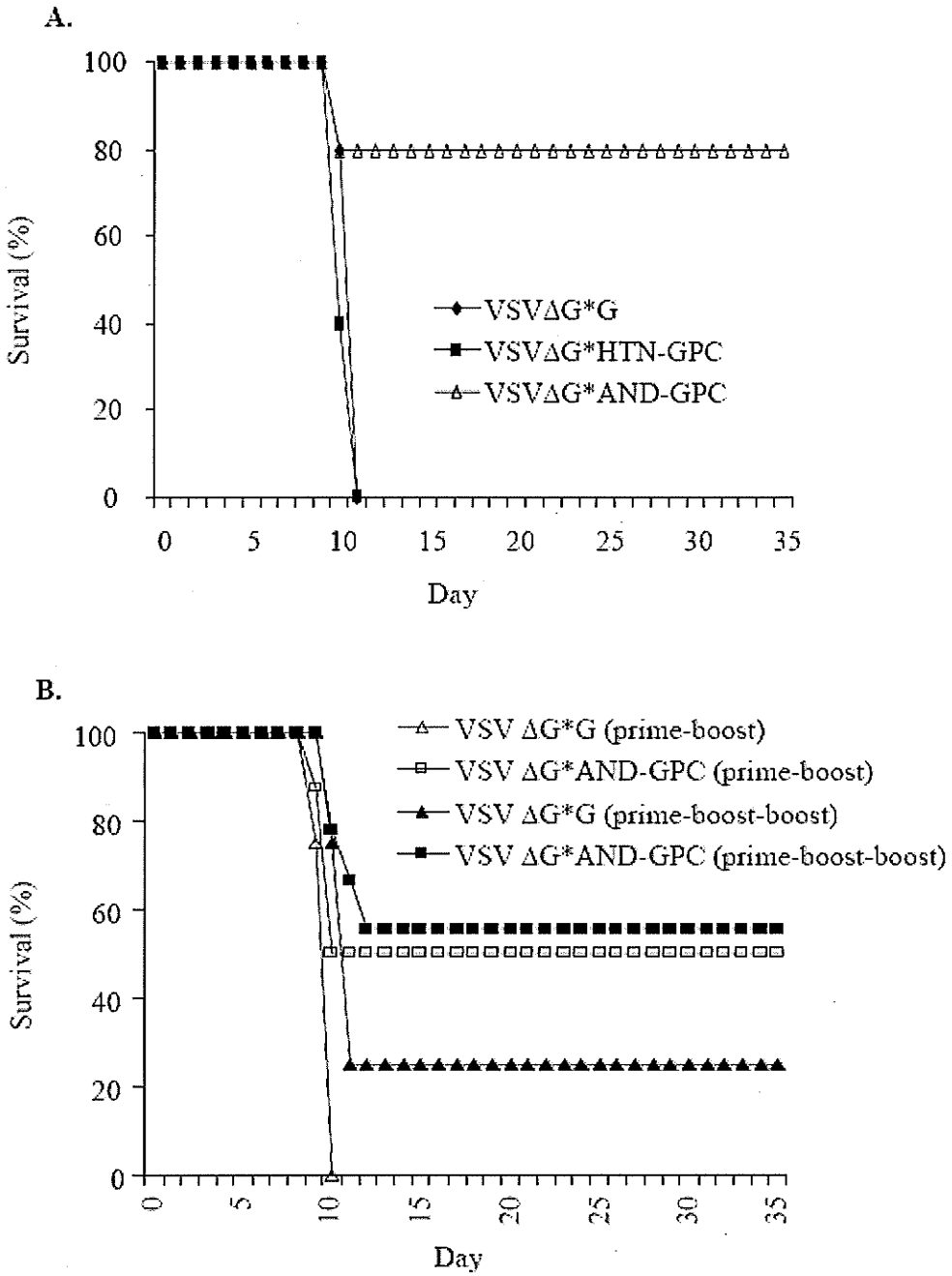


FIGURE 5.5. Protective efficacy of replication deficient VSVΔG*AND-GPC. (A) In the initial experiment, hamsters were immunized with VSVΔG*AND-GPC, VSVΔG*HTN-GPC, or VSVΔG*G three times at 21 day intervals prior to challenge with a lethal dose of ANDV. (B) In a follow-up hamsters were immunized with VSVΔG*ANDV-GPC or VSVΔG*G either twice (prime-boost) or three times (prime-boost-boost) and challenged 21 days after final immunization with ANDV. Percent survival is plotted against days post-challenge.

contrast 4 (80%) of 5 hamsters immunized with VSVΔG*AND-GPC survived the ANDV challenge without showing any signs of illness. The single hamster which died in this group was symptomatic at 10 dpi, and was found dead within 24 hrs.

In a repeat experiment, 2 groups of hamsters received VSVΔG*AND-GPC either twice (prime-boost) or three times (prime-boost-boost) before ANDV challenge. In the prime-boost group, 4 (50%) of 8 hamsters vaccinated with VSVΔG*AND-GPC survived lethal challenge with ANDV although all four showed moderate signs of illness. The prime-boost-boost group was only marginally better with 5 (55.5%) of 9 hamsters surviving, though none of these animals demonstrated any clinical signs of ANDV infection (Figure 5.5B).

Serological response in immunized hamsters:

The immunogenicity of VSVΔG*AND-GPC in hamsters was monitored using a surrogate group of 10 hamsters housed in a BSL 3 facility and immunized according the same schedule as the challenge group in the second challenge experiment. No neutralizing antibodies were detected in immunized hamsters until after the third vaccination, and even then low neutralizing titers (40) were observed in a minority (40%, 4/10) of immunized hamsters. Post-challenge, sera collected from the survivors had neutralizing activity, with titers as high as 640 (Figure 5.6).

Seroconversion was monitored in hamsters using a recombinant ELISA to detect hantavirus N specific antibodies. Prior to challenge hamster sera was uniformly negative for the presence of N specific antibodies. Serological analysis performed on sera samples collected from survivors demonstrated seroconversion to the N antigen in all but two

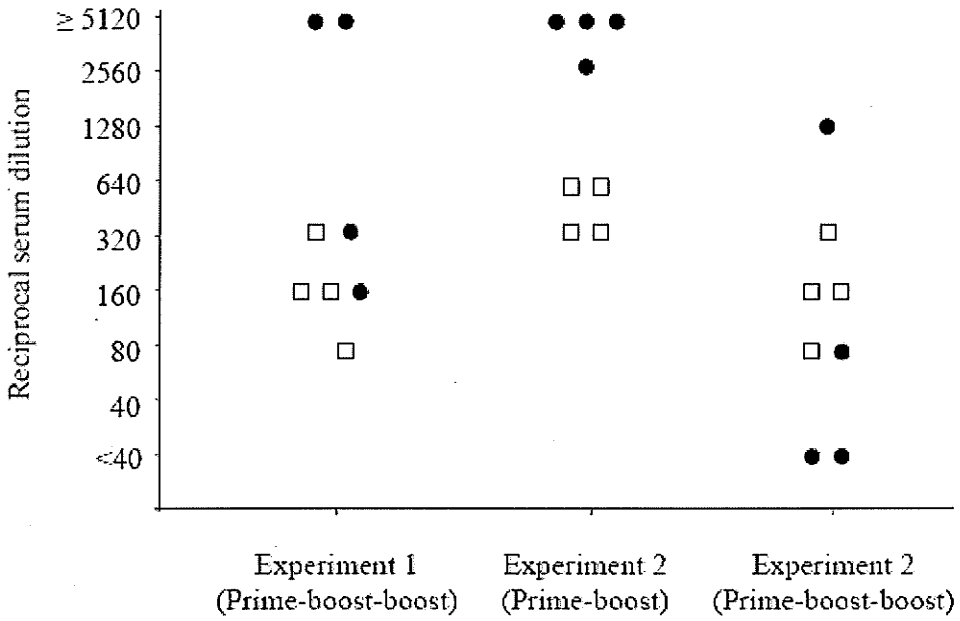


FIGURE 5.6. Post-challenge serological analysis of hamsters immunized with VSVΔG*AND-GPC. Sera was collected from hamsters approximately one month after ANDV challenge and tested for neutralizing (open boxes) and N specific (closed circles) antibody titers.

hamsters (Figure 5.6). In the first experiment, the four surviving hamsters had N antibody ELISA titers ranging between 160 and ≥ 5120. In the prime-boost group of experiment two, the four survivors had N antibody titers ranging from 2560 to ≥ 5120. In the prime-boost-boost, two survivors did not seroconvert, while the other two analyzed had titers of 80 and 1280. A serum sample from the fifth survivor in the prime-boost-boost group was unavailable.

Characterization of Ad-ANDV vectors:

Western blots were performed on infected cell pellets to confirm the expression individual ANDV proteins from the recAd vectors (data not shown). Following protein confirmation, titers of recAd vectors were calculated using Adeno-X rapid titer kits. With the exception of Ad-ANDV-G_N (6.5 x10^8 infectious particles per mL), the titers were comparable to those of Ad-EMPTY (8 x10^9, compared with 9 and 9.9 x10^9 infectious particles per mL for Ad-ANDV-G_C and Ad-ANDV-N respectively).

Assessing the protective efficacy of Ad-ANDV vectors:

In contrast to the partial protection afforded by VSVΔG*AND-GPC, the recAd vectors yielded complete protection against lethal ANDV challenge. When given alone, or in combination, Ad-ANDV-G_N, Ad-ANDV-G_C and Ad-ANDV-N were 100% protective, with immunized hamsters showing no obvious signs of infection post-challenge (Figure 5.7).

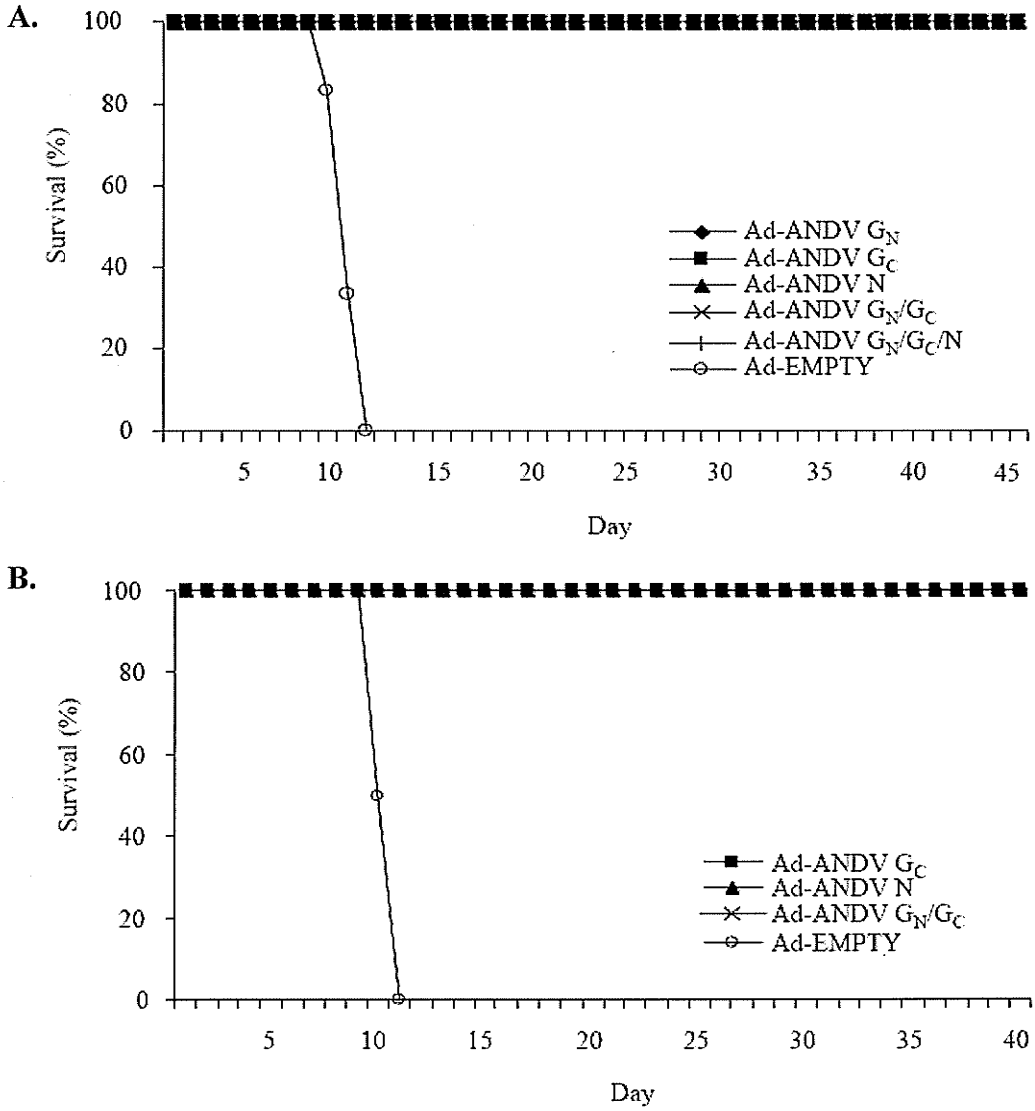


FIGURE 5.7. Protective efficacy of recombinant Adenovirus vectors expressing ANDV proteins. (A) Groups of six or seven hamsters were immunized with Ad-EMPTY (control group) or Ad-ANDV-N, Ad-ANDV-G_N, or Ad-ANDV-G_C alone, or in combination and challenged 28 days later with a lethal dose of ANDV. (B) As part a follow up study testing for sterile immunity, groups of six hamsters were immunized with Ad-EMPTY (control), Ad-ANDV-N or Ad-ANDV-G_C alone, or co-immunized with Ad-ANDV-G_N and Ad-ANDV-G_C and challenged as above. Animals were examined daily for signs of illness and survivors were maintained for approximately three times the average time to death of the control hamsters.

Serological responses in immunized hamsters:

Pre-challenge serum samples were tested for the presence of N specific and neutralizing antibodies. When administered alone, Ad-ANDV-N elicited a robust humoral immune response resulting in endpoint N antibody ELISA titers ranging between 40 and 640. When given in combination with Ad-ANDV-G_N and Ad-ANDV-G_C, N antibody titers were reduced to between 40 and 160. The remaining groups of hamsters had no detectable N antibodies prior to challenge. Neutralizing antibodies were not detected in hamsters immunized with any of the recAd vectors alone. Over the two experiments, modest neutralizing titers (40-80) were observed in 11 (45.8%) of 24 hamsters which received both the Ad-ANDV-G_N and Ad-ANDV-G_C (Figure 5.8).

Post-challenge, seroconversion was observed in all hamsters immunized with Ad-ANDV-N and Ad-ANDV-G_C alone (Figure 5.8). In contrast, half the animals immunized with Ad-ANDV-G_N, and the majority of those co-immunized with Ad-ANDV-G_N and Ad-ANDV-G_C or Ad-ANDV-G_N, Ad-ANDV-G_C and Ad-ANDV-N, did not seroconvert post-challenge, as suggested by relatively static N ELISA and neutralizing antibody titers (Figure 5.8).

Test for sterile immunity:

In a follow-up study, tissue samples (blood, lung, and liver) were collected from immunized animals at 6 and 9 days post ANDV challenge and analyzed for the presence of virus using real-time, quantitative RT-PCR. Control (Ad-EMPTY) immunized hamsters had considerable levels of ANDV RNA in all samples regardless of the time of collection (Figure 5.9). In contrast, samples collected from hamsters immunized with Ad-

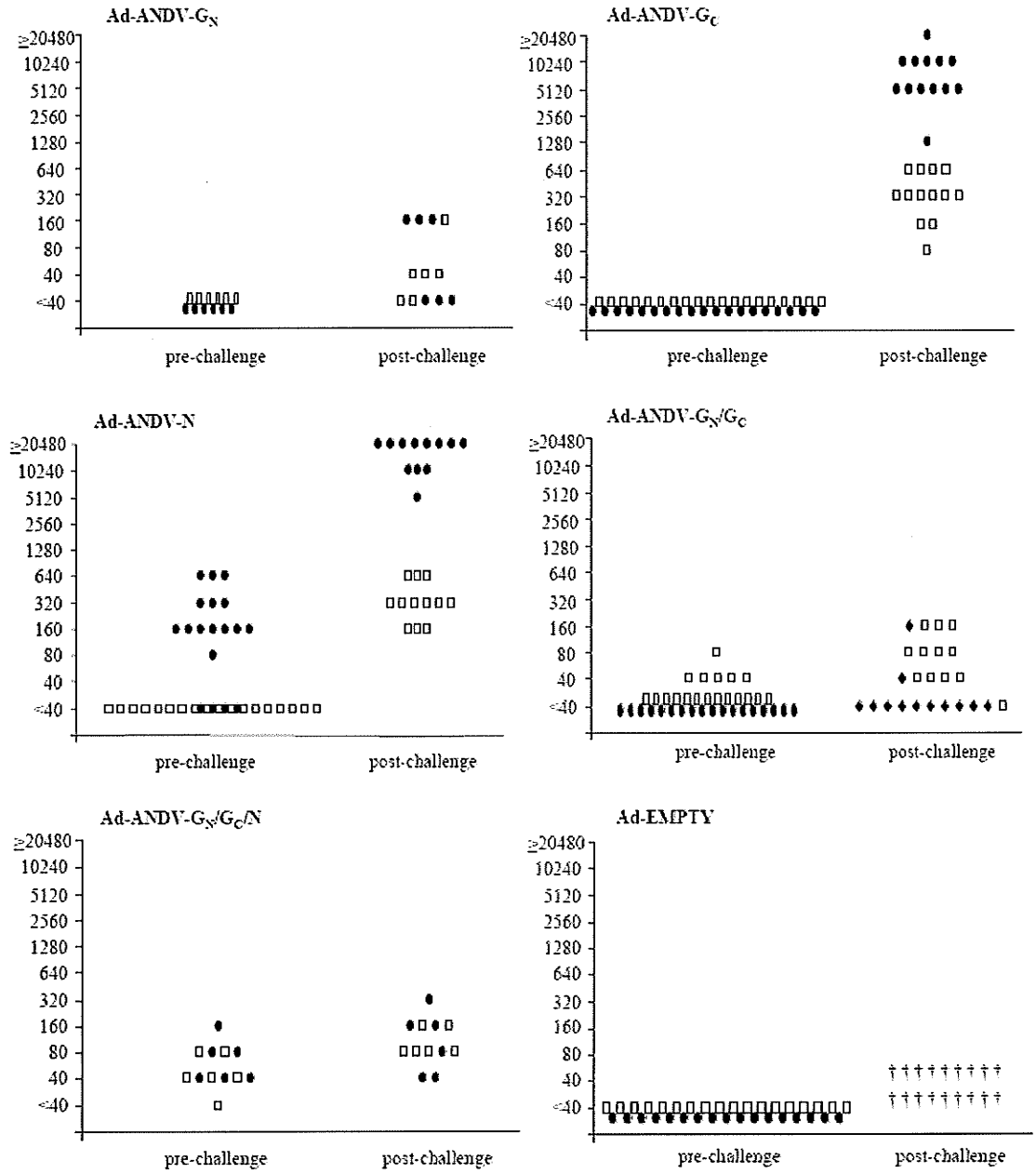


FIGURE 5.8. Pre- and post-challenge serological responses in hamsters immunized with Ad-ANDV vectors. End-point N specific and neutralizing antibody titers were determined for hamsters immunized with Ad-ANDV-G_N, Ad-ANDV-G_C and Ad-ANDV-N either alone or in combination. Closed circles represent ELISA N antibody titers, open boxes indicate neutralizing antibody titers, crosses represent hamsters which succumbed to infection (blood samples were not collected from these animals). Each point indicates a sample collected from a single hamster. Combined results of two protection studies are displayed.

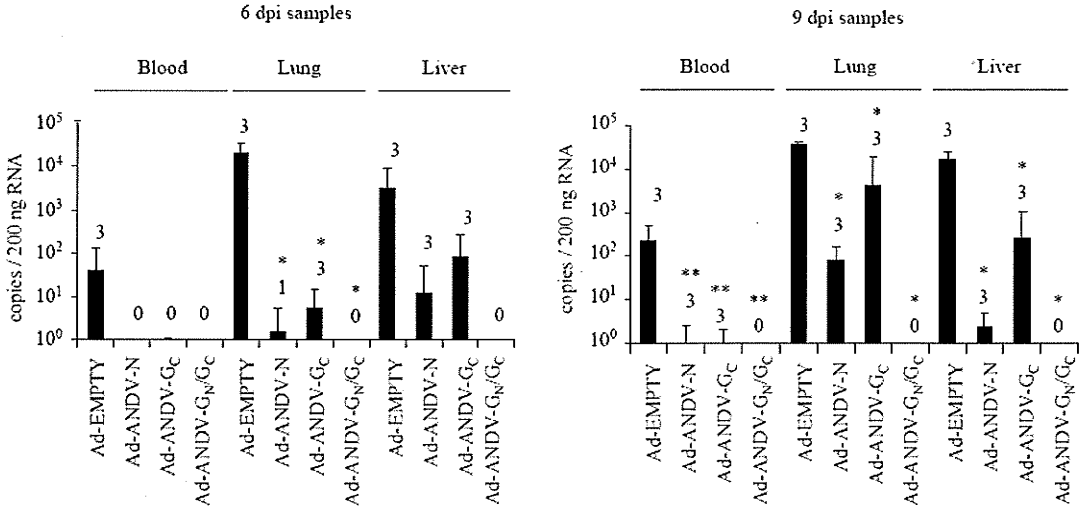


FIGURE 5.9. Test for sterile immunity in hamsters immunized with Ad-ANDV vectors. At 6 and 9 days post-challenge three hamsters per immunization group were euthanized and lung, liver and blood samples collected for real-time quantitative RT-PCR. Individual samples were quantified in triplicate. Data shown represents the average values for the three hamsters with numbers above bars indicating the number of hamsters with positive tissues. Asterisks are included above numbers where statistically significant differences were observed between the indicated group and control (Ad-EMPTY) immunized hamsters (* $p < 0.0001$; ** $p < 0.0096$). Error bars represent 2x SEM.

ANDV-N, Ad-ANDV-G_C or Ad-ANDV-G_N and Ad-ANDV-G_C had fewer positive tissues and appreciably lower amounts of detectable ANDV RNA in most samples collected at both time points (Figure 5.9). At 6 dpi, ANDV RNA was not detected in whole blood samples from hamsters immunized with either the N or G_C expressing recAd vectors. Despite this, ANDV RNA was detected at low levels in the lungs of 1 of 3 Ad-ANDV-N immunized hamsters and 3 of 3 Ad-ANDV-G_C immunized hamsters. All three liver samples analyzed from both groups at 6 dpi were also RT-PCR positive, albeit at lower levels than control animals. At 9 dpi low-level ANDV RNA was detected in whole blood samples from all three hamsters in both the N and G_C groups. Analysis of lung and liver samples from three hamsters in the G_C group showed increased amounts of ANDV RNA compared with samples tested at 6 dpi. Similar observations were made in lung samples from hamsters immunized with Ad-ANDV-N at 9 dpi, however, ANDV RNA in the liver samples from these animals decreased compared with levels calculated in the 6 dpi samples. In contrast to the other two groups, ANDV RNA was not detected in any samples collected from hamsters immunized with a combination of Ad-ANDV-G_N and Ad-ANDV-G_C at either the 6 or 9 dpi collection points.

Statistical analysis (ANOVA) indicated a significant effect of immunization on the detection of ANDV RNA in tissue samples. Tukey-Kramer tests demonstrated all pairwise comparisons conducted on lung, liver and blood samples collected at 9 dpi between the control group (Ad-EMPTY) and groups immunized with vectors expressing ANDV proteins (Ad-ANDV-N, Ad-ANDV-G_C alone or Ad-ANDV-G_N and Ad-ANDV-G_C in combination) were significant (lung and liver, $p < 0.0001$; blood, $p < 0.0096$) while

only comparisons between lung samples at 6 dpi were significant ($p < 0.0001$). There were no other pairwise differences.

DISCUSSION

In the present study two replication deficient viral vectors were examined for their ability to generate a specific immune response against ANDV proteins, which could protect hamsters from a lethal ANDV challenge. Although both vectors afforded at least partial protection in hamsters, the VSV pseudo-particles containing the ANDV G_N and G_C proteins were far less efficacious than any of the recAd vectors expressing ANDV N, G_N or G_C.

Previously, VSV pseudotypes bearing the surface proteins of HTNV or SEOV have been described as diagnostic tools useful for assessing neutralizing antibody responses in HFRS patients (Ogino et al. 2003). This is the first description of VSV pseudotypes with the glycoproteins of a New World hantavirus. The ANDV glycoproteins were efficiently incorporated into VSV particles and complemented VSV infectivity at levels similar to those reported for HTNV and SEOV (Ogino et al. 2003). The titers achieved for VSVΔG*AND-GPC (10^5-10^6 IU/mL) were 5-10 fold higher than those of native ANDV measured in the focus assay. Furthermore, within VSV pseudo-particles, the ANDV and HTNV glycoproteins were functional and defined the viral particles attachment and entry processes as demonstrated by the viral neutralization and receptor blocking experiments.

Despite this, VSV pseudo-particles were a weak immunogen and only partially protective against ANDV challenge. Serological analysis conducted on serial samples

from a surrogate group of hamsters immunized congruent to those of the challenge groups in experiment 2, revealed only weak neutralizing antibodies titers (reciprocal serum dilutions of 40) in 40% of hamsters and only in samples collected after three vaccinations. In spite of the apparently low response, in the initial protection study 80% (4 of 5) hamsters immunized three times with VSVΔG*AND-GPC were protected against an otherwise lethal challenge of ANDV. Unfortunately, pre-challenge sera samples were not collected as a part of the first protection experiment due to sensitivity of hamsters to blood collection techniques. Attempts to reproduce the results of the first protection study were not as successful with only half of hamsters receiving two or three immunizations of VSVΔG*AND-GPC surviving ANDV challenge. Furthermore, of the combined 13 surviving hamsters in the two experiments, 11 (84.6%) had documented seroconversion as demonstrated by the presence of N specific antibodies in serum from terminal blood samples. This strongly suggests in spite of repeated immunization with VSVΔG*AND-GPC, ANDV was able to infect and replicate within the hamsters.

Recently, a pseudotype VSV containing the HTNV glycoproteins (i.e., VSVΔG*HTN-GPC) was shown to be immunogenic and protective in mice against subsequent infection with HTNV. Following HTNV challenge, mice immunized with three doses of VSVΔG*HTN-GPC did not seroconvert to HTNV N antigen and had no detectable HTNV-specific CD8+ cell response compared with control (VSVΔG*G) immunized mice (Lee et al. 2006). Pseudo-particles of VSV have also been shown to be efficacious as potential vaccines for other infectious agents. A study by Majid et al. (2006) compared the immunogenicity of replication competent (i.e., VSV-C/E1/E2) and deficient (VSVΔG-C/E1/E2) chimeric VSV particles containing hepatitis C virus core

and envelope proteins. In BALB/c mice, the immunogenicty of the two VSV based constructs was similar as measured by antibody responses and specific interferon gamma producing CD8+ T cells (Majid et al. 2006). Mice immunized with VSVΔG-C/E1/E2 and subsequently challenged in tumor formation or surrogate HCV infection (recombinant vaccinia virus expressing HCV E1/E2/C) models showed significant differences in tumor growth and challenge virus titers (Majid et al. 2006).

It should be noted that the above mentioned studies were conducted in surrogate infection animal models and, as previously shown with DNA vaccines, may not reflect the results attained in the lethal hamster model (Custer et al. 2003). Nevertheless, the above studies support the use of VSV pseudotypes as vaccine candidates and suggest with minor alterations to the procedures outlined here, VSV-based replication deficient particles may be a suitable tool for studying the protective immune response against lethal ANDV challenge. Based on the results of Majid et al.(2006), future work should focus on incorporating the N protein into VSVΔG*AND-GPC. In addition, attempts should be made into boosting the immune response against the ANDV glycoproteins, perhaps through prime-boost strategies with other vaccine platforms or by aid of an adjuvant as used with VSVΔG*HTN-GPC (Lee et al. 2006). Efforts to increase the immunization dose in hamsters are also underway by attempting to enhance the maximum achievable titers of VSVΔG*AND-GPC in cell culture using codon optimization strategies. Although unsuccessful in generating a protective immune response in 100% of hamsters, the experiments outlined here using VSVΔG*AND-GPC as an immunogen demonstrate that an adaptive immune response can be produced in hamsters which is at least partially protective against lethal hantavirus challenge.

A major limitation of the VSV pseudotype based vaccines outlined above were relatively low attainable titers in tissue culture (resulting in lower immunization doses) which lead to inconsistent and generally insubstantial immune responses, even after three doses. With titers often approaching or exceeding 10^{10} infectious particles/mL, and the ability to express foreign proteins *in vivo*, recAd vectors have previously been demonstrated to be effective vaccine candidates for a myriad of infectious agents including hemorrhagic fever viruses (Kobinger et al. 2006, Wang et al. 2006a, 2006b). To-date, studies on recAd vectors expressing hantavirus proteins are limited. The immunogenicity of an adenovirus expressing the SNV N protein has been described in a comparative study of potential vaccines (Maeda et al. 2005). In the study, Maeda and colleagues demonstrated that a single dose of the recAd vaccine produced a robust N specific T-cell response which was greater than that produced by a recombinant vaccinia virus or DNA vaccine, even when the latter two were given in a prime-boost regime (Maeda et al. 2005). The ability of these vectors to express hantavirus glycoproteins has also been examined, although limited to G_N of SEOV and G_C of SNV, with neither being tested in animals for immunogenicity (Hayasaka et al. 2007, Yuan et al. 2008).

In the studies outlined here, the recAd vectors were far more effective as an immunogen than the VSV pseudotypes resulting in improved protection in hamsters from lethal ANDV challenge. Combining the results of the two protection studies, 100% of hamsters immunized with a recAd vector expressing either the ANDV G_N, G_C or N protein survived lethal challenge. After a single dose, a strong humoral immune response was generated in hamsters, as demonstrated in the Ad-ANDV-N vaccinated groups. Interestingly, when co-immunized with the two glycoprotein expressing vectors, the Ad-

ANDV-N elicited a reduced response. The reason for this is unknown, however it may be due to immune interference. A similar observation was reported with DNA based HIV vaccines, where the humoral and cellular immune responses to a Gag (p55) based immunogen were significantly reduced when mice were co-immunized with an envelope (gp120) plasmid based vaccine (Toapanta et al. 2007). Although unable to quantify the G_N and G_C specific antibody responses in the immunized hamsters, it seems likely a strong humoral response was also generated against them. The objective of upcoming studies will be to develop a glycoprotein ELISA to determine the non-neutralizing, glycoprotein-specific, antibody responses in these animals.

It is well established that the target of the neutralizing antibody response are the hantavirus glycoproteins. The induction of a strong neutralizing antibody response has previously been shown to correlate with protective and sterile immunity against hantaviruses in small animal infection models, including hamsters (Pensiero et al. 1988, Schmaljohn et al. 1990, Xu et al. 1992, Hooper et al. 1999,) and the detection of neutralizing antibodies in patients upon admission correlates with a clinically milder course of HPS (Bharadwaj et al. 2000). The location of neutralizing epitopes within the hantaviral glycoproteins is somewhat unclear, although neutralizing epitopes have been defined on both G_N and G_C proteins. Monoclonal antibodies specific for HTNV G_N or G_C have been shown to neutralize virus *in vitro*, using plaque reduction neutralization tests, and *in vivo* with mice protected from HTNV infection when antibodies were administered up to 2 dpi (Arikawa et al. 1989, Schmaljohn et al. 1990, Arikawa et al. 1992, Wang et al. 1993, Koch et al. 2003). Epitope mapping studies have also identified B-cell epitopes within the glycoproteins of New World hantaviruses, including G_N of SNV and ANDV

and G_C of ANDV (Jenison et al. 1994, Tischler et al. 2005a). The induction of a neutralizing antibody response is vague as well, though most studies observe neutralizing antibodies in animals only after immunization with constructs expressing both G_N and G_C proteins (Pensiero et al. 1988, Schmaljohn et al. 1990, Xu et al. 1992, Hooper et al. 1999). It has been suggested that the epitopes involved in virus neutralization are primarily conformational and may occur across the two proteins, though few studies have examined if individual G_N or G_C proteins are sufficient to invoke a neutralizing response in animals (Kikuchi et al. 1998). Bharadwaj and others (1999) documented a weak neutralizing response in mice following immunization with genetic vaccines containing fragments of SNV G_N or G_C, although subsequent studies were unable to reproduce these findings due to more stringent testing procedures (Bharadwaj et al. 2002). In the studies presented here, no neutralizing antibodies were detected in hamsters immunized with either the recAd vector expressing ANDV G_N or G_C alone, however, a weak response was observed in approximately half of the hamsters co-immunized with the two glycoprotein expressing vectors. While it is unlikely that G_N and G_C were expressed in the same cell allowing for the formation of the heterodimers predicted to be necessary for the generation of neutralizing antibodies, this cannot be ruled out. It is plausible though that at least some neutralizing epitopes on G_N and G_C are not conformational, and the induction of antibodies targeting both glycoproteins individually following co-immunization were sufficient to generate the low level neutralizing antibodies recorded here.

Passive transfer of immune serum can protect hamsters from lethal ANDV challenge, however to-date no vaccine candidate has generated a robust neutralizing

humoral immune response in hamsters (Custer et al. 2003, Hooper, Ferro & Wahl-Jensen 2008). It is possible that hamsters are deficient at producing strong neutralizing response against some *in vivo* expressed recombinant antigens. Custer and colleagues (2003) found an ANDV M segment based DNA vaccine was non-immunogenic in hamsters, despite producing a robust response in monkeys. Additional studies with recombinant vaccinia viruses expressing the Nipah virus glycoprotein and fusion proteins did not produce a measurable neutralizing response pre-challenge, although they were sufficient to induce a protective response (Guillaume et al. 2004). This may partially explain the weak neutralizing response generated in hamsters following immunization with recAd vectors expressing the ANDV glycoproteins, although it is most likely due to the choice of antigens (i.e., constructs expressing individual glycoproteins as opposed to the entire GPC from one recAd). Further studies are underway aimed at enhancing the neutralizing antibody response in hamsters using a recAd vector expressing the entire ANDV GPC.

The mechanism of protection in hamsters observed here with the recAd vectors is likely due to the generation of a potent cell-mediated immune response, although the current studies were unable to measure the T-cell response induced due to a lack of specific reagents available for hamsters. The ability of recAd based preventative vaccines to establish a strong, long lived T-cell response in animal models is well documented including against SNV N in mice (Maeda et al. 2005, Kobinger et al. 2006, 2007, Wang et al. 2006b).

Our understanding of the cellular mediated immune response against hantaviruses is rudimentary, however it is believed to play a role in the protective immune response and is hypothesized to be the underlying mechanism associated with protection observed

in animals immunized with N based vaccines (Schmaljohn et al. 1990, Xu et al. 1992, Lundkvist et al. 1996a, Ulrich et al. 1998). Despite being generally associated with the N protein, there is evidence that the glycoproteins can generate a strong T-cell response in animals capable of protecting them from infection. Hamsters which receive a heterotypic virus challenge are protected from lethal disease following a subsequent ANDV challenge (Hooper et al. 2001b, Safronetz, Ebihara, and Feldmann unpublished observations). Also, HTNV M segment based DNA vaccines have been shown to cross-protect a minority of hamsters against lethal ANDV challenge (Custer et al. 2003). While the mechanism of protection is unknown, the lack of ANDV neutralizing antibodies generated by heterotypic hantaviruses or vaccines suggest a role for cell-mediated immunity. Further, adaptive transfer studies of splenocytes from mice immunized with baculovirus derived N or glycoproteins were partially protective against lethal HTNV challenge in suckling mice (Yoshimatsu et al. 1993). While both these observation suggest a role for cell-mediate immunity targeting glycoproteins, it is unclear if both are involved or if the response preferentially targets one over the other. Recent studies by Bharadwaj and colleagues (1999, 2002) described protective T-cell epitopes in the SNV G_N and N protein using DNA based vaccines in mice. Similar to the findings here, deer mice were protected from SNV infection following vaccination with plasmids containing specific fragments of G_N or N, but not G_C. No neutralizing antibodies were detected in mice, but rather protection correlated with increase splenocyte proliferation following stimulation with cognate antigen (Bharadwaj et al. 1999, 2002). These findings are also supported by those of Van Epps and others (1999) who demonstrated the majority of memory T-cell responses in individuals who previously had HFRS were directed at the

HTNV N or G_N protein. Unfortunately the titers of Ad-ANDV-G_N were not sufficiently high enough to include an Ad-ANDV-G_N group in the second experiment looking at sterile immunity, although the data from the first experiment supports the findings of Bharadwaj and suggests an important role for G_N in the protective immune response. Based on seroconversion to the ANDV N protein, Ad-ANDV-G_N appeared more effective in generating a protective immune response in hamsters. Only three (50%) of six hamsters immunized with Ad-ANDV-G_N seroconverted to the N antigen compared with all six of the Ad-ANDV-G_C immunized hamsters. Furthermore, the endpoint ELISA titers achieved by the six Ad-ANDV-G_C hamsters were significantly higher than those from the three Ad-ANDV-G_N hamsters (ELISA titers 5120 to 10240 for G_C compared with 160 for G_N). Although ANDV was capable of infecting at least half of the hamsters immunized with Ad-ANDV-G_N in the first experiment (as suggested by seroconversion), it was likely replicating at far lower levels than those recorded in the Ad-ANDV-G_C immunized hamster from the second experiment. Unlike other studies, animals immunized with Ad-ANDV-G_C did not develop lethal ANDV disease, implying the presence of protective T-cell epitopes on G_C. Future studies using peptide libraries to stimulate splenocytes from animals immunized with the G_N, G_C and N constructs may provide a better understanding of the location of such epitopes.

The goal of preventative vaccines is not only to reduce morbidity and mortality, but to completely protect against viral infection. Viremia, as measured by the presence of ANDV RNA in whole blood samples, was delayed in the hamsters vaccinated with Ad-ANDV-N or Ad-ANDV-G_C alone compared with control (Ad-EMPTY) hamsters. Viral RNA was only detected in the blood collected at 9 dpi, though levels were significantly

lower than those of the control hamsters. Despite this, ANDV RNA was detectable in lung and liver samples collected at both 6 and 9 dpi from hamsters in these two groups, albeit at significantly lower levels than the control immunized hamsters. Nevertheless, the detection of increasing amounts ANDV RNA in these animals between 6 and 9 dpi demonstrates the presence of replicating virus. These data are further supported by documented seroconversion in both groups of hamsters post-challenge.

Previously, immunization of animals with N based vaccines alone has been reported to confer sterile protection (Schmaljohn et al. 1990, Lundkvist et al. 1996a). While this did not occur in the present study, this might be explained by differences in small animal infection models and the lethal disease model. In these experiments, sterile immunity was only observed in hamsters co-immunized with Ad-ANDV-G_N and Ad-ANDV-G_C, as determined by a lack of ANDV specific RNA in tissues samples collected at 6 and 9 dpi from these animals and no documented seroconversion in all but two hamsters combined over the both experiments.

Earlier studies have demonstrated that sterile immunity is highly associated with M segment (glycoprotein), based vaccines (Schmaljohn et al. 1990, Hooper et al. 1999, Kamrud et al. 1999). While the results presented here agree with these findings, it should be pointed out that only co-immunization with Ad-ANDV-G_N and Ad-ANDV-G_C was tested for the presence of ANDV RNA in hamster tissues post-challenge. Based on the results of the first experiment, it possible that co-immunization with other combinations may also confer sterile immunity (i.e., co-immunization with Ad-ANDV-G_N and Ad-ANDV-N).

Based on the data presented here, replication deficient Ad vectors expressing hantavirus proteins merit further study as potential vaccine candidates for hantavirus disease and as tools for dissecting the protective immune response in animal models.

CHAPTER VI:

Final Discussion.

It is estimated that 60% of human pathogens are of zoonotic origin. Moreover, approximately 75% of infectious diseases that emerged during the last few decades have a zoonotic reservoir, demonstrating the frequency with which they are appearing is increasing (Bengis et al. 2004, Woolhouse & Gowtage-Sequeria 2005). At a very basic level diseases of animal origin can be divided into two general categories based on characteristics of spillover events to humans (Bengis et al. 2004). Some agents (i.e., Ebola virus) spillover into human populations and are maintained for extended periods of time through human-to-human transmission. Methods of intervention for these agents typically rely on limiting disease post-spillover (i.e., controlling the outbreak). In contrast, other agents (i.e., hantaviruses) principally rely on transmission from infected animals. In practice, many different intervention strategies have been initiated to reduce the burden of disease associated with infectious agents maintained in, and predominantly transmitted from, animals. In theory, the majority of these strategies are aimed at reducing pre-spillover events and generally target one of three areas: the reservoir/vector, the point of spillover, or the susceptible (human) host (Childs 2004, 2007).

The most common point of intervention is at the reservoir/vector level with the objective of limiting the natural reservoir or vector and disrupting the natural enzootic cycle of the infectious agent. In practice this strategy often involves culling either infected animals (i.e., avian influenza positive flocks) or vectors (i.e., insecticides to control arboviruses); however, modern techniques are more commonly being employed,

and may include vaccination of primary reservoirs (i.e., bait laced with vaccine used to control rabies infections in feral animals) or secondary hosts (i.e., rabies vaccinations in domestic dogs), genetic modification or immuno-contraception to reduce reservoir breeding or induce vector resistance (both generally associated with arboviral vectors). While highly effective for some zoonotic diseases, this scenario is impractical for rodent-borne diseases for several reasons. Individual rodent species act as both reservoir and vector for specific hantaviruses, meaning interventions following this strategy would solely target rodents. Large scale efforts to control rodent populations or infection levels in the wild would prove to be a futile event due to the tremendous reproductive ability of rodents and the vast geographical areas rodents inhabit, and in which virus has been confirmed. In addition, unlike diseases such as rabies, the vectors do not demonstrate any ill effects of virus infection, making it impossible to specifically target infected rodents. The remaining two points of intervention are more practical for the control of hantaviruses, and are the topics of work described within this thesis.

The second target of intervention is at the point of spillover with the objective of reducing the likelihood of humans contracting the disease by limiting contact with reservoirs. This generally involves barriers (i.e., quarantine or import bans to prevent exotic diseases) or public education aimed at risk reduction. Without specific therapies, the only effective means for preventing hantavirus infection in humans is reducing the likelihood of contact with rodents and/or virus laden excreta/secreta, which is often the underlying message of promotional campaigns intended to reduce HPS or HFRS. The most common methods for this is rodent proofing homes and other dwellings to minimize the extent of rodent infestation in conjunction with safe methods of cleaning outlying and

previously vacant buildings, as suggested by the Centers for Disease Control and Prevention (Mills et al. 2002).

A primary component of controlling the point of spillover is understanding the enzootic cycle of the agent. Monitoring prevalence and enzootic activity in nature, with the goal of forecasting human disease presents some unique problems with hantaviruses. Unlike many other zoonotic agents (e.g., West Nile virus infection in horses or birds), sentinel animals to serve as local indicators of epizootic transmission do not exist for hantaviruses. Further, while human-to-human transmission has been demonstrated with specific viral species, cases of disease are generally sporadic and occur as single cases, meaning the occurrence of one case in a specific area does not necessarily predict the occurrence of more cases. The only mechanism to monitor viral prevalence and assess the risk of human infections is directly through the rodent host, which has proven to be an arduous task with limited predicative value.

Over a decade of continuous field research has been conducted in several locations of the United States with the overall intent of understanding hantavirus maintenance in nature and factors influencing transmission, particularly spillover to humans. While patterns are beginning to form with respect to infection dynamics in rodents (i.e., age and sex biased, seasonal variation in prevalence, correlations with density and low biodiversity, to name a few), the overall understanding of the mechanisms involved in transmission remain rudimentary. The enzootic cycle is undoubtedly complicated and probably involves several intrinsic and extrinsic influences, yet in its simplest form virus maintenance and transmission in nature has three requirements: an infected rodent, a susceptible rodent, and a means to transmit the virus

between the two. The majority of field studies conducted thus far only monitor the first two requirements, almost exclusively through serological analysis, without considering the third factor.

The objectives of the field studies presented in this thesis were to attempt to address transmission between rodents and define when infected deer mice were infectious using the presence of viral RNA in urine and OPF as a surrogate for transmissible virus. While these studies only occurred for a short duration they offer some insight into the mechanisms of transmission and supply a framework that could be applied to large-scale studies, possibly retrospectively if appropriate samples were collected, to provide a better understanding of natural transmission of virus. The findings here suggest that recently infected mice represent a greater risk to humans than do chronically infected rodents, although both appear capable of periodically shedding virus. Considering overwinter maintenance of virus likely occurs in chronically infected rodents (due to reduced activity and high mortality rates), it seems necessary that virus in these individuals can reactivate, making them a suitable vector, if only for a defined period of time.

The use of field studies for defining transmission characteristics of any zoonotic biological agent is difficult due to the various factors which cannot be controlled. Naturally occurring cycles such as predator-prey relationships, food availability (i.e., mast years and the effect on bank vole populations), and climate (including spontaneously occurring floods such as the one observed in the CMR study outlined here), all influence the enzootic/epizootic cycles of the biological agent being observed. The slightest ecological changes can drastically influence many aspects of the natural cycle of hosts and/or biological agents in ways we have yet to understand. In addition,

geographical diversity and the selection of an appropriate study site(s) can account for large differences in observations.

Due to the inherent variability in data collected in the natural setting, experimental studies are generally established to refine hypotheses, patterns and observations gathered, though even these are not without problems. In North America, experimental transmission studies with pathogenic hantaviruses require BSL-4 containment, which limits the availability of such studies. Furthermore, to-date the few laboratory studies conducted on transmission of HPS-causing viruses have not been able to effectively reproduce the conditions observed in nature (i.e., viral transmission between rodents) suggesting that experimental infections (i.e., injection of virus) in natural hosts are not reproducing natural modes of infection.

The use of field studies to understand natural transmission of hantaviruses, particularly those indigenous to the Americas, is further complicated by the lack of a reliable infectious virus assay. A limitation of the studies here is the inability to undertake isolation studies to demonstrate infectious SNV from OPF, urine or blood from naturally infected rodents. However, current methods of tissue culture (i.e., passage on Vero E6 cells) for SNV and other hantaviruses are insensitive and unreliable as demonstrated by the studies of Fulhorst and others (2002). Due to these problems, few studies have evaluated virus transmission rates or incidence of infection in rodents beyond changes in serological prevalence and, rarely, documented seroconversion. Application of the avidity assay to these studies may provide a better assessment of overall human risk since it not only allows for the determination of prevalence, but the differentiation of recently from

chronically infected deer mice which can indirectly gauge recent levels of viral transmission within rodent populations.

Based on the sporadic occurrence of HPS in Canada and throughout most of the United States a better understanding of the ecological parameters associated with hantavirus maintenance and transmission, coupled with public education aimed at reducing human contact with rodents, may be sufficient measures to counter the disease in the general public. However, in countries with higher incidence of HPS (i.e., areas of South America) and HFRS (i.e., multiple regions within Eurasia), medical intervention is required to reduce morbidity and mortality associated with human infections.

The final intervention point is aimed at the susceptible (human) host and involves medical initiatives. The most common preventative measure against infectious disease is vaccination, however like many zoonotic diseases, effective and safe vaccines do not exist for hantaviruses. In 1990, a killed HTNV vaccine (Hantavax) was approved for use in some Asian countries. The efficacy of this vaccine is still under debate with at least one study finding the level of protection afforded by the vaccine was not statistically significant (Park, Kim & Moon 2004). Regardless, similar inactivated virus vaccines for HPS are not likely to be produced based on safety concerns.

Over the last few decades several recombinant viruses have been studied as potential vaccines for infectious diseases, with poxvirus and adenovirus based platforms receiving the most attention. Post smallpox eradication, recombinant vaccinia viruses were the first generation of viral vectors for vaccines to be studied and even today remain the best characterized and most commonly used (Souza et al. 2005). The first recombinant hantavirus vaccine to be tested in phase I and II clinical trials was a vaccinia

virus expressing HTNV N, G_N and G_C proteins (Schmaljohn, Hasty & Dalrymple 1992, McClain et al. 2000). Although the vaccine was safe with no apparent side effects, and showed promising results in immunologically-naïve volunteers, its efficacy was drastically reduced in individuals with pre-existing immunity to vaccinia virus (McClain et al. 2000).

Adenovirus-based, recombinant vaccine platforms, similar to those described here, offer several advantages making them an attractive vaccine platform for the prevention of hantavirus diseases (Tatsis & Ertl 2004, Souza et al. 2005, Bangari & Mittal 2006b). The recAd vectors are relatively easy to manipulate allowing for easy construction of recombinant viruses expressing specific transgenes. Unlike the pseudotyped VSV particles, they are capable of high titer growth in appropriate cell lines, and can be lyophilized rendering them thermostabile. Adenoviruses have a broad tropism and are able to transduce both dividing and non-dividing cells. Moreover, other recAd based studies have shown a potent immune response is generated regardless of the route of administration be it via parenteral or mucosal routes. When given orally or intranasally, recAd vectors induce a strong mucosal immune response, which, in theory, may enhance the protective response against hantaviruses, since the majority of human infections occur via inhalation of virus (Santosuosso, McCormick & Xing 2005).

The safety of these viruses as vaccines in humans has been well studied, with no adverse side effects or illnesses reported following vaccination with live oral vaccines in military personnel (Top et al. 1971, Chaloner-Larsson et al. 1986). Replication deficient adenovirus platforms are also well tolerated, as has been demonstrated in clinical trials of adenovirus based HIV vaccines (Catanzaro et al. 2006, Priddy et al. 2008). When

introduced into animals (or humans) replication deficient recAd vectors result in abortive, non-lytic, infections (since the E1 gene products are not supplied) meaning the potential of horizontal transmission of vaccines is abrogated. Furthermore, the adenovirus genome is well characterized and persists episomally thereby eliminating the possibility of insertional oncogenesis or mutagenesis associated with integration into the host DNA.

Several recAd vectors have been shown to elicit a strong humoral and cellular immune response targeting the inserted foreign proteins after a single dose without the use of exogenous adjuvants and, in appropriate animal models, this response has correlated with protection from lethal infection (Sullivan et al. 2006). The data presented here supports this, while demonstrating the utility of recAd vectors for hantavirus vaccines. Currently, no other vaccine platform has resulted in substantial levels of protection in the lethal animal model making comparisons difficult. Nevertheless, the observation that individually expressed hantavirus antigens were completely protective against lethal disease, including G_C which was not previously reported to be a suitable antigen of HPS causing viruses for the induction of a protective immune response, argues the strength of the recAd platform.

While the hamster model is an important milestone for the study of HPS, its application to the study of therapeutics, vaccines and pathogenesis is currently restricted by the limited amount of commercially available hamster-specific reagents. The studies presented here demonstrate the potential of recAd vectors for both hantavirus vaccines as well as tools to dissect the protective immune response, however further studies into the mechanisms of protection requires the development of such reagents to use in specific proliferation or cytokine secretion assays. Hamster-specific reagents would also be of

value to assess the level of cross-protection afforded by the recAd based vectors. Currently, only ANDV has been described to cause lethal HPS-like disease in hamsters, which limits the usefulness of heterotypic challenge experiments. However, splenocyte proliferation assays performed on immunized hamsters using antigens of other HPS causing hantaviruses as a stimulant may be able to address this question. Additionally, the development of such reagents would greatly improve the model by allowing new insight into immune-mediate aspects of disease progression.

In spite of promising results from several studies conducted in a variety of animal models, the use of recAd vaccines have to overcome some potential disadvantages before being accepted as viable candidates approved for use in humans (Bangari & Mittal 2006a, Tatsis & Ertl 2004). The major limitation to the use of any viral vector as a vaccine is pre-existing immunity. It has been estimated that between 30-50% of the population in the USA have neutralizing antibodies against human adenvirus serotype 5 (AdHu5), which is the predominant vector used in experimental vaccines, including those described here, and the prevalence in developing countries may be significantly higher (Chirmule et al. 1999, Souza et al. 2005). Similar to the situation observed in clinical trials with recombinant vaccinia viruses, pre-existing immunity to recAd vectors compromises the efficacy of developing an immune response against the foreign proteins (Kobinger et al. 2006, McClain et al. 2000). However, mucosal immunization with recAd vectors has been shown to evade pre-existing neutralizing serum antibodies, resulting in the generation of a trans-gene specific immune response (Xiang et al. 2003). Alternatively, non-human adenovirus based platforms are also being investigated as methods to circumvent the issue of pre-existing immunity (Bangari & Mittal 2006a, 2006b). Recently

Kobinger and others (2006) demonstrated a Chimpanzee adenovirus based Ebola vaccine produced an immune response equivalent to that of the same vaccine in an AdHu5 background, however unlike the AdHu5 platform, the Chimpanzee constructs were impervious to pre-existing (AdHu5) immunity.

For any viral vaccines, replication deficient or otherwise, safety is the major concern. Although AdHu5 is associated with only mild upper respiratory tract infections, typically in children, some adenovirus serogroups are tumorgenic in experimental animal models. While no human cancers have been linked to these viruses, the use of animal derived adenovirus vectors requires further study prior to testing in humans. Perhaps the biggest obstacle to overcome is the recent failure of the recAd based HIV vaccine in clinical trials. Although it can be argued that currently no effective HIV vaccine has been produced, the failure of the recAd based vaccine was associated with increased HIV infection in treatment groups, a point that the vaccine appears to be liable for (Anonymous 2008, Lu 2008). While damaging to the use of AdHu5 based vaccines in humans, other less prevalent adenovirus vectors should not be discounted based on these results. Further, the failure of the HIV vaccine demonstrates the importance of developing and testing multiple vaccine platforms since the protective efficacy of individual candidates in humans may not reflect pre-clinical results obtained in rodent or NHP models.

The incidence of emerging infectious diseases has been steadily increasing for several decades. The majority of these newly recognized pathogens are classified as zoonotic diseases, with most having a wildlife reservoir (Jones et al. 2008). With an estimated 2000 unique species, rodentia is the largest order of mammals accounting for

approximately 40% of all mammalian species (Wilson & Reeder 1993). Currently, only a fraction (approximately 100) of the known rodent species have been thoroughly tested for the presence of hantaviruses, with roughly 25% of these demonstrated as a reservoir for a unique hantavirus, suggesting the potential for the emergence of novel species is high (Henttonen, Vapalahti & Vaheri 1995). Hantaviruses already represent a constant and significant threat to human health and the burden of disease associated with them, along with other zoonotic infections, is predicted to continually increase with escalating human populations and changes in land-use practices, not to mention the potential effect that climate change may have on higher order reservoirs and vectors, such as rodents. Combined, this highlights the need for the development of predictive models and effective medical interventions to circumvent their impact on public health. Since 1993, intense research has been conducted in both field and laboratory settings to develop a means to reduce the morbidity and mortality associated with hantaviruses. The long-term goal for the prevention of HPS and HFRS is effective and safe vaccines, however until then, ecological studies aimed at understanding maintenance and transmission of these viruses in nature are imperative to reducing human infections.

TABLE 6
Oligo Seqeunces

Oligo	Sequence (5'-3')	Reference
	SNV G_N nested primers	
SM 1687C	ACAATGGGITCIATGGTITGTGA	Johnson et al. 1997
SM 2255R	TTIAATITIICATCCATCCA	
SM 1723C	GAITGIGAIACAGCAAAAGA	
ASM 2016R	TCIGCACTIGCIGCCCA	
	SNV Real-time primers/probe	
SNV-S179f	GCAGACGGGCAGCTGTG	Botten et al. 2000
SNV-S245r	AGATCAGCCAGTTCCCGCT	
SNV TM	(FAM)GCATTGGAGACCAAACTCGGAGAACTT-(TAMRA)	
	ANDV Real-time primers/probe	
ANDV S129f	AAGGCAGTGGAGGTGGAC	
ANDV S291r	CCCTGTTGGATCAACTGGTT	
ANDV TM	(FAM)ACGGGCAGCTGTGTCTACATTGGA-(TAMRA)	
	ANDV GPC sequencing primers	
ANDV M56f	AAGGGTGGTATCTGGTTGTTC	
ANDV M662r	GTGTGAGCAGGCTGAGATAATGTC	
ANDV M771f	GTCGGAGCAACTAAAAG	
ANDV M1342r	CTACCCGCTGACAGATAAAAT	
ANDV M1368f	TGGGCAAAAGAAAGTCATA	
ANDV M1957r	ATACCAAACCCACATAACATCTAC	
ANDV M1904f	ACAGAACACTCGGGGTATTTAGAT	
ANDV M2442r	ATAGGCCTTCCCAACAGATTTTAG	
ANDV M2392f	ACTGCTTGTGGTGTTTATCTCG	
ANDV M2908r	GGTCTCTTGTATTCCCATCT	
ANDV M3003f	ATGGGGTTCTGGTGTAGGGTTTA	
ANDV M3593r	AAGTGAGGTTAGTTCCATA	
	ANDV M segment / GPC cloning primers	
SN-Fj	GGCCGCGGCCGCGGATCTGCAGGAATTCGGCAC GAGAGTAGTAGACTCCGCACGAAGAAGC	Custer et al. 2003
PUUM-R	GCGCGGATCCTAGTAGTATGCTCCGCAGGAAC	
ANDGPC BmsBI f	CGTCTCCTCGAGATGGAAGGGTGGTATCTGGTT GCTCTT	
ANDGPC BglII r	GAAGATCTTAAGACAGTTTTCTTGTGTCCTCT	

REFERENCES CITED

Anonymous. 2008. Failed HIV vaccine may have increased vulnerability to infection. Clin. Infect. Dis. **46:**iii.

Abbott, K.D., Ksiazek, T.G. & Mills, J.N. 1999. Long-term hantavirus persistence in rodent populations in central Arizona. Emerg. Infect. Dis. **5:**102-112.

Alff, P.J., Gavrilovskaya, I.N., Gorbunova, E., Endriss, K., Chong, Y., Geimonen, E., Sen, N., Reich, N.C. & Mackow, E.R. 2006. The pathogenic NY-1 hantavirus G1 cytoplasmic tail inhibits RIG-I- and TBK-1-directed interferon responses. J. Virol. **80:**9676-9686.

Arai, S., Song, J.W., Sumibcay, L., Bennett, S.N., Nerurkar, V.R., Parmenter, C., Cook, J.A., Yates, T.L. & Yanagihara, R. 2007. Hantavirus in northern short-tailed shrew, United States. Emerg. Infect. Dis. **13:**1420-1423.

Arai, S., Bennett, S.N., Sumibcay, L., Cook, J.A., Song, J.W., Hope, A., Parmenter, C., Nerurkar, V.R., Yates, T.L. & Yanagihara, R. 2008. Phylogenetically distinct hantaviruses in the masked shrew (*Sorex cinereus*) and dusky shrew (*Sorex monticolus*) in the United States. Am. J. Trop. Med. Hyg. **78:**348-351.

Arikawa, J., Schmaljohn, A.L., Dalrymple, J.M. & Schmaljohn, C.S. 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. J. Gen. Virol. **70:**615-624.

Arikawa, J., Yao, J.S., Yoshimatsu, K., Takashima, I. & Hashimoto, N. 1992. Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. Arch. Virol. **126:**271-281.

Armstrong, L.R., Bryan, R.T., Sarisky, J., Khan, A.S., Rowe, T., Ettestad, P.J., Cheek, J.E., Peters, C.J., Rollin, P. & Martin, M.L. 1995. Mild hantaviral disease caused by Sin Nombre virus in a four-year-old child. Pediatr. Infect. Dis. J. **14:**1108-1110.

Banfield, A.W.F. 1974. Deer Mouse in *The Mammals of Canada* University of Toronto Press, Toronto, Canada, pp. 164-170.

Bangari, D.S. & Mittal, S.K. 2006a. Current strategies and future directions for eluding adenoviral vector immunity. Curr. Gene Ther. **6:**215-226.

Bangari, D.S. & Mittal, S.K. 2006b. Development of nonhuman adenoviruses as vaccine vectors. Vaccine. **24:**849-862.

Bengis, R.G., Leighton, F.A., Fischer, J.R., Artois, M., Morner, T. & Tate, C.M. 2004. The role of wildlife in emerging and re-emerging zoonoses. Rev. Sci. Tech. **23:**497-511.

Bernshtein, A.D., Apekina, N.S., Mikhailova, T.V., Myasnikov, Y.A., Khlyap, L.A., Korotkov, Y.S. & Gavrilovskaya, I.N. 1999. Dynamics of Puumala hantavirus infection in naturally infected bank voles (*Clethrinomys glareolus*). Arch. Virol. **144:**2415-2428.

Bharadwaj, M., Lyons, C.R., Wortman, I.A. & Hjelle, B. 1999. Intramuscular inoculation of Sin Nombre hantavirus cDNAs induces cellular and humoral immune responses in BALB/c mice. Vaccine. **17:**2836-2843.

Bharadwaj, M., Nofchissey, R., Goade, D., Koster, F. & Hjelle, B. 2000. Humoral immune responses in the hantavirus cardiopulmonary syndrome. J. Infect. Dis. **182:**43-48.

Bharadwaj, M., Mirowsky, K., Ye, C., Botten, J., Masten, B., Yee, J., Lyons, C.R. & Hjelle, B. 2002. Genetic vaccines protect against Sin Nombre hantavirus challenge in the deer mouse (*Peromyscus maniculatus*). J. Gen. Virol. **83:**1745-1751.

Boone, J.D., Otteson, E.W., McGwire, K.C., Villard, P., Rowe, J.E. & St Jeor, S.C. 1998. Ecology and demographics of hantavirus infections in rodent populations in the Walker River Basin of Nevada and California. Am. J. Trop. Med. Hyg. **59:**445-451.

Borges, A.A., Campos, G.M., Moreli, M.L., Souza, R.L., Aquino, V.H., Saggioro, F.P. & Figueiredo, L.T. 2006. Hantavirus cardiopulmonary syndrome: immune response and pathogenesis. Microbes Infect. **8:**2324-2330.

Borucki, M.K., Boone, J.D., Rowe, J.E., Bohlman, M.C., Kuhn, E.A., DeBaca, R. & St Jeor, S.C. 2000. Role of maternal antibody in natural infection of *Peromyscus maniculatus* with Sin Nombre virus. J. Virol. **74:**2426-2429.

Botten, J., Mirowsky, K., Kusewitt, D., Bharadwaj, M., Yee, J., Ricci, R., Feddersen, R.M. & Hjelle, B. 2000. Experimental infection model for Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*). Proc. Natl. Acad. Sci. U.S.A. **97:**10578-10583.

Botten, J., Ricci, R. & Hjelle, B. 2001. Establishment of a deer mouse (*Peromyscus maniculatus rufinus*) breeding colony from wild-caught founders: comparison of reproductive performance of wild-caught and laboratory-reared pairs. Comp. Med. **51:**314-318.

Botten, J., Mirowsky, K., Ye, C., Gottlieb, K., Saavedra, M., Ponce, L. & Hjelle, B. 2002. Shedding and intracage transmission of Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*) model. J. Virol. **76:**7587-7594.

Botten, J., Mirowsky, K., Kusewitt, D., Ye, C., Gottlieb, K., Prescott, J. & Hjelle, B. 2003. Persistent Sin Nombre virus infection in the deer mouse (*Peromyscus maniculatus*) model: sites of replication and strand-specific expression. J. Virol. **77:**1540-1550.

Bridgen, A., Weber, F., Fazakerley, J.K. & Elliott, R.M. 2001. Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. Proc. Natl. Acad. Sci. U.S.A. **98:**664-669.

Calisher, C.H., Sweeney, W., Mills, J.N. & Beaty, B.J. 1999. Natural history of Sin Nombre virus in western Colorado. Emerg. Infect. Dis. **5:**126-134.

Calisher, C.H., Root, J.J., Mills, J.N., Rowe, J.E., Reeder, S.A., Jentes, E.S., Wagoner, K. & Beaty, B.J. 2005. Epizootiology of Sin Nombre and El Moro Canyon hantaviruses, southeastern Colorado, 1995-2000. J. Wildl. Dis. **41:**1-11.

Calisher, C.H., Wagoner, K.D., Amman, B.R., Root, J.J., Douglass, R.J., Kuenzi, A.J., Abbott, K.D., Parmenter, C., Yates, T.L., Ksiazek, T.G., Beaty, B.J. & Mills, J.N. 2007. Demographic factors associated with prevalence of antibody to Sin Nombre virus in deer mice in the western United States. J. Wildl. Dis. **43:**1-11.

Campen, M.J., Milazzo, M.L., Fulhorst, C.F., Obot Akata, C.J. & Koster, F. 2006. Characterization of shock in a hamster model of hantavirus infection. Virology. **356:**45-49.

Campos, G.M., Moro de Sousa, R.L., Badra, S.J., Pane, C., Gomes, U.A. & Figueiredo, L.T. 2003. Serological survey of hantavirus in Jardinopolis County, Brazil. J. Med. Virol. **71:**417-422.

Cantoni, G., Padula, P., Calderon, G., Mills, J., Herrero, E., Sandoval, P., Martinez, V., Pini, N. & Larrieu, E. 2001. Seasonal variation in prevalence of antibody to hantaviruses in rodents from southern Argentina. Trop. Med. Int. Health. **6:**811-816.

Carey, D.E., Reuben, R., Panicker, K.N., Shope, R.E. & Myers, R.M. 1971. Thottapalayam virus: a presumptive arbovirus isolated from a shrew in India. Indian J. Med. Res. **59:**1758-1760.

Casals, J., Henderson, B.E., Hoogstraal, H., Johnson, K.M. & Shelokov, A. 1970. A review of Soviet viral hemorrhagic fevers, 1969. J. Infect. Dis. **122:**437-453.

Castillo, C., Sanhueza, L., Tager, M., Munoz, S., Ossa, G. & Vial, P. 2002. Seroprevalence of antibodies against hantavirus in 10 communities of the IX Region of Chile where hantavirus infection were diagnosed. Rev. Med. Chil. **130:**251-258.

Catanzaro, A.T., Koup, R.A., Roederer, M., Bailer, R.T., Enama, M.E., Moodie, Z., Gu, L., Martin, J.E., Novik, L., Chakrabarti, B.K., Butman, B.T., Gall, J.G., King, C.R., Andrews, C.A., Sheets, R., Gomez, P.L., Mascola, J.R., Nabel, G.J., Graham, B.S. & Vaccine Research Center 006 Study Team. 2006. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. J. Infect. Dis. **194:**1638-1649.

CDC 1994. Laboratory management of agents associated with hantavirus pulmonary syndrome: interim biosafety guidelines. MMWR Morb. Mortal. Wkly. Rep. **43**(RR-7):1-7.

CDC 2004. Hantavirus pulmonary syndrome (HPS): 1996 case definition. [Homepage of US Department of Health and Human Services, CDC]. Available at: http://www.cdc.gov/epo/dphsi/casedef/hantaviruscurrent.htm. [accessed 1 April 2008].

Chaloner-Larsson, G., Contreras, G., Furesz, J., Boucher, D.W., Krepps, D., Humphreys, G.R. & Mohanna, S.M. 1986. Immunization of Canadian Armed Forces personnel with live types 4 and 7 adenovirus vaccines. Can. J. Public Health. **77:**367-370.

Childs, J.E., Glass, G.E., Korch, G.W. & LeDuc, J.W. 1989. Effects of hantaviral infection on survival, growth and fertility in wild rat (*Rattus norvegicus*) populations of Baltimore, Maryland. J. Wildl. Dis. **25:**469-476.

Childs, J.E., Ksiazek, T.G., Spiropoulou, C.F., Krebs, J.W., Morzunov, S., Maupin, G.O., Gage, K.L., Rollin, P.E., Sarisky, J. & Enscore, R.E. 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J. Infect. Dis. **169:**1271-1280.

Childs, J.E. 2004. Zoonotic viruses of wildlife: hither from yon. Arch. Virol. Suppl. **18:**1-11.

Childs, J.E. 2007. Pre-spillover prevention of emerging zoonotic diseases: what are the targets and what are the tools? Curr. Top. Microbiol. Immunol. **315:**389-443.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R. & Wilson, J. 1999. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. **6:**1574-1583.

Chitty, D. & Phipps, E. 1966. Seasonal Change in Survival in mixed populations of two species of vole. J. Anim. Ecol. **35:**313-331.

Chu, Y.K., Jennings, G.B. & Schmaljohn, C.S. 1995. A vaccinia virus-vectored Hantaan virus vaccine protects hamsters from challenge with Hantaan and Seoul viruses but not Puumala virus. J. Virol. **69:**6417-6423.

Chu, Y.K., Owen, R.D., Gonzalez, L.M. & Jonsson, C.B. 2003. The complex ecology of hantavirus in Paraguay. Am. J. Trop. Med. Hyg. **69:**263-268.

Chu, Y.K., Owen, R.D., Sanchez-Hernandez, C., Romero-Almaraz Mde, L. & Jonsson, C.B. 2008. Genetic characterization and phylogeny of a hantavirus from Western Mexico. Virus Res. **131:**180-188.

Custer, D.M., Thompson, E., Schmaljohn, C.S., Ksiazek, T.G. & Hooper, J.W. 2003. Active and passive vaccination against hantavirus pulmonary syndrome with Andes virus M genome segment-based DNA vaccine. J. Virol. **77:**9894-9905.

Dargeviciute, A., Brus Sjolander, K., Sasnauskas, K., Kruger, D.H., Meisel, H., Ulrich, R. & Lundkvist, A. 2002. Yeast-expressed Puumala hantavirus nucleocapsid protein induces protection in a bank vole model. Vaccine. **20:**3523-3531.

de Carvalho Nicacio, C., Sallberg, M., Hultgren, C. & Lundkvist, A. 2001. T-helper and humoral responses to Puumala hantavirus nucleocapsid protein: identification of T-helper epitopes in a mouse model. J. Gen. Virol. **82:**129-138.

Dietl, C.A., Wernly, J.A., Pett, S.B., Yassin, S.F., Sterling, J.P., Dragan, R., Milligan, K. & Crowley, M.R. 2008. Extracorporeal membrane oxygenation support improves survival of patients with severe Hantavirus cardiopulmonary syndrome. J. Thorac. Cardiovasc. Surg. **135:**579-584.

Dohmae, K., Koshimizu, U. & Nishimune, Y. 1993. *In utero* and mammary transfer of hantavirus antibody from dams to infant rats. Lab. Anim. Sci. **43:**557-561.

Douglass, R.J., Wilson, T., Semmens, W.J., Zanto, S.N., Bond, C.W., Van Horn, R.C. & Mills, J.N. 2001. Longitudinal studies of Sin Nombre virus in deer mouse-dominated ecosystems of Montana. Am. J. Trop. Med. Hyg. **65:**33-41.

Douglass, R.J., Kuenzi, A.J., Williams, C.Y., Douglass, S.J. & Mills, J.N. 2003. Removing deer mice from buildings and the risk for human exposure to Sin Nombre virus. Emerg. Infect. Dis. **9:**390-392.

Douglass, R.J., Calisher, C.H. & Bradley, K.C. 2005. State-by-state incidences of hantavirus pulmonary syndrome in the United States, 1993-2004. Vector Borne Zoonotic Dis. **5:**189-192.

Douglass, R.J., Calisher, C.H., Wagoner, K.D. & Mills, J.N. 2007. Sin Nombre virus infection of deer mice in Montana: characteristics of newly infected mice, incidence, and temporal pattern of infection. J. Wildl. Dis. **43:**12-22.

Drebot, M.A., Artsob, H. & Werker, D. 2000. Hantavirus pulmonary syndrome in Canada, 1989-1999. Can. Commun. Dis. Rep. **26:**65-69.

Drebot, M.A., Gavrilovskaya, I., Mackow, E.R., Chen, Z., Lindsay, R., Sanchez, A.J., Nichol, S.T. & Artsob, H. 2001. Genetic and serotypic characterization of Sin Nombre-like viruses in Canadian *Peromyscus maniculatus* mice. Virus Res. **75:**75-86.

Duchin, J.S., Koster, F.T., Peters, C.J., Simpson, G.L., Tempest, B., Zaki, S.R., Ksiazek, T.G., Rollin, P.E., Nichol, S. & Umland, E.T. 1994. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. The Hantavirus Study Group. N. Engl. J. Med. **330:**949-955.

Easterbrook, J.D., Zink, M.C. & Klein, S.L. 2007. Regulatory T cells enhance persistence of the zoonotic pathogen Seoul virus in its reservoir host. Proc. Natl. Acad. Sci. U.S.A. **104:**15502-15507.

Ebihara, H., Takada, A., Kobasa, D., Jones, S., Neumann, G., Theriault, S., Bray, M., Feldmann, H. & Kawaoka, Y. 2006. Molecular determinants of Ebola virus virulence in mice. PLoS Pathog. **2:**e73.

Eisen, H.N. & Siskind, G.W. 1964. Variations in Affinities of Antibodies during the Immune Response. Biochemistry. **3:**996-1008.

Ennis, F.A., Cruz, J., Spiropoulou, C.F., Waite, D., Peters, C.J., Nichol, S.T., Kariwa, H. & Koster, F.T. 1997. Hantavirus pulmonary syndrome: CD8+ and CD4+ cytotoxic T lymphocytes to epitopes on Sin Nombre virus nucleocapsid protein isolated during acute illness. Virology. **238:**380-390.

Enria, D.A., Briggiler, A.M., Pini, N. & Levis, S. 2001. Clinical manifestations of New World hantaviruses. Curr. Top. Microbiol. Immunol. **256:**117-134.

Eyzaguirre, E.J., Milazzo, M.L., Koster, F.T. & Fulhorst, C.F. 2008. Choclo virus infection in the Syrian golden hamster. Am. J. Trop. Med. Hyg. **78:**669-674.

Feldmann, H., Sanchez, A., Morzunov, S., Spiropoulou, C.F., Rollin, P.E., Ksiazek, T.G., Peters, C.J. & Nichol, S.T. 1993. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. Virus Res. **30:**351-367.

Ferres, M., Vial, P., Marco, C., Yanez, L., Godoy, P., Castillo, C., Hjelle, B., Delgado, I., Lee, S.J., Mertz, G.J. & Andes Virus Household Contacts Study Group 2007. Prospective evaluation of household contacts of persons with hantavirus cardiopulmonary syndrome in chile. J. Infect. Dis. **195:**1563-1571.

Flick, K., Hooper, J.W., Schmaljohn, C.S., Pettersson, R.F., Feldmann, H. & Flick, R. 2003. Rescue of Hantaan virus minigenomes. Virology. **306:**219-224.

French, G.R., Foulke, R.S., Brand, O.A., Eddy, G.A., Lee, H.W. & Lee, P.W. 1981. Korean hemorrhagic fever: propagation of the etiologic agent in a cell line of human origin. Science. **211:**1046-1048.

Frese, M., Kochs, G., Feldmann, H., Hertkorn, C. & Haller, O. 1996. Inhibition of bunyaviruses, phleboviruses, and hantaviruses by human MxA protein. J. Virol. **70:**915-923.

Fritz, C.L., Fulhorst, C.F., Enge, B., Winthrop, K.L., Glaser, C.A. & Vugia, D.J. 2002. Exposure to rodents and rodent-borne viruses among persons with elevated occupational risk. J. Occup. Environ. Med. **44:**962-967.

Fulhorst, C.F., Milazzo, M.L., Duno, G. & Salas, R.A. 2002. Experimental infection of the *Sigmodon alstoni* cotton rat with Cano Delgadito virus, a South American hantavirus. Am. J. Trop. Med. Hyg. **67:**107-111.

Fulhorst, C.F., Cajimat, M.N., Utrera, A., Milazzo, M.L. & Duno, G.M. 2004. Maporal virus, a hantavirus associated with the fulvous pygmy rice rat (*Oligoryzomys fulvescens*) in western Venezuela. Virus Res. **104:**139-144.

Garcin, D., Lezzi, M., Dobbs, M., Elliott, R.M., Schmaljohn, C., Kang, C.Y. & Kolakofsky, D. 1995. The 5' ends of Hantaan virus (*Bunyaviridae*) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. J. Virol. **69:**5754-5762.

Gardner, S.L., Von Essen, S., Berger, J. & Hjelle, B. 2005. Low seroprevalence among farmers from Nebraska and vicinity suggests low level of human exposure to Sin Nombre virus. J. Agromedicine. **10:**59-61.

Gavrilovskaya, I.N., Apekina, N.S., Myasnikov, Y., Bernshtein, A.D., Ryltseva, E.V., Gorbachkova, E.A. & Chumakov, M.P. 1983. Features of circulation of hemorrhagic fever with renal syndrome (HFRS) virus among small mammals in the European U.S.S.R. Arch. Virol. **75:**313-316.

Gavrilovskaya, I., Apekina, N., Okulova, N., Demina, V., Bernshtein, A. & Myasnikov, Y. 1993. IgG avidity assay for estimation of the time after onset of hantavirus infection in colonized and wild bank voles. Arch. Virol. **132:**359-367.

Gavrilovskaya, I.N., Shepley, M., Shaw, R., Ginsberg, M.H. & Mackow, E.R. 1998. Beta3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure. Proc. Natl. Acad. Sci. U.S.A. **95:**7074-7079.

Gavrilovskaya, I.N., Brown, E.J., Ginsberg, M.H. & Mackow, E.R. 1999. Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. J. Virol. **73:**3951-3959.

Geimonen, E., Neff, S., Raymond, T., Kocer, S.S., Gavrilovskaya, I.N. & Mackow, E.R. 2002. Pathogenic and nonpathogenic hantaviruses differentially regulate endothelial cell responses. Proc. Natl. Acad. Sci. U.S.A. **99:**13837-13842.

Glass, G.E., Childs, J.E., Korch, G.W. & LeDuc, J.W. 1988. Association of intraspecific wounding with hantaviral infection in wild rats (*Rattus norvegicus*). Epidemiol. Infect. **101:**459-472.

Glass, G.E., Yates, T.L., Fine, J.B., Shields, T.M., Kendall, J.B., Hope, A.G., Parmenter, C.A., Peters, C.J., Ksiazek, T.G., Li, C.S., Patz, J.A. & Mills, J.N. 2002. Satellite imagery characterizes local animal reservoir populations of Sin Nombre virus in the southwestern United States. Proc. Natl. Acad. Sci. U.S.A. **99:**16817-16822.

Goldsmith, C.S., Elliott, L.H., Peters, C.J. & Zaki, S.R. 1995. Ultrastructural characteristics of Sin Nombre virus, causative agent of hantavirus pulmonary syndrome. Arch. Virol. **140:**2107-2122.

Gonzalez, L.M., Lindsey, A.E., Hjelle, B., Dominguez, D., Brown, J., Goade, D. & Jonsson, C.B. 2001. Prevalence of antibodies to Sin Nombre virus in humans living in rural areas of southern New Mexico and western Texas. Virus Res. **74:**177-179.

Gowen, B.B., Wong, M-H., Jung, K-H., Sanders, A.B., Mendenhall, M., Bailey, K.W., Furuta, Y. & Sidwell, R.W. 2007. In Vitro and in vivo activities of T-705 against arenavirus and bunyavirus infections. Antimicrob. Agents Chemother. **51:**3168-3176.

Groen, J. & Osterhsus, A.D.M.E. 1999. Monkeys in hantavirus research in *Manual of Hemorrhagic Fever with Renal Syndome and Hantavirus Pulmonary Syndrome*, eds. H.W. Lee, C. Calisher & C. Schmaljohn, WHO Collaborating Center for Virus Reference and Research (Hantaviruses), Asian Institute for Life Sciences, Seoul, pp. 186-190.

Guillaume, V., Contamin, H., Loth, P., Georges-Courbot, M.C., Lefeuvre, A., Marianneau, P., Chua, K.B., Lam, S.K., Buckland, R., Deubel, V. & Wild, T.F. 2004. Nipah virus: vaccination and passive protection studies in a hamster model. J. Virol. **78:**834-840.

Hardestam, J., Klingstrom, J., Mattsson, K. & Lundkvist, A. 2005. HFRS causing hantaviruses do not induce apoptosis in confluent Vero E6 and A-549 cells. J. Med. Virol. **76:**234-240.

Hayasaka, D., Maeda, K., Ennis, F.A. & Terajima, M. 2007. Increased permeability of human endothelial cell line EA.hy926 induced by hantavirus-specific cytotoxic T lymphocytes. Virus Res. **123:**120-127.

Hedman, K., Vaheri, A. & Brummer-Korvenkontio, M. 1991. Rapid diagnosis of hantavirus disease with an IgG-avidity assay. Lancet. **338:**1353-1356.

Henttonen, H., Vapalahti, O. & Vaheri, A. 1995. How many kinds of hantaviruses? Trends Ecol. Evol. **11:**7-8.

Hinson, E.R., Shone, S.M., Zink, M.C., Glass, G.E. & Klein, S.L. 2004. Wounding: the primary mode of Seoul virus transmission among male Norway rats. Am. J. Trop. Med. Hyg. **70:**310-317.

Hjelle, B., Lee, S.W., Song, W., Torrez-Martinez, N., Song, J.W., Yanagihara, R., Gavrilovskaya, I. & Mackow, E.R. 1995. Molecular linkage of hantavirus pulmonary syndrome to the white-footed mouse, Peromyscus leucopus: genetic characterization of the M genome of New York virus. J. Virol. **69:**8137-8141.

Hjelle, B., Goade, D., Torrez-Martinez, N., Lang-Williams, M., Kim, J., Harris, R.L. & Rawlings, J.A. 1996. Hantavirus pulmonary syndrome, renal insufficiency, and myositis associated with infection by Bayou hantavirus. Clin. Infect. Dis. **23:**495-500.

Hjelle, B. & Glass, G.E. 2000. Outbreak of hantavirus infection in the Four Corners region of the United States in the wake of the 1997-1998 El Nino-southern oscillation. J. Infect. Dis. **181:**1569-1573.

Hjelle, B. & Yates, T. 2001. Modeling hantavirus maintenance and transmission in rodent communities. Curr. Top. Microbiol. Immunol. **256:**77-90.

Hooper, J.W., Kamrud, K.I., Elgh, F., Custer, D. & Schmaljohn, C.S. 1999. DNA vaccination with hantavirus M segment elicits neutralizing antibodies and protects against Seoul virus infection. Virology. **255:**269-278.

Hooper, J.W., Custer, D.M., Thompson, E. & Schmaljohn, C.S. 2001a. DNA vaccination with the Hantaan virus M gene protects Hamsters against three of four HFRS hantaviruses and elicits a high-titer neutralizing antibody response in Rhesus monkeys. J. Virol. **75:**8469-8477.

Hooper, J.W., Larsen, T., Custer, D.M. & Schmaljohn, C.S. 2001b. A lethal disease model for hantavirus pulmonary syndrome. Virology. **289:**6-14.

Hooper, J.W., Custer, D.M., Smith, J. & Wahl-Jensen, V. 2006. Hantaan/Andes virus DNA vaccine elicits a broadly cross-reactive neutralizing antibody response in nonhuman primates. Virology. **347:**208-216.

Hooper, J.W., Ferro, A.M. & Wahl-Jensen, V. 2008. Immune serum produced by DNA vaccination protects hamsters against lethal respiratory challenge with Andes virus. J. Virol. **82:**1332-1338.

Houck, M.A., Qin, H. & Roberts, H.R. 2001. Hantavirus transmission: potential role of ectoparasites. Vector Borne Zoonotic Dis. **1:**75-79.

Huang, C., Jin, B., Wang, M., Li, E. & Sun, C. 1994. Hemorrhagic fever with renal syndrome: relationship between pathogenesis and cellular immunity. J. Infect. Dis. **169:**868-870.

Huggins, J.W., Kim, G.R., Brand, O.M. & McKee, K.T.,Jr 1986. Ribavirin therapy for Hantaan virus infection in suckling mice. J. Infect. Dis. **153:**489-497.

Huggins, J.W., Hsiang, C.M., Cosgriff, T.M., Guang, M.Y., Smith, J.I., Wu, Z.O., LeDuc, J.W., Zheng, Z.M., Meegan, J.M. & Wang, Q.N. 1991. Prospective, double-blind, concurrent, placebo-controlled clinical trial of intravenous ribavirin therapy of hemorrhagic fever with renal syndrome. J. Infect. Dis. **164:**1119-1127.

Hutchinson, K.L., Peters, C.J. & Nichol, S.T. 1996. Sin Nombre virus mRNA synthesis. Virology. **224:**139-149.

Hutchinson, K.L., Rollin, P.E. & Peters, C.J. 1998. Pathogenesis of a North American hantavirus, Black Creek Canal virus, in experimentally infected *Sigmodon hispidus*. Am. J. Trop. Med. Hyg. **59:**58-65.

Hutchinson, K.L., Rollin, P.E., Shieh, W.J., Zaki, S., Greer, P.W. & Peters, C.J. 2000. Transmission of Black Creek Canal virus between cotton rats. J. Med. Virol. **60:**70-76.

Jääskeläinen, K.M., Kaukinen, P., Minskaya, E.S., Plyusnina, A., Vapalahti, O., Elliott, R.M., Weber, F., Vaheri, A. & Plyusnin, A. 2007. Tula and Puumala hantavirus NSs ORFs are functional and the products inhibit activation of the interferon-beta promoter. J. Med. Virol. **79:**1527-1536.

Jääskeläinen, K.M., Plyusnina, A., Lundkvist, A., Vaheri, A. & Plyusnin, A. 2008. Tula hantavirus isolate with the full-length ORF for nonstructural protein NSs survives for more consequent passages in interferon-competent cells than the isolate having truncated NSs ORF. Virol. J. **5:**3.

Jenison, S., Yamada, T., Morris, C., Anderson, B., Torrez-Martinez, N., Keller, N. & Hjelle, B. 1994. Characterization of human antibody responses to Four Corners hantavirus infections among patients with hantavirus pulmonary syndrome. J. Virol. **68:**3000-3006.

Jiang, J.F., Zhang, W.Y., Wu, X.M., Zhang, P.H. & Cao, W.C. 2007. Soochong virus and Amur virus might be the same entities of hantavirus. J. Med. Virol. **79:**1792-1795.

Johnson, A.M., Bowen, M.D., Ksiazek, T.G., Williams, R.J., Bryan, R.T., Mills, J.N., Peters, C.J. & Nichol, S.T. 1997. Laguna Negra virus associated with HPS in western Paraguay and Bolivia. Virology. **238:**115-127.

Johnson, A.M., de Souza, L.T., Ferreira, I.B., Pereira, L.E., Ksiazek, T.G., Rollin, P.E., Peters, C.J. & Nichol, S.T. 1999. Genetic investigation of novel hantaviruses causing fatal HPS in Brazil. J. Med. Virol. **59:**527-535.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. & Daszak, P. 2008. Global trends in emerging infectious diseases. Nature. **451:**990-994.

Jonsson, C.B. & Schmaljohn, C.S. 2001. Replication of hantaviruses. Curr. Top. Microbiol. Immunol. **256:**15-32.

Jonsson, C.B., Hooper, J. & Mertz, G. 2008. Treatment of hantavirus pulmonary syndrome. Antiviral Res. **78:**162-169.

Kallio, E.R., Klingstrom, J., Gustafsson, E., Manni, T., Vaheri, A., Henttonen, H., Vapalahti, O. & Lundkvist, A. 2006. Prolonged survival of Puumala hantavirus outside the host: evidence for indirect transmission via the environment. J. Gen. Virol. **87:**2127-2134.

Kallio, E.R., Voutilainen, L., Vapalahti, O., Vaheri, A., Henttonen, H., Koskela, E. & Mappes, T. 2007. Endemic hantavirus infection impairs the winter survival of its rodent host. Ecology. **88:**1911-1916.

Kallio-Kokko, H., Vapalahti, O., Hedman, K., Brummer-Korvenkontio, M. & Vaheri, A. 1993. Puumala virus antibody and immunoglobulin G avidity assays based on a recombinant nucleocapsid antigen. J. Clin. Microbiol. **31:**677-680.

Kallio-Kokko, H., Leveelahti, R., Brummer-Korvenkontio, M., Lundkvist, A., Vaheri, A. & Vapalahti, O. 2001. Human immune response to Puumala virus glycoproteins and nucleocapsid protein expressed in mammalian cells. J. Med. Virol. **65:**605-613.

Kamrud, K.I., Hooper, J.W., Elgh, F. & Schmaljohn, C.S. 1999. Comparison of the protective efficacy of naked DNA, DNA-based Sindbis replicon, and packaged Sindbis replicon vectors expressing Hantavirus structural genes in hamsters. Virology. **263:**209-219.

Kang, J.I., Park, S.H., Lee, P.W. & Ahn, B.Y. 1999. Apoptosis is induced by hantaviruses in cultured cells. Virology. **264:**99-105.

Kariwa, H., Fujiki, M., Yoshimatsu, K., Arikawa, J., Takashima, I. & Hashimoto, N. 1998. Urine-associated horizontal transmission of Seoul virus among rats. Arch. Virol. **143:**365-374.

Kariwa, H., Yoshimatsu, K., Sawabe, J., Yokota, E., Arikawa, J., Takashima, I., Fukushima, H., Lundkvist, A., Shubin, F.N., Isachkova, L.M., Slonova, R.A., Leonova, G.N. & Hashimoto, N. 1999. Genetic diversities of hantaviruses among rodents in Hokkaido, Japan and Far East Russia. Virus Res. **59:**219-228.

Kaukinen, P., Vaheri, A. & Plyusnin, A. 2005. Hantavirus nucleocapsid protein: a multifunctional molecule with both housekeeping and ambassadorial duties. Arch. Virol. **150:**1693-1713.

Kawamura, K., Zhang, X.K., Arikawa, J., Takashima, I., Dempo, K. & Hashimoto, N. 1991. Susceptibility of laboratory and wild rodents to *Rattus* or *Apodemus*-type hantaviruses. Acta Virol. **35:**54-63.

Kehm, R., Jakob, N.J., Welzel, T.M., Tobiasch, E., Viczian, O., Jock, S., Geider, K., Sule, S. & Darai, G. 2001. Expression of immunogenic Puumala virus nucleocapsid protein in transgenic tobacco and potato plants. Virus Genes. **22:**73-83.

Khaiboullina, S.F., Rizvanov, A.A., Otteson, E., Miyazato, A., Maciejewski, J. & St Jeor, S. 2004. Regulation of cellular gene expression in endothelial cells by Sin Nombre and Prospect Hill viruses. Viral Immunol. **17:**234-251.

Khaiboullina, S.F., Morzunov, S.P. & St Jeor, S.C. 2005. Hantaviruses: molecular biology, evolution and pathogenesis. Curr. Mol. Med. **5:**773-790.

Khaiboullina, S.F., Rizvanov, A.A., Deyde, V.M. & St Jeor, S.C. 2005. Andes virus stimulates interferon-inducible MxA protein expression in endothelial cells. J. Med. Virol. **75:**267-275.

Khan, A.S., Spiropoulou, C.F., Morzunov, S., Zaki, S.R., Kohn, M.A., Nawas, S.R., McFarland, L. & Nichol, S.T. 1995. Fatal illness associated with a new hantavirus in Louisiana. J. Med. Virol. **46:**281-286.

Khan, A.S., Khabbaz, R.F., Armstrong, L.R., Holman, R.C., Bauer, S.P., Graber, J., Strine, T., Miller, G., Reef, S., Tappero, J., Rollin, P.E., Nichol, S.T., Zaki, S.R., Bryan, R.T., Chapman, L.E., Peters, C.J. & Ksiazek, T.G. 1996. Hantavirus pulmonary syndrome: the first 100 US cases. J. Infect. Dis. **173:**1297-1303.

Kikuchi, M., Yoshimatsu, K., Arikawa, J., Yoshida, R., Yoo, Y.C., Isegawa, Y., Yamanishi, K., Tono-oka, S. & Azuma, I. 1998. Characterization of neutralizing monoclonal antibody escape mutants of Hantaan virus 76118. Arch. Virol. **143:**73-83.

Kilpatrick, E.D., Terajima, M., Koster, F.T., Catalina, M.D., Cruz, J. & Ennis, F.A. 2004. Role of specific CD8+ T cells in the severity of a fulminant zoonotic viral hemorrhagic fever, hantavirus pulmonary syndrome. J. Immunol. **172:**3297-3304.

Kim, T.Y., Choi, Y., Cheong, H.S. & Choe, J. 2002. Identification of a cell surface 30 kDa protein as a candidate receptor for Hantaan virus. J. Gen. Virol. **83:**767-773.

Klein, S.L., Bird, B.H. & Glass, G.E. 2000. Sex differences in Seoul virus infection are not related to adult sex steroid concentrations in Norway rats. J. Virol. **74:**8213-8217.

Klein, S.L., Cernetich, A., Hilmer, S., Hoffman, E.P., Scott, A.L. & Glass, G.E. 2004. Differential expression of immunoregulatory genes in male and female Norway rats following infection with Seoul virus. J. Med. Virol. **74:**180-190.

Klein, S.L. & Calisher, C.H. 2007. Emergence and persistence of hantaviruses. Curr. Top. Microbiol. Immunol. **315:**217-252.

Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., Denys, C., Koivogui, L., ter Meulen, J. & Kruger, D.H. 2006. Hantavirus in African wood mouse, Guinea. Emerg. Infect. Dis. **12:**838-840.

Klempa, B., Fichet-Calvet, E., Lecompte, E., Auste, B., Aniskin, V., Meisel, H., Barriere, P., Koivogui, L., ter Meulen, J. & Kruger, D.H. 2007. Novel hantavirus sequences in Shrew, Guinea. Emerg. Infect. Dis. **13:**520-522.

Kobinger, G.P., Feldmann, H., Zhi, Y., Schumer, G., Gao, G., Feldmann, F., Jones, S. & Wilson, J.M. 2006. Chimpanzee adenovirus vaccine protects against Zaire Ebola virus. Virology. **346:**394-401.

Kobinger, G.P., Figueredo, J.M., Rowe, T., Zhi, Y., Gao, G., Sanmiguel, J.C., Bell, P., Wivel, N.A., Zitzow, L.A., Flieder, D.B., Hogan, R.J. & Wilson, J.M. 2007. Adenovirus-based vaccine prevents pneumonia in ferrets challenged with the SARS coronavirus and stimulates robust immune responses in macaques. Vaccine. **25:**5220-5231.

Koch, J., Liang, M., Queitsch, I., Kraus, A.A. & Bautz, E.K. 2003. Human recombinant neutralizing antibodies against Hantaan virus G2 protein. Virology. **308:**64-73.

Krakauer, T., Leduc, J.W., Morrill, J.C., Anderson, A.O. & Krakauer, H. 1994. Serum levels of alpha and gamma interferons in hemorrhagic fever with renal syndrome. Viral Immunol. **7:**97-101.

Kraus, A.A., Raftery, M.J., Giese, T., Ulrich, R., Zawatzky, R., Hippenstiel, S., Suttorp, N., Kruger, D.H. & Schonrich, G. 2004. Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses. J. Virol. **78:**6143-6150.

Kuenzi, A.J., Douglass, R.J., Bond, C.W., Calisher, C.H. & Mills, J.N. 2005. Long-term dynamics of Sin Nombre viral RNA and antibody in deer mice in Montana. J. Wildl. Dis. **41:**473-481.

Kukkonen, S.K., Vaheri, A. & Plyusnin, A. 2005. L protein, the RNA-dependent RNA polymerase of hantaviruses. Arch. Virol. **150:**533-556.

Larson, R.S., Brown, D.C., Ye, C. & Hjelle, B. 2005. Peptide antagonists that inhibit Sin Nombre virus and Hantaan virus entry through the beta3-integrin receptor. J. Virol. **79:**7319-7326.

Lee, B.H., Yoshimatsu, K., Maeda, A., Ochiai, K., Morimatsu, M., Araki, K., Ogino, M., Morikawa, S. & Arikawa, J. 2003. Association of the nucleocapsid protein of the Seoul and Hantaan hantaviruses with small ubiquitin-like modifier-1-related molecules. Virus Res. **98:**83-91.

Lee, B.H., Yoshimatsu, K., Araki, K., Okumura, M., Nakamura, I. & Arikawa, J. 2006. A pseudotype vesicular stomatitis virus containing Hantaan virus envelope glycoproteins G1 and G2 as an alternative to hantavirus vaccine in mice. Vaccine. **24:**2928-2934.

Lee, H.W., Lee, P.W. & Johnson, K.M. 1978. Isolation of the etiological agent of Korean hemorrhagic fever. J. Infect. Dis. **137:**298-308.

Lee, H.W., Lee, P.W., Baek, L.J., Song, C.K. & Seong, I.W. 1981. Intraspecific transmission of Hantaan virus, etiologic agent of Korean hemorrhagic fever, in the rodent *Apodemus agrarius*. Am. J. Trop. Med. Hyg. **30:**1106-1112.

Lee, H.W., Baek, L.J. & Johnson, K.M. 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever, from wild urban rats. J. Infect. Dis. **146:**638-644.

Lee, H.W. 1989. Hemorrhagic fever with renal syndrome in Korea. Rev. Infect. Dis. **11** (Suppl 4):S864-76.

Lee, H.W. 1996. Epidemiology and pathogenesis of hemorrhagic fever with renal syndrome in *The Bunyaviridae*, ed. Elliott RM, Plenum Press, New York, pp. 253-267.

Leighton, F.A., Artsob, H.A., Chu, M.C. & Olson, J.G. 2001. A serological survey of rural dogs and cats on the southwestern Canadian prairie for zoonotic pathogens. Can. J. Public Health. **92:**67-71.

Levis, S., Morzunov, S.P., Rowe, J.E., Enria, D., Pini, N., Calderon, G., Sabattini, M. & St Jeor, S.C. 1998. Genetic diversity and epidemiology of hantaviruses in Argentina. J. Infect. Dis. **177:**529-538.

Li, X.D., Lankinen, H., Putkuri, N., Vapalahti, O. & Vaheri, A. 2005. Tula hantavirus triggers pro-apoptotic signals of ER stress in Vero E6 cells. Virology. **333:**180-189.

Linderholm, M., Ahlm, C., Settergren, B., Waage, A. & Tarnvik, A. 1996. Elevated plasma levels of tumor necrosis factor (TNF)-alpha, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients with hemorrhagic fever with renal syndrome. J. Infect. Dis. **173:**38-43.

Linderholm, M. & Elgh, F. 2001. Clinical characteristics of hantavirus infections on the Eurasian continent. Curr. Top. Microbiol. Immunol. **256:**135-151.

Lindsay, L.R., Drebot, M.A., Weiss, E. & Artsob, H. 2001. Hantavirus pulmonary syndrome in Manitoba. Can. J. Infect. Dis. **12:**169-173.

Lober, C., Anheier, B., Lindow, S., Klenk, H.D. & Feldmann, H. 2001. The Hantaan virus glycoprotein precursor is cleaved at the conserved pentapeptide WAASA. Virology. **289:**224-229.

Lu, S. 2008. Human versus HIV: round 2 defeat in AIDS vaccine development. Expert Rev. Vaccines. **7:**151-153.

Lundkvist, A., Horling, J. & Niklasson, B. 1993. The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays. Arch. Virol. **130:**121-130.

Lundkvist, A., Kallio-Kokko, H., Sjolander, K.B., Lankinen, H., Niklasson, B., Vaheri, A. & Vapalahti, O. 1996a. Characterization of Puumala virus nucleocapsid protein: identification of B-cell epitopes and domains involved in protective immunity. Virology. **216:**397-406.

Lundkvist, A., Vapalahti, O., Plyusnin, A., Sjolander, K.B., Niklasson, B. & Vaheri, A. 1996b. Characterization of Tula virus antigenic determinants defined by monoclonal antibodies raised against baculovirus-expressed nucleocapsid protein. Virus Res. **45:**29-44.

Lyubsky, S., Gavrilovskaya, I., Luft, B. & Mackow, E. 1996. Histopathology of *Peromyscus leucopus* naturally infected with pathogenic NY-1 hantaviruses: pathologic markers of HPS viral infection in mice. Lab. Invest. **74:**627-633.

Mackow, E.R. & Gavrilovskaya, I.N. 2001. Cellular receptors and hantavirus pathogenesis. Curr. Top. Microbiol. Immunol. **256:**91-115.

Madhav, N.K., Wagoner, K.D., Douglass, R.J. & Mills, J.N. 2007. Delayed density-dependent prevalence of Sin Nombre virus antibody in Montana deer mice (*Peromyscus maniculatus*) and implications for human disease risk. Vector Borne Zoonotic Dis. **7:**353-364.

Maeda, A., Lee, B.H., Yoshimatsu, K., Saijo, M., Kurane, I., Arikawa, J. & Morikawa, S. 2003. The intracellular association of the nucleocapsid protein (NP) of Hantaan virus (HTNV) with small ubiquitin-like modifier-1 (SUMO-1) conjugating enzyme 9 (Ubc9). Virology. **305:**288-297.

Maeda, K., West, K., Hayasaka, D., Ennis, F.A. & Terajima, M. 2005. Recombinant adenovirus vector vaccine induces stronger cytotoxic T-cell responses than recombinant vaccinia virus vector, plasmid DNA, or a combination of these. Viral Immunol. **18:**657-667.

Mailles, A., Sin, M.A., Ducoffre, G., Heyman, P., Koch, J. & Zeller, H. 2005. Larger than usual increase in cases of hantavirus infections in Belgium, France and Germany, June 2005. Euro Surveill. **10:**E050721.4.

Majid, A.M., Ezelle, H., Shah, S. & Barber, G.N. 2006. Evaluating replication-defective vesicular stomatitis virus as a vaccine vehicle. J. Virol. **80:**6993-7008.

Markotic, A., Hensley, L., Geisbert, T., Spik, K. & Schmaljohn, C. 2003. Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells. J. Gen. Virol. **84:**2197-2202.

Martinez, V.P., Bellomo, C., San Juan, J., Pinna, D., Forlenza, R., Elder, M. & Padula, P.J. 2005. Person-to-person transmission of Andes virus. Emerg. Infect. Dis. **11:**1848-1853.

Matthews, D.A., Cummings, D., Evelegh, C., Graham, F.L. & Prevec, L. 1999. Development and use of a 293 cell line expressing lac repressor for the rescue of recombinant adenoviruses expressing high levels of rabies virus glycoprotein. J. Gen. Virol. **80:**345-353.

McClain, D.J., Summers, P.L., Harrison, S.A., Schmaljohn, A.L. & Schmaljohn, C.S. 2000. Clinical evaluation of a vaccinia-vectored Hantaan virus vaccine. J. Med. Virol. **60:**77-85.

McCormick, J.B., Sasso, D.R., Palmer, E.L. & Kiley, M.P. 1982. Morphological identification of the agent of Korean haemorrhagic fever (Hantaan virus) as a member of the Bunyaviridae. Lancet. **1:**765-768.

McElroy, A.K., Bray, M., Reed, D.S. & Schmaljohn, C.S. 2002. Andes virus infection of cynomolgus macaques. J. Infect. Dis. **186:**1706-1712.

McElroy, A.K., Smith, J.M., Hooper, J.W. & Schmaljohn, C.S. 2004. Andes virus M genome segment is not sufficient to confer the virulence associated with Andes virus in Syrian hamsters. Virology. **326:**130-139.

McKee, K.T.,Jr, Kim, G.R., Green, D.E. & Peters, C.J. 1985. Hantaan virus infection in suckling mice: virologic and pathologic correlates. J. Med. Virol. **17:**107-117.

Medina, R.A., Mirowsky-Garcia, K., Hutt, J. & Hjelle, B. 2007. Ribavirin, human convalescent plasma and anti-beta3 integrin antibody inhibit infection by Sin Nombre virus in the deer mouse model. J. Gen. Virol. **88:**493-505.

Mertz, G.J., Miedzinski, L., Goade, D., Pavia, A.T., Hjelle, B., Hansbarger, C.O., Levy, H., Koster, F.T., Baum, K., Lindemulder, A., Wang, W., Riser, L., Fernandez, H., Whitley, R.J. & Collaborative Antiviral Study Group 2004. Placebo-controlled, double-blind trial of intravenous ribavirin for the treatment of hantavirus cardiopulmonary syndrome in North America. Clin. Infect. Dis. **39:**1307-1313.

Meissner, J.D., Rowe, J.E., Borucki, M.K., & St. Joer, S.C. 2002. Complete nucleotide sequence of a Chilean hantavirus. Virus Res. **89:**131-143.

Milazzo, M.L., Eyzaguirre, E.J., Molina, C.P. & Fulhorst, C.F. 2002. Maporal viral infection in the Syrian golden hamster: a model of hantavirus pulmonary syndrome. J. Infect. Dis. **186:**1390-1395.

Milazzo, M.L., Cajimat, M.N., Hanson, J.D., Bradley, R.D., Quintana, M., Sherman, C., Velasquez, R.T. & Fulhorst, C.F. 2006. Catacamas virus, a hantaviral species naturally associated with *Oryzomys couesi* (Coues' oryzomys) in Honduras. Am. J. Trop. Med. Hyg. **75:**1003-1010.

Mills, J.N., Ksiazek, T.G., Ellis, B.A., Rollin, P.E., Nichol, S.T., Yates, T.L., Gannon, W.L., Levy, C.E., Engelthaler, D.M., Davis, T., Tanda, D.T., Frampton, J.W., Nichols, C.R., Peters, C.J. & Childs, J.E. 1997. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am. J. Trop. Med. Hyg. **56:**273-284.

Mills, J.N., Johnson, J.M., Ksiazek, T.G., Ellis, B.A., Rollin, P.E., Yates, T.L., Mann, M.O., Johnson, M.R., Campbell, M.L., Miyashiro, J., Patrick, M., Zyzak, M., Lavender, D., Novak, M.G., Schmidt, K., Peters, C.J. & Childs, J.E. 1998. A survey of hantavirus antibody in small-mammal populations in selected United States National Parks. Am. J. Trop. Med. Hyg. **58:**525-532.

Mills, J.N., Corneli, A., Young, J.C., Garrison, L.E., Khan, A.S. & Ksiazek, T.G. 2002. Hantavirus pulmonary syndrome--United States: updated recommendations for risk reduction. Centers for Disease Control and Prevention. MMWR Recomm. Rep. **51:**1-12.

Mills, J.N. 2005. Regulation of rodent-borne viruses in the natural host: implications for human disease. Arch. Virol. Suppl. **(19):**45-57.

Mills, J.N., Schmidt, K., Ellis, B.A., Calderon, G., Enria, D.A. & Ksiazek, T.G. 2007. A longitudinal study of hantavirus infection in three sympatric reservoir species in agroecosystems on the Argentine Pampa. Vector Borne Zoonotic Dis. **7:**229-240.

Mir, M.A. & Panganiban, A.T. 2005. The hantavirus nucleocapsid protein recognizes specific features of the viral RNA panhandle and is altered in conformation upon RNA binding. J. Virol. **79:**1824-1835.

Monroe, M.C., Morzunov, S.P., Johnson, A.M., Bowen, M.D., Artsob, H., Yates, T., Peters, C.J., Rollin, P.E., Ksiazek, T.G. & Nichol, S.T. 1999. Genetic diversity and distribution of *Peromyscus*-borne hantaviruses in North America. Emerg. Infect. Dis. **5:**75-86.

Mori, M., Rothman, A.L., Kurane, I., Montoya, J.M., Nolte, K.B., Norman, J.E., Waite, D.C., Koster, F.T. & Ennis, F.A. 1999. High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. J.Infect.Dis. **179:**295-302.

Morzunov, S.P., Rowe, J.E., Ksiazek, T.G., Peters, C.J., St Jeor, S.C. & Nichol, S.T. 1998. Genetic analysis of the diversity and origin of hantaviruses in *Peromyscus leucopus* mice in North America. J. Virol. **72:**57-64.

Murgue, B., Domart, Y., Coudrier, D., Rollin, P.E., Darchis, J.P., Merrien, D. & Zeller, H.G. 2002. First reported case of imported hantavirus pulmonary syndrome in Europe. Emerg. Infect. Dis. **8:**106-107.

Murphy, M.E., Kariwa, H., Mizutani, T., Tanabe, H., Yoshimatsu, K., Arikawa, J. & Takashima, I. 2001. Characterization of in vitro and in vivo antiviral activity of lactoferrin and ribavirin upon hantavirus. J. Vet. Med. Sci. **63:**637-645.

Mustonen, J., Partanen, J., Kanerva, M., Pietila, K., Vapalahti, O., Pasternack, A. & Vaheri, A. 1996. Genetic susceptibility to severe course of nephropathia epidemica caused by Puumala hantavirus. Kidney Int. **49:**217-221.

Nasci, R.S., Gottfried, K.L., Burkhalter, K.L., Kulasekera, V.L., Lambert, A.J., Lanciotti, R.S., Hunt, A.R. & Ryan, J.R. 2002. Comparison of vero cell plaque assay, TaqMan reverse transcriptase polymerase chain reaction RNA assay, and VecTest antigen assay for detection of West Nile virus in field-collected mosquitoes. J. Am. Mosq. Control Assoc. **18**:294-300.

Netski, D., Thran, B.H. & St Jeor, S.C. 1999. Sin Nombre virus pathogenesis in *Peromyscus maniculatus*. J. Virol. **73:**585-591.

Nichol, S.T., Spiropoulou, C.F., Morzunov, S., Rollin, P.E., Ksiazek, T.G., Feldmann, H., Sanchez, A., Childs, J., Zaki, S. & Peters, C.J. 1993. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science. **262:**914-917.

Nichol, S.T., Elliott, R.M., Goldbach, R., Plyusnin, A., Schmaljohn, C.S. & Tesh, R.B. 2005. Bunyaviridae in *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses.*, eds. C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger & L.A. Ball, Elsevier Academic Press, pp. 695-716.

Niklasson, B., Hornfeldt, B., Mullaart, M., Settergren, B., Tkachenko, E., Myasnikov, Y., Ryltceva, E.V., Leschinskaya, E., Malkin, A. & Dzagurova, T. 1993. An epidemiologic study of hemorrhagic fever with renal syndrome in Bashkirtostan (Russia) and Sweden. Am. J. Trop. Med. Hyg. **48:**670-675.

Niklasson, B., Hellsten, G. & LeDuc, J. 1994. Hemorrhagic fever with renal syndrome: a study of sequelae following nephropathia epidemica. Arch. Virol. **137:**241-247.

Niklasson, B., Hornfeldt, B., Lundkvist, A., Bjorsten, S. & Leduc, J. 1995. Temporal dynamics of Puumala virus antibody prevalence in voles and of nephropathia epidemica incidence in humans. Am. J. Trop. Med. Hyg. **53:**134-140.

Nolte, K.B., Feddersen, R.M., Foucar, K., Zaki, S.R., Koster, F.T., Madar, D., Merlin, T.L., McFeeley, P.J., Umland, E.T. & Zumwalt, R.E. 1995. Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. Hum. Pathol. **26:**110-120.

Ogino, M., Ebihara, H., Lee, B.H., Araki, K., Lundkvist, A., Kawaoka, Y., Yoshimatsu, K. & Arikawa, J. 2003. Use of vesicular stomatitis virus pseudotypes bearing Hantaan or Seoul virus envelope proteins in a rapid and safe neutralization test. Clin. Diagn. Lab. Immunol. **10:**154-160.

Ogino, M., Yoshimatsu, K., Ebihara, H., Araki, K., Lee, B.H., Okumura, M. & Arikawa, J. 2004. Cell fusion activities of Hantaan virus envelope glycoproteins. J. Virol. **78:**10776-10782.

Olsson, G.E., White, N., Ahlm, C., Elgh, F., Verlemyr, A.C., Juto, P. & Palo, R.T. 2002. Demographic factors associated with hantavirus infection in bank voles (*Clethrionomys glareolus*). Emerg. Infect. Dis. **8:**924-929.

Padula, P.J., Rossi, C.M., Della Valle, M.O., Martinez, P.V., Colavecchia, S.B., Edelstein, A., Miguel, S.D., Rabinovich, R.D. & Segura, E.L. 2000. Development and evaluation of a solid-phase enzyme immunoassay based on Andes hantavirus recombinant nucleoprotein. J. Med. Microbiol. **49:**149-155.

Padula, P.J., Sanchez, A.J., Edelstein, A. & Nichol, S.T. 2002. Complete nucleotide sequence of the M RNA segment of Andes virus and analysis of the variability of the termini of the virus S, M and L RNA segments. J. Gen. Virol. **83:**2177-2122.

Padula, P., Figueroa, R., Navarrete, M., Pizarro, E., Cadiz, R., Bellomo, C., Jofre, C., Zaror, L., Rodriguez, E. & Murua, R. 2004. Transmission study of Andes hantavirus infection in wild sigmodontine rodents. J. Virol. **78:**11972-11979.

Park, K., Kim, C.S. & Moon, K.T. 2004. Protective effectiveness of hantavirus vaccine. Emerg. Infect. Dis. **10:**2218-2220.

Pearce-Duvet, J.M., St Jeor, S.C., Boone, J.D. & Dearing, M.D. 2006. Changes in Sin Nombre virus antibody prevalence in deer mice across seasons: the interaction between habitat, sex, and infection in deer mice. J. Wildl. Dis. **42:**819-824.

Pensiero, M.N., Jennings, G.B., Schmaljohn, C.S. & Hay, J. 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. J. Virol. **62:**696-702.

Pettersson, R.F. 1991. Protein localization and virus assembly at intracellular membranes. Curr. Top. Microbiol. Immunol. **170:**67-106.

Pini, N., Levis, S., Calderon, G., Ramirez, J., Bravo, D., Lozano, E., Ripoll, C., St Jeor, S., Ksiazek, T.G., Barquez, R.M. & Enria, D. 2003. Hantavirus infection in humans and rodents, northwestern Argentina. Emerg. Infect. Dis. **9:**1070-1076.

Plyusnin, A., Cheng, Y., Vapalahti, O., Pejcoch, M., Unar, J., Jelinkova, Z., Lehvaslaiho, H., Lundkvist, A. & Vaheri, A. 1995. Genetic variation in Tula hantaviruses: sequence analysis of the S and M segments of strains from Central Europe. Virus Res. **39:**237-250.

Plyusnin, A., Vapalahti, O. & Vaheri, A. 1996. Hantaviruses: genome structure, expression and evolution. J. Gen. Virol. **77:**2677-2687.

Powers, A.M., Mercer, D.R., Watts, D.M., Guzman, H., Fulhorst, C.F., Popov, V.L. & Tesh, R.B. 1999. Isolation and genetic characterization of a hantavirus (*Bunyaviridae*: Hantavirus) from a rodent, *Oligoryzomys microtis* (Muridae), collected in northeastern Peru. Am. J. Trop. Med. Hyg. **61:**92-98.

Prescott, J., Ye, C., Sen, G. & Hjelle, B. 2005. Induction of innate immune response genes by Sin Nombre hantavirus does not require viral replication. J. Virol. **79:**15007-15015.

Priddy, F.H., Brown, D., Kublin, J., Monahan, K., Wright, D.P., Lalezari, J., Santiago, S., Marmor, M., Lally, M., Novak, R.M., Brown, S.J., Kulkarni, P., Dubey, S.A., Kierstead, L.S., Casimiro, D.R., Mogg, R., DiNubile, M.J., Shiver, J.W., Leavitt, R.Y., Robertson, M.N., Mehrotra, D.V., Quirk, E. & Merck V520-016 Study Group 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clin. Infect. Dis. **46:**1769-1781.

Raboni, S.M., Probst, C.M., Bordignon, J., Zeferino, A. & dos Santos, C.N. 2005. Hantaviruses in Central South America: phylogenetic analysis of the S segment from HPS cases in Parana, Brazil. J. Med. Virol. **76:**553-562.

Raftery, M.J., Kraus, A.A., Ulrich, R., Kruger, D.H. & Schonrich, G. 2002. Hantavirus infection of dendritic cells. J. Virol. **76:**10724-10733.

Ravkov, E.V., Nichol, S.T. & Compans, R.W. 1997. Polarized entry and release in epithelial cells of Black Creek Canal virus, a New World hantavirus. J. Virol. **71:**1147-1154.

Raymond, T., Gorbunova, E., Gavrilovskaya, I.N. & Mackow, E.R. 2005. Pathogenic hantaviruses bind plexin-semaphorin-integrin domains present at the apex of inactive, bent alphavbeta3 integrin conformers. Proc. Natl. Acad. Sci. U.S.A. **102:**1163-1168.

Reynolds, S., Galanis, E., Krajden, M., Morshed, M., Bowering, D., Abelson, W. & Kollmann, T.R. 2007. Imported fatal hantavirus pulmonary syndrome. Emerg. Infect. Dis. **13:**1424-1425.

Rooney, J., McCombs, K., Pavlin, B. & Sinclair, J. 2004. Two Cases of Hantavirus Pulmonary Syndrome --- Randolph County, West Virginia, July 2004. MMWR. **53:**1086-1089.

Rosa, E.S., Mills, J.N., Padula, P.J., Elkhoury, M.R., Ksiazek, T.G., Mendes, W.S., Santos, E.D., Araujo, G.C., Martinez, V.P., Rosa, J.F., Edelstein, A. & Vasconcelos, P.F. 2005. Newly recognized hantaviruses associated with hantavirus pulmonary syndrome in northern Brazil: partial genetic characterization of viruses and serologic implication of likely reservoirs. Vector Borne Zoonotic Dis. **5:**11-19.

Ruusala, A., Persson, R., Schmaljohn, C.S. & Pettersson, R.F. 1992. Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. Virology. **186:**53-64.

Safronetz, D., Lindsay, R., Dibernardo, A., Hjelle, B., Xiao, R., Artsob, H. & Drebot, M.A. 2005. A preliminary study of the patterns of Sin Nombre viral infection and shedding in naturally infected deer mice (*Peromyscus maniculatus*). Vector Borne Zoonotic Dis. **5:**127-132.

Safronetz, D., Lindsay, R., Hjelle, B., Medina, R.A., Mirowsky-Garcia, K. & Drebot, M.A. 2006. Use of IgG avidity to indirectly monitor epizootic transmission of Sin Nombre virus in deer mice (*Peromyscus maniculatus*). Am. J. Trop. Med. Hyg. **75:**1135-1139.

Safronetz, D., Drebot, M.A., Artsob, H., Cote, T., Makowski, K. & Lindsay, L.R. 2008. Sin Nombre Virus shedding patterns in naturally infected deer mice (*Peromyscus maniculatus*) in relation to duration of infection. Vector Borne Zoonotic Dis. **8:**97-100.

Sanchez, A.J., Abbott, K.D. & Nichol, S.T. 2001. Genetic identification and characterization of limestone canyon virus, a unique *Peromyscus*-borne hantavirus. Virology. **286:**345-353.

Santosuosso, M., McCormick, S. & Xing, Z. 2005. Adenoviral vectors for mucosal vaccination against infectious diseases. Viral Immunol. **18:**283-291.

Sauvage, F., Penalba, C., Vuillaume, P., Boue, F., Coudrier, D., Pontier, D. & Artois, M. 2002. Puumala hantavirus infection in humans and in the reservoir host, Ardennes region, France. Emerg. Infect. Dis. **8:**1509-1511.

Schmaljohn, C.S. & Dalrymple, J.M. 1983. Analysis of Hantaan virus RNA: evidence for a new genus of bunyaviridae. Virology. **131:**482-491.

Schmaljohn, C.S., Hasty, S.E., Dalrymple, J.M., LeDuc, J.W., Lee, H.W., von Bonsdorff, C.H., Brummer-Korvenkontio, M., Vaheri, A., Tsai, T.F. & Regnery, H.L. 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science. **227:**1041-1044.

Schmaljohn, C.S., Schmaljohn, A.L. & Dalrymple, J.M. 1987. Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order. Virology. **157:**31-39.

Schmaljohn, C.S., Chu, Y.K., Schmaljohn, A.L. & Dalrymple, J.M. 1990. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. J. Virol. **64:**3162-3170.

Schmaljohn, C.S., Hasty, S.E. & Dalrymple, J.M. 1992. Preparation of candidate vaccinia-vectored vaccines for haemorrhagic fever with renal syndrome. Vaccine. **10:**10-13.

Schmaljohn, C.S. & Hooper, J.W. 2001, Bunyaviridae: The Viruses and Their Replication in *Fundamental Virology*, eds. D.M. Knipe & P.M. Howley, Fourth edn, Lippincott Williams & Wilkins, Philadelphia, PA, pp. 771-792.

Schountz, T., Prescott, J., Cogswell, A.C., Oko, L., Mirowsky-Garcia, K., Galvez, A.P. & Hjelle, B. 2007. Regulatory T cell-like responses in deer mice persistently infected with Sin Nombre virus. Proc. Natl. Acad. Sci. U.S.A. **104:**15496-15501.

Sen, N., Sen, A. & Mackow, E.R. 2007. Degrons at the C terminus of the pathogenic but not the nonpathogenic hantavirus G1 tail direct proteasomal degradation. J. Virol. **81:**4323-4330.

Shi, X. & Elliott, R.M. 2002. Golgi localization of Hantaan virus glycoproteins requires coexpression of G1 and G2. Virology. **300:**31-38.

Shi, X. & Elliott, R.M. 2004. Analysis of N-linked glycosylation of Hantaan virus glycoproteins and the role of oligosaccharide side chains in protein folding and intracellular trafficking. J. Virol. **78:**5414-5422.

Sidwell, R.W. & Smee, D.F. 2003. Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control. Antiviral Res. **57:**101-111.

Song, G., Huang, Y.C., Hang, C.S., Hao, F.Y., Li, D.X., Zheng, X.L., Liu, W.M., Li, S.L., Huo, Z.W. & Huei, L.J. 1992. Preliminary human trial of inactivated golden hamster kidney cell (GHKC) vaccine against haemorrhagic fever with renal syndrome (HFRS). Vaccine. **10:**214-216.

Song, J.W., Gu, S.H., Bennett, S.N., Arai, S., Puorger, M., Hilbe, M. & Yanagihara, R. 2007a. Seewis virus, a genetically distinct hantavirus in the Eurasian common shrew (*Sorex araneus*). Virol. J. **4:**114.

Song, J.W., Kang, H.J., Song, K.J., Truong, T.T., Bennett, S.N., Arai, S., Truong, N.U. & Yanagihara, R. 2007b. Newfound hantavirus in Chinese mole shrew, Vietnam. Emerg. Infect. Dis. **13:**1784-1787.

Souza, A.P., Haut, L., Reyes-Sandoval, A. & Pinto, A.R. 2005. Recombinant viruses as vaccines against viral diseases. Braz. J. Med. Biol. Res. **38:**509-522.

Spiropoulou, C.F., Morzunov, S., Feldmann, H., Sanchez, A., Peters, C.J. & Nichol, S.T. 1994. Genome structure and variability of a virus causing hantavirus pulmonary syndrome. Virol. **200:**715-723.

Spiropoulou, C.F., Albarino, C.G., Ksiazek, T.G. & Rollin, P.E. 2007. Andes and Prospect Hill hantaviruses differ in early induction of interferon although both can downregulate interferon signaling. J. Virol. **81:**2769-2776.

Sullivan, N.J., Geisbert, T.W., Geisbert, J.B., Shedlock, D.J., Xu, L., Lamoreaux, L., Custers, J.H., Popernack, P.M., Yang, Z.Y., Pau, M.G., Roederer, M., Koup, R.A., Goudsmit, J., Jahrling, P.B. & Nabel, G.J. 2006. Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. PLoS Med. **3:**e177.

Sundstrom, J.B., McMullan, L.K., Spiropoulou, C.F., Hooper, W.C., Ansari, A.A., Peters, C.J. & Rollin, P.E. 2001. Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. J. Virol. **75:**6070-6085.

Suzuki, A., Bisordi, I., Levis, S., Garcia, J., Pereira, L.E., Souza, R.P., Sugahara, T.K., Pini, N., Enria, D. & Souza, L.T. 2004. Identifying rodent hantavirus reservoirs, Brazil. Emerg. Infect. Dis. **10:**2127-2134.

Takada, A., Robison, C., Goto, H., Sanchez, A., Murti, K.G., Whitt, M.A. & Kawaoka, Y. 1997. A system for functional analysis of Ebola virus glycoprotein. Proc. Natl. Acad. Sci. U.S.A. **94:**14764-14769.

Tamura, M., Ogino, S., Matsunaga, T., Asada, H., Kondo, K., Kurata, T. & Yamanishi, K. 1991. Experimental labyrinthitis in guinea pigs caused by a hantavirus. ORL J. Otorhinolaryngol. Relat. Spec. **53:**1-5.

Tatsis, N. & Ertl, H.C. 2004. Adenoviruses as vaccine vectors. Mol. Ther. **10:**616-629.

Temonen, M., Mustonen, J., Helin, H., Pasternack, A., Vaheri, A. & Holthofer, H. 1996. Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. Clin. Immunol. Immunopathol. **78:**47-55.

Terajima, M., Hayasaka, D., Maeda, K. & Ennis, F.A. 2007. Immunopathogenesis of hantavirus pulmonary syndrome and hemorrhagic fever with renal syndrome: Do CD8(+) T cells trigger capillary leakage in viral hemorrhagic fevers? Immunol. Lett. **113:**117-120.

Tischler, N.D., Fernandez, J., Muller, I., Martinez, R., Galeno, H., Villagra, E., Mora, J., Ramirez, E., Rosemblatt, M. & Valenzuela, P.D. 2003. Complete sequence of the genome of the human isolate of Andes virus CHI-7913: comparative sequence and protein structure analysis. Biol. Res. **36:**201-210.

Tischler, N.D., Galeno, H., Rosemblatt, M. & Valenzuela, P.D. 2005a. Human and rodent humoral immune responses to Andes virus structural proteins. Virology. **334:**319-326.

Tischler, N.D., Gonzalez, A., Perez-Acle, T., Rosemblatt, M. & Valenzuela, P.D. 2005b. Hantavirus Gc glycoprotein: evidence for a class II fusion protein. J. Gen. Virol. **86:**2937-2947.

Tischler, N.D., Rosemblatt, M. & Valenzuela, P.D. 2008. Characterization of cross-reactive and serotype-specific epitopes on the nucleocapsid proteins of hantaviruses. Virus Res. **135:**1-9.

Toapanta, F.R., Craigo, J.K., Montelaro, R.C. & Ross, T.M. 2007. Reduction of anti-HIV-1 Gag immune responses during co-immunization: immune interference by the HIV-1 envelope. Curr. HIV. Res. **5:**199-209.

Top, F.H.J., Grossman, R.A., Bartelloni, P.J., Segal, H.E., Dudding, B.H., Russel, P.K. & Buescher, E.L. 1971. Immunization with live types 7 and 4 adenovirus vaccines. I. Safety, infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. J. Infect. Dis. **124:**148-154.

Toro, J., Vega, J.D., Khan, A.S., Mills, J.N., Padula, P., Terry, W., Yadon, Z., Valderrama, R., Ellis, B.A., Pavletic, C., Cerda, R., Zaki, S., Shieh, W.J., Meyer, R., Tapia, M., Mansilla, C., Baro, M., Vergara, J.A., Concha, M., Calderon, G., Enria, D., Peters, C.J. & Ksiazek, T.G. 1998. An outbreak of hantavirus pulmonary syndrome, Chile, 1997. Emerg. Infect. Dis. **4:**687-694.

Tsai, T.F. 1987. Hemorrhagic fever with renal syndrome: mode of transmission to humans. Lab. Anim. Sci. **37:**428-430.

Ulrich, R., Lundkvist, A., Meisel, H., Koletzki, D., Sjolander, K.B., Gelderblom, H.R., Borisova, G., Schnitzler, P., Darai, G. & Kruger, D.H. 1998. Chimaeric HBV core particles carrying a defined segment of Puumala hantavirus nucleocapsid protein evoke protective immunity in an animal model. Vaccine. **16:**272-280.

Ulrich, R., Koletzki, D., Lachmann, S., Lundkvist, A., Zankl, A., Kazaks, A., Kurth, A., Gelderblom, H.R., Borisova, G., Meisel, H. & Kruger, D.H. 1999. New chimaeric hepatitis B virus core particles carrying hantavirus (serotype Puumala) epitopes: immunogenicity and protection against virus challenge. J. Biotechnol. **73:**141-153.

Valdivieso, F., Vial, P., Ferres, M., Ye, C., Goade, D., Cuiza, A. & Hjelle, B. 2006. Neutralizing antibodies in survivors of Sin Nombre and Andes hantavirus infection. Emerg. Infect. Dis. **12:**166-168.

Van Epps, H.L., Schmaljohn, C.S. & Ennis, F.A. 1999. Human memory cytotoxic T-lymphocyte (CTL) responses to Hantaan virus infection: identification of virus-specific and cross-reactive CD8(+) CTL epitopes on nucleocapsid protein. J. Virol. **73:**5301-5308.

Vapalahti, O., Kallio-Kokko, H., Salonen, E.M., Brummer-Korvenkontio, M. & Vaheri, A. 1992. Cloning and sequencing of Puumala virus Sotkamo strain S and M RNA segments: evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. J. Gen. Virol. **73:**829-838.

Vapalahti, O., Lundkvist, A., Kukkonen, S.K., Cheng, Y., Gilljam, M., Kanerva, M., Manni, T., Pejcoch, M., Niemimaa, J., Kaikusalo, A., Henttonen, H., Vaheri, A. & Plyusnin, A. 1996. Isolation and characterization of Tula virus, a distinct serotype in the genus Hantavirus, family Bunyaviridae. J. Gen. Virol. **77:**3063-3067.

Vapalahti, O., Lundkvist, A. & Vaheri, A. 2001. Human immune response, host genetics, and severity of disease. Curr. Top. Microbiol. Immunol. **256:**153-169.

Verity, R., Prasad, E., Grimsrud, K., Artsob, H., Drebot, M., Miedzinski, L. & Preiksaitis, J. 2000. Hantavirus pulmonary syndrome in northern Alberta, Canada: clinical and laboratory findings for 19 cases. Clin. Infect. Dis. **31:**942-946.

Vial, P.A., Valdivieso, F., Mertz, G., Castillo, C., Belmar, E., Delgado, I., Tapia, M. & Ferres, M. 2006. Incubation period of hantavirus cardiopulmonary syndrome. Emerg. Infect. Dis. **12:**1271-1273.

Vincent, M.J., Quiroz, E., Gracia, F., Sanchez, A.J., Ksiazek, T.G., Kitsutani, P.T., Ruedas, L.A., Tinnin, D.S., Caceres, L., Garcia, A., Rollin, P.E., Mills, J.N., Peters, C.J. & Nichol, S.T. 2000. Hantavirus pulmonary syndrome in Panama: identification of novel hantaviruses and their likely reservoirs. Virology. **277:**14-19.

Wahl-Jensen, V., Chapman, J., Asher, L., Fisher, R., Zimmerman, M., Larsen, T. & Hooper, J.W. 2007. Temporal analysis of Andes virus and Sin Nombre virus infections of Syrian hamsters. J. Virol. **81:**7449-7462.

Wang, D., Raja, N.U., Trubey, C.M., Juompan, L.Y., Luo, M., Woraratanadharm, J., Deitz, S.B., Yu, H., Swain, B.M., Moore, K.M., Pratt, W.D., Hart, M.K. & Dong, J.Y. 2006a. Development of a cAdVax-based bivalent Ebola virus vaccine that induces immune responses against both the Sudan and Zaire species of Ebola virus. J. Virol. **80:**2738-2746.

Wang, D., Schmaljohn, A.L., Raja, N.U., Trubey, C.M., Juompan, L.Y., Luo, M., Deitz, S.B., Yu, H., Woraratanadharm, J., Holman, D.H., Moore, K.M., Swain, B.M., Pratt, W.D. & Dong, J.Y. 2006b. De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses. Vaccine. **24:**2975-2986.

Wang, H., Kumiko, Y. & Zhang, Q. 2000. Discovery of a new subtype of SEO virus. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi. **14:**317-321.

Wang, M., Pennock, D.G., Spik, K.W. & Schmaljohn, C.S. 1993. Epitope mapping studies with neutralizing and non-neutralizing monoclonal antibodies to the G1 and G2 envelope glycoproteins of Hantaan virus. Virology. **197:**757-766.

Webster, D., Lee, B., Joffe, A., Sligl, W., Dick, D., Grolla, A., Feldmann, H., Yacoub, W., Grimsrud, K., Safronetz, D. & Lindsay, R. 2007. Cluster of cases of hantavirus pulmonary syndrome in Alberta, Canada. Am. J. Trop. Med. Hyg. **77:**914-918.

Wichmann, D., Grone, H.J., Frese, M., Pavlovic, J., Anheier, B., Haller, O., Klenk, H.D. & Feldmann, H. 2002. Hantaan virus infection causes an acute neurological disease that is fatal in adult laboratory mice. J. Virol. **76:**8890-8899.

Wilson, D.E. & Reeder, D.M. (eds) 1993, *Mammal species of the world: a taxonomic and geographical reference.*, Smithsonian Institution Press, Washington.

Woolhouse, M.E. & Gowtage-Sequeria, S. 2005. Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. **11:**1842-1847.

Xiang, Z.Q., Gao, G.P., Reyes-Sandoval, A., Li, Y., Wilson, J.M. & Ertl, H.C. 2003. Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier. J. Virol. **77:**10780-10789.

Xu, X., Ruo, S.L., McCormick, J.B. & Fisher-Hoch, S.P. 1992. Immunity to Hantavirus challenge in *Meriones unguiculatus* induced by vaccinia-vectored viral proteins. Am. J. Trop. Med. Hyg. **47:**397-404.

Yadav, P.D., Vincent, M.J. & Nichol, S.T. 2007. Thottapalayam virus is genetically distant to the rodent-borne hantaviruses, consistent with its isolation from the Asian house shrew (*Suncus murinus*). Virol. J. **4:**80.

Yamanishi, K., Tanishita, O., Tamura, M., Asada, H., Kondo, K., Takagi, M., Yoshida, I., Konobe, T. & Fukai, K. 1988. Development of inactivated vaccine against virus causing haemorrhagic fever with renal syndrome. Vaccine. **6:**278-282.

Yanagihara, R., Amyx, H.L. & Gajdusek, D.C. 1985. Experimental infection with Puumala virus, the etiologic agent of nephropathia epidemica, in bank voles (*Clethrionomys glareolus*). J. Virol. **55:**34-38.

Yanagihara, R., Amyx, H.L., Lee, P.W., Asher, D.M., Gibbs, C.J.,Jr & Gajdusek, D.C. 1988. Experimental hantavirus infection in nonhuman primates. Arch. Virol. **101:**125-130.

Yashina, L.N., Patrushev, N.A., Ivanov, L.I., Slonova, R.A., Mishin, V.P., Kompanez, G.G., Zdanovskaya, N.I., Kuzina, I.I., Safronov, P.F., Chizhikov, V.E., Schmaljohn, C. & Netesov, S.V. 2000. Genetic diversity of hantaviruses associated with hemorrhagic fever with renal syndrome in the far east of Russia. Virus Res. **70:**31-44.

Yoshimatsu, K., Yoo, Y.C., Yoshida, R., Ishihara, C., Azuma, I. & Arikawa, J. 1993. Protective immunity of Hantaan virus nucleocapsid and envelope protein studied using baculovirus-expressed proteins. Arch. Virol. **130:**365-376.

Young, J.C., Hansen, G.R., Graves, T.K., Deasy, M.P., Humphreys, J.G., Fritz, C.L., Gorham, K.L., Khan, A.S., Ksiazek, T.G., Metzger, K.B. & Peters, C.J. 2000. The incubation period of hantavirus pulmonary syndrome. Am. J. Trop. Med. Hyg. **62:**714-717.

Yuan, Z., Zhang, X., Zhang, S., Liu, Y., Gao, S., Zhang, F., Xu, H., Wang, X. & Hu, R. 2008. Development of recombinant canine adenovirus type-2 expressing the Gn glycoprotein of Seoul virus. Biologicals. **36:**162-167.

Zaki, S.R., Greer, P.W., Coffield, L.M., Goldsmith, C.S., Nolte, K.B., Foucar, K., Feddersen, R.M., Zumwalt, R.E., Miller, G.L. & Khan, A.S. 1995. Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am. J. Pathol. **146:**552-579.

Zhang, Y.Z., Zou, Y., Yan, Y.Z., Hu, G.W., Yao, L.S., Du, Z.S., Jin, L.Z., Liu, Y.Y., Li, M.H., Chen, H.X. & Fu, Z.F. 2007. Detection of phylogenetically distinct Puumala-like viruses from red-grey vole *Clethrionomys rufocanus* in China. J. Med. Virol. **79:**1208-1218.