Ecology of Hantaviruses in Rodent Reservoirs and Their Early Innate Immune Responses in Human Model Systems

Evan Peter Williams
University of Tennessee Health Science Center

Follow this and additional works at: https://dc.uthsc.edu/dissertations

Part of the Animal Diseases Commons, Disease Modeling Commons, Environmental Public Health Commons, Epidemiology Commons, Investigative Techniques Commons, Medical Immunology Commons, and the Virus Diseases Commons

Recommended Citation
Ecology of Hantaviruses in Rodent Reservoirs and Their Early Innate Immune Responses in Human Model Systems

Abstract
The spillover of zoonotic RNA viruses is responsible for a great deal of the disease outbreaks in human populations. These spillover events are set to continue due to anthropogenic and environmental changes that impact the distribution of these viruses. The viruses in the family Hantaviridae are classified as one of these emerging zoonotic RNA viruses. The spillover of the viruses in this family are responsible for two severe human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). These viruses are distributed across the globe and are responsible for a large number of human disease cases with potentially high mortality rates each year. Unfortunately, there is a lack of surveillance efforts to identify hantaviruses in most countries making accurate diagnosis or recognition of hantavirus cases complicated. To address the potential public health impact of hantaviruses, we surveyed rodents in a rural region of Ukraine, and identified a high prevalence which underscores the potential for human disease in this country. As it is challenging to address how hantaviruses infect humans, I established approaches to evaluate the early innate immune response in primary lung microvascular endothelial cells (HLMVECs) with pathogenic and nonpathogenic hantaviruses. Surprisingly, my findings challenged some of the current dogma in that there were not dramatic difference between pathogenic and nonpathogenic viruses. This work highlights the critical need for advancement of cell culture models to probe the immune response.

To understand the ecology of hantaviruses in their reservoirs their prevalence was studied in northwestern Ukraine. A field capture study was conducted at two sites which each had distinct habitats and contained nine capture lines. During this survey, we captured 424 small mammals, consisting of species across three orders. The most abundant species were Myodes glareolus, the bank vole (45%); Apodemus flavicollis, the yellow-necked mouse (29%); and Apodemus agrarius, the striped field mouse (14.6%). Out of the collection, it was determined that 79 animals were seropositive by immunofluorescent assay (IFA), from which 15.7% were M. glareolus, 20.5% A. flavicollis, and 33.9% A. agrarius. These finding were of interest as M. glareolus and Apodemus spp. harbor Puumala orthohantavirus and Dobrava-Belgrade orthohantavirus viruses, respectively, which are responsible for causing HFRS in humans. IFA reciprocal titer showed a wide distribution indicating new infections are occurring. No relationship was found between species diversity and the proportion of hantavirus seropositive animals captured at these sites. Population analysis on M. glareolus and Apodemus spp. revealed that neither sex nor age was associated with being seropositive.

To define the early innate immune responses during human infection by hantaviruses, this research studies the responses in HLMVECs, the primary cells of infection in humans, infected by the pathogenic viruses, Andes orthohantavirus (ANDV) and Hantaan orthohantavirus (HTNV), and the nonpathogenic virus, Prospect Hill orthohantavirus (PHV). A curated list of 39 host genes were studied across multiple time points during the first 72 hours of infection of HLMVECs from a male donor by these three viruses. mRNA level analysis revealed the mRNA levels of only CCL5, CXCL10, CXCL11, IDO1, IFNB1, IRF7, and TLR3 we increased during infection of each viruses. The measurement of CCL5, CXCL10, CXCL11, IDO, and IFN-β secreted protein levels in the same HLMVEC donor during infection confirmed gene expression findings. The study of host immune responses to hantavirus infection was expanded to include HLMVECs from an additional male and two female donors. Measurement of secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β by each of the four donors revealed that levels of these proteins are upregulated during infection by each of the viruses. Pair wise analysis on these secreted protein levels by each of the donors during hantavirus infection suggests that donor characteristics and virus species together drive different outcomes. However, female donors had higher levels of CXCL10, IDO, and IFN-β
and these increased protein levels were species specific. Lastly, the suppression of immune response involved in cell death were examined and it was found that ANDV is capable of inhibiting cell death in HLMVECs.

In summary, the findings presented, show the critical need to understand and define the early innate immune responses to hantaviral infection in human models as well as the necessity of understanding the ecology of hantaviruses in their reservoir hosts.

**Document Type**
Dissertation

**Degree Name**
Doctor of Philosophy (PhD)

**Program**
Biomedical Sciences

**Research Advisor**
Colleen B. Jonsson, PhD

**Subject Categories**
Analytical, Diagnostic and Therapeutic Techniques and Equipment | Animal Diseases | Disease Modeling | Diseases | Environmental Public Health | Epidemiology | Investigative Techniques | Medical Immunology | Medical Sciences | Medicine and Health Sciences | Public Health | Virus Diseases

This dissertation is available at UTHSC Digital Commons: [https://dc.uthsc.edu/dissertations/572](https://dc.uthsc.edu/dissertations/572)
Ecology of Hantaviruses in Rodent Reservoirs and Their Early Innate Immune Responses in Human Model Systems.

Author: Evan Peter Williams

Advisor: Colleen B. Jonsson, PhD

A Dissertation Presented for The Graduate Studies Council of The University of Tennessee Health Science Center in Partial Fulfillment of the Requirements for the Doctor of Philosophy degree from The University of Tennessee in Biomedical Sciences: Microbiology, Immunology, and Biochemistry College of Graduate Health Sciences

December 2021
Copyright © 2021 by Evan Peter Williams.
All rights reserved.
DEDICATION

To my parents Piet and Amanda Williams—
for their unconditional love, inspiration, support,
and for instilling the importance of education in me.
ACKNOWLEDGMENTS

I sincerely thank my mentor Dr. Colleen Beth Jonsson for guiding me through my time as a graduate student. Dr Jonsson allowed me to grow as a scientist and provided me countless opportunities to conduct research of interest to me, may it be in the laboratory, the jungles of Paraguay, or the woods of Ukraine.

I would like to thank my committee members, Dr. Michele M. Kosiewicz, Dr. Kui Li, Dr. Elizabeth A. Fitzpatrick, and especially Dr. Amber M. Smith. I appreciate your guidance, time and effort you have bestowed upon me as you served as my committee members.

I thank the past and present members of the Jonsson laboratory, including, Dr. Mariah Taylor, Dr. Jasper Lee, Dr. Leonardo Valdivieso-Torres, Dr. Yi Xue, Briana Spruill-Harrell, Jacob Nichols and Walter Reichard for their companionship, friendship and support throughout the years in the Jonsson laboratory.

Lastly, I wish to thank my family for their endless love and support as I ventured across the Atlantic Ocean in the pursuit of my dreams.

This work was funded by the National Research Foundation 1517719 and United Stated Defense Threat Reduction Agency.
ABSTRACT

The spillover of zoonotic RNA viruses is responsible for a great deal of the disease outbreaks in human populations. These spillover events are set to continue due to anthropogenic and environmental changes that impact the distribution of these viruses. The viruses in the family Hantaviridae are classified as one of these emerging zoonotic RNA viruses. The spillover of the viruses in this family are responsible for two severe human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). These viruses are distributed across the globe and are responsible for a large number of human disease cases with potentially high mortality rates each year. Unfortunately, there is a lack of surveillance efforts to identify hantaviruses in most countries making accurate diagnosis or recognition of hantavirus cases complicated. To address the potential public health impact of hantaviruses, we surveyed rodents in a rural region of Ukraine, and identified a high prevalence which underscores the potential for human disease in this country. As it is challenging to address how hantaviruses infect humans, I established approaches to evaluate the early innate immune response in primary lung microvascular endothelial cells (HLMVECs) with pathogenic and nonpathogenic hantaviruses. Surprisingly, my findings challenged some of the current dogma in that there were not dramatic difference between pathogenic and nonpathogenic viruses. This work highlights the critical need for advancement of cell culture models to probe the immune response.

To understand the ecology of hantaviruses in their reservoirs their prevalence was studied in northwestern Ukraine. A field capture study was conducted at two sites which each had distinct habitats and contained nine capture lines. During this survey, we captured 424 small mammals, consisting of species across three orders. The most abundant species were Myodes glareolus, the bank vole (45%); Apodemus flavicollis, the yellow-necked mouse (29%); and Apodemus agrarius, the striped field mouse (14.6%). Out of the collection, it was determined that 79 animals were seropositive by immunofluorescent assay (IFA), from which 15.7% were M. glareolus, 20.5% A. flavicollis, and 33.9% A. agrarius. These finding were of interest as M. glareolus and Apodemus spp. harbor Puumala orthohantavirus and Dobrava-Belgrade orthohantavirus viruses, respectively, which are responsible for causing HFRS in humans. IFA reciprocal titer showed a wide distribution indicating new infections are occurring. No relationship was found between species diversity and the proportion of hantavirus seropositive animals captured at these sites. Population analysis on M. glareolus and Apodemus spp. revealed that neither sex nor age was associated with being seropositive.

To define the early innate immune responses during human infection by hantaviruses, this research studies the responses in HLMVECs, the primary cells of infection in humans, infected by the pathogenic viruses, Andes orthohantavirus (ANDV) and Hantaan orthohantavirus (HTNV), and the nonpathogenic virus, Prospect Hill orthohantavirus (PHV). A curated list of 39 host genes were studied across multiple time points during the first 72 hours of infection of HLMVECs from a male donor by these three viruses. mRNA level analysis revealed the mRNA levels of only CCL5, CXCL10,
CXCL11, IDO1, IFNB1, IRF7, and TLR3 we increased during infection of each viruses. The measurement of CCL5, CXCL10, CXCL11, IDO, and IFN-β secreted protein levels in the same HLMVEC donor during infection confirmed gene expression findings. The study of host immune responses to hantavirus infection was expanded to include HLMVECs from an additional male and two female donors. Measurement of secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β by each of the four donors revealed that levels of these proteins are upregulated during infection by each of the viruses. Pair wise analysis on these secreted protein levels by each of the donors during hantavirus infection suggests that donor characteristics and virus species together drive different outcomes. However, female donors had higher levels of CXCL10, IDO, and IFN-β and these increased protein levels were species specific. Lastly, the suppression of immune response involved in cell death were examined and it was found that ANDV is capable of inhibiting cell death in HLMVECs.

In summary, the findings presented, show the critical need to understand and define the early innate immune responses to hantaviral infection in human models as well as the necessity of understanding the ecology of hantaviruses in their reservoir hosts.
# TABLE OF CONTENTS

## CHAPTER 1. HANTAVIRUSES AND THEIR INTERACTIONS WITH HOSTS DURING INFECTION ................................................................. 1

- Taxonomic and Disease Classifications ................................................................. 1
- Reservoir Hosts of Hantaviruses .............................................................................. 1
- Genomes, Genes, Proteins, and Morphology of Hantaviruses ................................. 4
- Replication Cycle of Hantaviruses ........................................................................... 6
- Human Host Responses to Infection by Hantaviruses ............................................. 10
- Regulation of Human Host Responses by Hantaviruses .......................................... 13

## CHAPTER 2. COMMON THEMES IN ZOONOTIC SPILLOVER AND DISEASE EMERGENCE: LESSONS LEARNED FROM BAT- AND RODENT-BORNE RNA VIRUSES* .......................................................... 16

- Introduction ........................................................................................................... 16
  - What Biological Strategies Have Evolved That Allow RNA Viruses to Reside in Bats and Rodents? ................................................................. 17
  - Pathogenesis of the Viral Infection .................................................................. 20
  - The Host Immune Response ............................................................................ 22
  - Behavioral and Demographic Characteristics .................................................. 25
  - The Inherent Genetic Plasticity of RNA Viruses ............................................... 27
  - A Look at Environmental Factors That Drive Spillover of Viruses in Bat and Rodent Populations ........................................................................... 28
  - How Do Viruses Spillover from Bats or Rodents to Humans? ......................... 29
    - Spillover Associated with Direct Animal Reservoir Contact ....................... 31
    - Spillover Associated with Direct Contact: Intermediate Hosts .................... 32
    - Spillover Associated with Indirect Animal Reservoir Contact ..................... 33
  - Conclusions ........................................................................................................... 36

## CHAPTER 3. PREVALENCE OF HANTAVIRUSES HARBORRED BY MURID RODENTS IN NORTHWESTERN UKRAINE* .......................................................... 39

- Introduction ........................................................................................................... 39
- Materials and Methods .......................................................................................... 41
  - Small Mammal Collection ............................................................................... 41
  - Immunofluorescence Assay (IFA) ...................................................................... 41
  - Statistical Analyses ............................................................................................ 42
  - Ethics Statements ............................................................................................... 42
- Results ...................................................................................................................... 42
  - Distribution of Small Mammal Species .............................................................. 42
  - Evidence and Distribution of Antibodies to Hantavirus by Line ....................... 44
  - Rodent Diversity Within Each Line .................................................................... 44
  - Association of Population Structure and Prevalence of Antibody to Hantavirus in *Myodes glareolus* and *Apodemus* Species ............................................ 44
  - Discussion ............................................................................................................. 49
LIST OF TABLES

Table 1-1. Species of Mammantavirinae and their natural reservoirs .......................2

Table 2-1. Examples of outbreaks in human populations by RNA viruses harbored by rodents .................................................................18

Table 2-2. Examples of outbreaks in human populations by RNA viruses harbored by bats ...........................................................................19

Table 3-1. Distribution of captured small mammal species by line .........................43

Table 3-2. Distribution of antibody-positive small mammal species by line ............45

Table 3-3. Distribution of IFA reciprocal titers in rodent reservoir species of PUUV and DOBV .......................................................................46

Table 3-4. Overall Hantaviral Ab status and Shannon diversity in captured rodents by line ............................................................................47

Table 3-5. A. agrarius, A. flavicollis, and M. glareolus captured according to age, sex, and Ab prevalence .................................................................48

Table 4-1. Characteristics of HLMVEC donors .....................................................54
# LIST OF FIGURES

| Figure 1-1. | Trisegmented genomes of hantaviruses | 5 |
| Figure 1-2. | Virion structure of hantaviruses | 5 |
| Figure 1-3. | Polymorphic virion ultrastructures of Old and New World | 7 |
| Figure 1-4. | Replication cycle of hantaviruses | 8 |
| Figure 1-5. | Infection routes of hantaviruses | 11 |
| Figure 1-6. | Multiple mechanisms utilized by hantaviruses to suppress early innate immune responses | 14 |
| Figure 2-1. | Major routes of spillover transmission of viruses harbored by bats and rodents | 30 |
| Figure 3-1. | Survey location in northwestern Ukraine | 40 |
| Figure 3-2. | Distribution of captured small mammal species by line | 43 |
| Figure 3-3. | Distribution of hantavirus seropositive small mammal species | 45 |
| Figure 3-4. | Distribution of IFA reciprocal titers in rodent reservoir species of PUUV and DOBV | 46 |
| Figure 3-5. | Relationship between proportion of seropositive animals at each capture line and Shannon Index | 47 |
| Figure 3-6. | Distribution of male and female hantavirus antibody positive small mammals | 48 |
| Figure 3-7. | Weight range of rodents and percent hantavirus for A. agrarius, A. flavicollis and M. glareolus | 50 |
| Figure 4-1. | HLMVECs infected by ANDV, HTNV, or PHV at 24 hpi and 72 hpi | 58 |
| Figure 4-2. | Selected PRR, cytokines and chemokines showing upregulation at 12, 24, 36, 48, 60, and 72 hpi in HLMVECs following infection with ANDV, HTNV, or PHV | 59 |
| Figure 4-3. | Principle component analysis of normalized mRNA levels of 39 genes in HLMVECs at 12, 24, 36, 48, 60, and 72 hpi following infection with ANDV, HTNV, or PHV | 60 |
Figure 4-4. Secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β at 48 hpi and 60 hpi by four HLMVEC donors following infection with ANDV, HTNV, or PHV .................................................................63

Figure 4-5. Principle component analysis of the protein levels of CCL5, CXCL10, CXCL11, IDO, and IFNB-β secreted at 48 and 60 hpi by four HLMVEC donors following infection with ANDV, HTNV, or PHV ...........64

Figure 4-6. Secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β at 48 hpi and 60 hpi by male and female HLMVECs donors following infection with ANDV, HTNV, or PHV .................................................................65

Figure 4-7. The inhibition of cell death of ANDV-infected HLMVECs following staurosporine treatment .................................................................66
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACUP</td>
<td>Animal Care and Use Procedure</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin Converting Enzyme 2</td>
</tr>
<tr>
<td>AHF</td>
<td>Argentine Hemorrhagic Fever</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ANDV</td>
<td><em>Andes Orthohantavirus</em></td>
</tr>
<tr>
<td>BCCV</td>
<td><em>Black Creek Canal Orthohantavirus</em></td>
</tr>
<tr>
<td>BHF</td>
<td>Bolivian Hemorrhagic Fever</td>
</tr>
<tr>
<td>BSL</td>
<td>Biosafety Level</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C Motif Chemokine Ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>COVID-19</td>
<td>Coronavirus Disease 2019</td>
</tr>
<tr>
<td>CFR</td>
<td>Case Fatality Rate</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CTL-associated antigen 4</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C Motif Chemokine</td>
</tr>
<tr>
<td>DDT</td>
<td>dichloro-diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DOBV</td>
<td><em>Dobrava orthohantavirus</em></td>
</tr>
<tr>
<td>DPI</td>
<td>Days Post-Infection</td>
</tr>
<tr>
<td>EBOV</td>
<td><em>Ebola virus</em></td>
</tr>
<tr>
<td>ENSO</td>
<td>El Niño-Southern Oscillation</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GnGc</td>
<td>Envelope Glycoproteins</td>
</tr>
<tr>
<td>HCV</td>
<td><em>Hepatitis C Virus</em></td>
</tr>
<tr>
<td>HCPS</td>
<td>Hantavirus Cardiopulmonary Syndrome</td>
</tr>
<tr>
<td>HeV</td>
<td><em>Hendra henipavirus</em></td>
</tr>
<tr>
<td>HFRS</td>
<td>Hemorrhagic Fever with Renal Syndrome</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HLMVEC</td>
<td>Human Lung Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours Post-Infection</td>
</tr>
<tr>
<td>HPS</td>
<td>Hantavirus Pulmonary Syndrome</td>
</tr>
<tr>
<td>HTNV</td>
<td><em>Hantaan orthohantavirus</em></td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial</td>
</tr>
<tr>
<td>HV</td>
<td>Hantavirus</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care &amp; Use Committee</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon-Alpha/Beta Receptor</td>
</tr>
<tr>
<td>IKKε</td>
<td>Inhibitor-κB kinase ε</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon Stimulating Gene</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-Dioxygenase</td>
</tr>
<tr>
<td>IFNB1</td>
<td>Interferon Beta 1</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JUNV</td>
<td>Argentinian mammarenavirus</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte Activation Gene-3</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LMVEC</td>
<td>Lung Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>LASV</td>
<td>Lassa mammarenavirus</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial Antiviral-Signaling Protein</td>
</tr>
<tr>
<td>MACV</td>
<td>Machupo mammarenavirus</td>
</tr>
<tr>
<td>MAPV</td>
<td>Maporel orthohantavirus</td>
</tr>
<tr>
<td>MARV</td>
<td>Marburg marburgvirus</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>Middle East Respiratory Syndrome-Coronavirus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histone Complex</td>
</tr>
<tr>
<td>MYA</td>
<td>Million Years Ago</td>
</tr>
<tr>
<td>Mx</td>
<td>Myxovirus Resistance</td>
</tr>
<tr>
<td>NE</td>
<td>Nephropathia Epidemica</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>NiV</td>
<td>Nipah henipavirus</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like Receptor</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleocapsid Protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCDH1</td>
<td>Protocadherin-1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Death-1</td>
</tr>
<tr>
<td>PHV</td>
<td>Prospect Hill Virus</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RER</td>
<td>Rough Endoplasmic Reticulum</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PUUV</td>
<td>Puumala Orthohantavirus</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-Dependent RNA Polymerase</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic Acid Inducible Gene I</td>
</tr>
<tr>
<td>RVFV</td>
<td>Rift Valley Fever Phlebovirus</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe Acute Respiratory Syndrome-Coronavirus</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>Severe Acute Respiratory Syndrome-Coronavirus-2</td>
</tr>
<tr>
<td>SEOV</td>
<td>Seoul Orthohantavirus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SNV</td>
<td>Sin Nombre Orthohantavirus</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transduction and Activators of Transcription Factors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SUDV</td>
<td><em>Sudan ebolavirus</em></td>
</tr>
<tr>
<td>SWSV</td>
<td><em>Seewis orthohantavirus</em></td>
</tr>
<tr>
<td>TBK</td>
<td>TANK-Binding Kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TCRV</td>
<td><em>Tacaribe mammarenavirus</em></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor-Associated Factor</td>
</tr>
<tr>
<td>TOX</td>
<td>Thymocyte Selection Associated High Mobility Group Box</td>
</tr>
<tr>
<td>TULV</td>
<td><em>Tula Orthohantavirus</em></td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Regions</td>
</tr>
<tr>
<td>vRNA</td>
<td>Virus Ribonucleic Acid</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular Endothelial Growth Factor A</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZEBOV</td>
<td><em>Zaire ebolavirus</em></td>
</tr>
</tbody>
</table>
CHAPTER 1. HANTAVIRUSES AND THEIR INTERACTIONS WITH HOSTS DURING INFECTION

Taxonomic and Disease Classifications

Hantaviruses are group V negative-sense, tri-segmented, single-stranded RNA viruses. As of 2021 there are 38 species in the genus Orthohantavirus, family Hantaviridae, order Bunyavirales, which are classified in the subfamily Mammantavirinae, based on the taxonomic order of their reservoir host (Schmaljohn and Dalrymple, 1983; Maes et al., 2018; Walker et al., 2020). Hantavirus species that circulate in mammals in Africa, Asia and Europe are classified as Old World hantaviruses, whereas species circulating in the America’s are referred to as New World hantaviruses. However, there are Old World hantaviruses such as Seoul Orthohantavirus (SEOV) present in the New World. Transmission of hantaviruses from reservoir hosts to humans occur through the inhalation of rodent excreta from infected individuals. Human infection by some hantavirus circulating in the Old World cause hemorrhagic fever with renal syndrome (HFRS) or nephropathic epidemica (NE) a milder form of HFRS (Lee et al., 1978), whereas some of the New World hantaviruses cause hantavirus pulmonary syndrome (HPS) (Nichol et al., 1993).

Reservoir Hosts of Hantaviruses

The viruses within the family Hantaviridae are harbored by a number of different reservoir host, these include fish, reptiles and small mammals which include bats, moles, shrews, and rodents. Viruses in the genus Orthohantavirus are harbored by mice, moles, rats, bats, and shrews (Table 1-1) (Jonsson et al., 2010; Schountz and Prescott, 2014; Reuter and Krüger, 2018). Within the rodent family Cricetidae, hantaviruses are harbored by Old World rodents in the subfamilies Arvicolinae and Murinae as well as the New World rodents in the subfamilies Neotominae and Sigmodontinae. Hantaviruses are also harbored by the small mammals in the order Eulipotyphla which include two families of moles and shrews. Each species and in some cases lineages of hantavirus has a unique small mammal species that serves as its reservoir host (Hughes and Friedman, 2000; Plyusnin and Sironen, 2014). Phylogenetic analysis of hantaviruses and their host suggest a long history of co-evolution that has occurred between viruses and their host (Bennett et al., 2014).

Hantaviruses persistently infect their reservoir with a limited immune response and no pathology, but infections are not without consequences as fecundity is decreased in female rodents (Easterbrook and Klein, 2008b; Schountz et al., 2012; Kallio et al., 2015). Spillover infections are acute and can potentially cause mild pathology other rodents (Botten et al., 2000; Spengler et al., 2013; McGuire et al., 2016). Interestingly, it is only hantavirus species harbored by rodents that infect humans with a disease outcome.
Table 1-1. Species of *Mammantavirinae* and their natural reservoirs

<table>
<thead>
<tr>
<th>Virus Species</th>
<th>Reservoir Host Species</th>
<th>Reservoir Host (Common Name)</th>
<th>Geographical Distribution</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Loanvirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brno loanvirus</td>
<td>Nyctalus noctula</td>
<td>European noctules</td>
<td>Europe</td>
<td>(Straková et al., 2017)</td>
</tr>
<tr>
<td>Longquan loanvirus</td>
<td>Rhinolophus spp.</td>
<td>Horseshoe bats</td>
<td>Asia</td>
<td>(Guo et al., 2013)</td>
</tr>
<tr>
<td><strong>Mobatvirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laibin mobatvirus</td>
<td>Taphozous melanopogon</td>
<td>Black-bearded tomb bats</td>
<td>China</td>
<td>(Xu et al., 2015)</td>
</tr>
<tr>
<td>Lena mobatvirus</td>
<td>Sorex caecatus</td>
<td>Laxmann's shrew</td>
<td>Europe</td>
<td>(Yashina et al., 2019)</td>
</tr>
<tr>
<td>Nova mobatvirus</td>
<td>Talpa europaea</td>
<td>European moles</td>
<td>Europe</td>
<td>(Kang et al., 2009b)</td>
</tr>
<tr>
<td>Quezon mobatvirus</td>
<td>Rousettus amplexicaudatus</td>
<td>Geoffroy’s rousette</td>
<td>Asia</td>
<td>(Arat et al., 2016)</td>
</tr>
<tr>
<td>Xuan Son mobatvirus</td>
<td>Hipposideros Pomona</td>
<td>Pomona leaf-nosed bat</td>
<td>Asia</td>
<td>(Arat et al., 2013)</td>
</tr>
<tr>
<td><strong>Orthohantavirus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Andes orthohantavirus</td>
<td>Oligoryzomys longicaudatus</td>
<td>Long-tailed pygmy rice rat</td>
<td>South America</td>
<td>(López et al., 1996)</td>
</tr>
<tr>
<td>Asama orthohantavirus</td>
<td>Urotrichus talpoides</td>
<td>Japanese shrewy mouse</td>
<td>Asia</td>
<td>(Arat et al., 2008)</td>
</tr>
<tr>
<td>Asikala orthohantavirus</td>
<td>Sorex araneus</td>
<td>Pygmy shrew</td>
<td>Europe</td>
<td>(Radosa et al., 2013)</td>
</tr>
<tr>
<td>Bayou orthohantavirus</td>
<td>Orzyromys palustris</td>
<td>Marsh rice rat</td>
<td>North America</td>
<td>(Morzuov et al., 1995)</td>
</tr>
<tr>
<td>Black Creek Canal orthohantavirus</td>
<td>Sigmodon hispidus</td>
<td>Hispid cotton rat</td>
<td>North America</td>
<td>(Rollin et al., 1995)</td>
</tr>
<tr>
<td>Bowe orthohantavirus</td>
<td>Crocidura douceti</td>
<td>Doucet’s musk shrew</td>
<td>Africa</td>
<td>(Gu et al., 2013)</td>
</tr>
<tr>
<td>Brages orthohantavirus</td>
<td>Talpa europaea</td>
<td>European moles</td>
<td>Europe</td>
<td>(Laenen et al., 2018)</td>
</tr>
<tr>
<td>Cano Delgado orthohantavirus</td>
<td>Sigmodon alstoni</td>
<td>Alston’s cotton rat</td>
<td>South America</td>
<td>(Fulhorst et al., 1997b)</td>
</tr>
<tr>
<td>Cao Bang orthohantavirus</td>
<td>Anuurosorex squamipes</td>
<td>Chinese mole shrew</td>
<td>Asia</td>
<td>(Song et al., 2007b)</td>
</tr>
<tr>
<td>Choco orthohantavirus</td>
<td>Zygodontomys brevicauda</td>
<td>Common cane mouse</td>
<td>South America</td>
<td>(Vincent et al., 2000)</td>
</tr>
<tr>
<td>Dabieshan orthohantavirus</td>
<td>Niviventer confucianus</td>
<td>Chinese white-bellied rat</td>
<td>Asia</td>
<td>(Wang et al., 2000a)</td>
</tr>
<tr>
<td>Dobrava-Belgrade orthohantavirus</td>
<td>Apodemus flavicollis</td>
<td>Yellow-necked mouse</td>
<td>Europe</td>
<td>(Avsic-Zupanc et al., 1992)</td>
</tr>
<tr>
<td>El Moro Canyon orthohantavirus</td>
<td>Reithrodontomys megalotis</td>
<td>Western harvest mouse</td>
<td>North America</td>
<td>(Torrez-Martinez et al., 1995)</td>
</tr>
<tr>
<td>Fugong orthohantavirus</td>
<td>Eothenomys eleusis</td>
<td>Small oriental vole</td>
<td>Asia</td>
<td>(Ge et al., 2016)</td>
</tr>
<tr>
<td>Fusong orthohantavirus</td>
<td>Myodes rafocanus</td>
<td>Grey red-backed vole</td>
<td>Asia</td>
<td>(Zou et al., 2008)</td>
</tr>
<tr>
<td>Hantau orthohantavirus</td>
<td>Apodemus agrarius</td>
<td>Stripped field mouse</td>
<td>Eurasia</td>
<td>(Lee et al., 1978)</td>
</tr>
<tr>
<td>Jeju orthohantavirus</td>
<td>Crocidura shantungensis</td>
<td>Asian lesser white-toothed</td>
<td>Asia</td>
<td>(Arat et al., 2012)</td>
</tr>
<tr>
<td>Kenkeme orthohantavirus</td>
<td>Sorex roboratus</td>
<td>Flat-skulled shrew</td>
<td>Asia</td>
<td>(Kang et al., 2010)</td>
</tr>
<tr>
<td>Khabarovsky orthohantavirus</td>
<td>Microtus fortis</td>
<td>Reed vole</td>
<td>Asia</td>
<td>(Hörining et al., 1996)</td>
</tr>
<tr>
<td>Laguna Negra orthohantavirus</td>
<td>Calomys spp.</td>
<td>vespers mice</td>
<td>South America</td>
<td>(Johnson et al., 1997)</td>
</tr>
<tr>
<td>Luxi orthohantavirus</td>
<td>Eothenomys miletus</td>
<td>Yunnan red-backed vole</td>
<td>Asia</td>
<td>(Zhang et al., 2011)</td>
</tr>
<tr>
<td>Maporal orthohantavirus</td>
<td>Sigmodon alstoni</td>
<td>Alston’s cotton rat</td>
<td>South America</td>
<td>(Fulhorst et al., 2004)</td>
</tr>
<tr>
<td>Montano orthohantavirus</td>
<td>Peromyscus aztecus</td>
<td>Aztec mouse</td>
<td>North America</td>
<td>(Kariwa et al., 2012)</td>
</tr>
<tr>
<td>Necoči orthohantavirus</td>
<td>Zygodontomys brevicauda</td>
<td>short-tailed cane mouse</td>
<td>South America</td>
<td>(Londoño et al., 2011)</td>
</tr>
<tr>
<td>Virus Species</td>
<td>Reservoir Host Species</td>
<td>Reservoir Host (Common Name)</td>
<td>Geographical Distribution</td>
<td>Citations</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------</td>
<td>------------------------------</td>
<td>---------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Oxbow orthohantavirus</td>
<td>Neurotrichus gibbsii</td>
<td>American shrew mole</td>
<td>North America</td>
<td>(Kang et al., 2009a)</td>
</tr>
<tr>
<td>Prospect Hill orthohantavirus</td>
<td>Microtus pennsylvanicus</td>
<td>Meadow vole</td>
<td>North America</td>
<td>(Lee et al., 1982b)</td>
</tr>
<tr>
<td>Puumala orthohantavirus</td>
<td>Myodes glareolus</td>
<td>Bank vole</td>
<td>Europe</td>
<td>(Brummer-Korvenkontio et al., 1980)</td>
</tr>
<tr>
<td>Robina orthohantavirus</td>
<td>Pteropus alecto</td>
<td>Black flying fox</td>
<td>Australia</td>
<td></td>
</tr>
<tr>
<td>Rockport orthohantavirus</td>
<td>Scalopus aquaticus</td>
<td>Eastern mole</td>
<td>North America</td>
<td>(Kang et al., 2011)</td>
</tr>
<tr>
<td>Sangassou orthohantavirus</td>
<td>Hylomyscus alleni</td>
<td>Allen's wood mouse</td>
<td>Africa</td>
<td>(Klempp et al., 2006)</td>
</tr>
<tr>
<td>Seewis orthohantavirus</td>
<td>Sorex daphaenodon</td>
<td>Siberian large-toothed shrew</td>
<td>Eurasia</td>
<td>(Song et al., 2007a)</td>
</tr>
<tr>
<td>Seoul orthohantavirus</td>
<td>Rattus norvegicus</td>
<td>Brown rat</td>
<td>Eurasia</td>
<td>(Lee et al., 1982a)</td>
</tr>
<tr>
<td>Sin Nombre orthohantavirus</td>
<td>Peromyscus maniculatus</td>
<td>Deer mouse</td>
<td>North America</td>
<td>(Nichol et al., 1993)</td>
</tr>
<tr>
<td>Tatenale orthohantavirus</td>
<td>Microtus agrestis</td>
<td>Field vole</td>
<td>Europe</td>
<td>(Pounder et al., 2013)</td>
</tr>
<tr>
<td>Thailand orthohantavirus</td>
<td>Bandicota indica</td>
<td>Greater bandicoot rat</td>
<td>Asia</td>
<td>(Elwell et al., 1985)</td>
</tr>
<tr>
<td>Tigray orthohantavirus</td>
<td>Stenocaphelemys alipes</td>
<td>Ethiopian white-footed mouse</td>
<td>Africa</td>
<td>(Meheretu et al., 2012)</td>
</tr>
<tr>
<td>Tula orthohantavirus</td>
<td>Microtus arvalis</td>
<td>Common vole</td>
<td>Europe</td>
<td>(Plyusnin et al., 1994)</td>
</tr>
<tr>
<td>Yakeshi orthohantavirus</td>
<td>Sorex isodon</td>
<td>Taiga shrew</td>
<td>Asia</td>
<td>(Guo et al., 2013)</td>
</tr>
<tr>
<td>Thottimivirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imjin thottimivirus</td>
<td>Crocidura lasiura</td>
<td>Ussuri white-toothed shrew</td>
<td>Asia</td>
<td>(Song et al., 2009a)</td>
</tr>
<tr>
<td>Thottopalayam thottimivirus</td>
<td>Suncus murinus</td>
<td>Asian house shrew</td>
<td>Asia</td>
<td>(Carey et al., 1971)</td>
</tr>
</tbody>
</table>
Genomes, Genes, Proteins, and Morphology of Hantaviruses

The tri-segmented genomes of hantaviruses are classified based on their differences in length; Large (L), Medium (M), and Small (S) segment (Jonsson and Schmaljohn, 2001) (Figure 1-1).

The L-segment is approximately 6500-6590 nt in length and encodes for the 250 kDa RNA-dependent RNA polymerase (RdRp) (Schmaljohn, 1990). The polymerase exhibits replicase, transcriptase and endonuclease activity but lacks any exonuclease activity. The polymerase has an error rate of 1x10^-3 - 1x10^-4 substitutions/nucleotide site/year which is comparable to that of other group IV and V viruses (Jenkins et al., 2002; Chung et al., 2007; Ramsden et al., 2008). Along with this RdRp shares other hallmark characteristics observed in that of group V viruses such as the polymerase domain as well as the six motifs, Pre-A, A, B, C, D, and E (Kukkonen et al., 2005). The RdRp may form a ribonucleoprotein (RNP) complex with the nucleocapsid protein (NP), through an interaction by RdRp’s C terminus and the NP’s N terminus (Jonsson and Schmaljohn, 2001).

The M-segment is approximately 3570-3710 nt in length and encodes for the two glycoproteins, Gc and Gn, that mediate binding, entry and fusion of virions to host cells and dictate tissue tropism (Schmaljohn et al., 1987). The Gc is classified as class II membrane fusion protein (Tischler et al., 2005), where Gn mediates receptor binding and the fusion peptide in Gc enables fusion. GcGc are coexpressed as a precursor protein that is cleaved into Gn and Gc with their molecular weight being 68-76 kDa and 52-58 kDa, respectively (Figure 1-2). The glycoproteins protrude from the phospholipid bilayer of the virus as a tetramer consisting of GcGc (Huiskonen et al., 2010; Battisti et al., 2011). The Gc contains three domains, I, II, and III, these domains are arranged such that domain I is centered and flanked by domain II and III. Domain II contains both the fusion loop as well as the endosomal membrane anchor whereas domain III contains the stem region of the protein. (Willensky et al., 2016). The interactions of domain I and III stabilizes the protein in its pre-fusion conformation (Serris et al., 2020). The glycoproteins have been suggested to regulate host immune responses during infection (Alff et al., 2006; Alff et al., 2008; Matthys et al., 2011).

The S-segment is approximately 1871-2059 nt in length and encodes for the nucleocapsid (NP), with a molecular weight of 50 kDa (Schmaljohn et al., 1986). The protein has been observed as mono- to multimeric (Alfadhl et al., 2001; Kaukinen et al., 2001; Alfadhl et al., 2002). NP encapsidates genomic and antigenomic RNA which may protect the viral RNA from various host degradation proteins such as RNases as well as preventing triggering the host’s pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) (Habjan et al., 2008). The NP bind selectively to vRNA and cRNA and has a very high affinity to vRNA. The recognition of vRNA and cRNA by the NP occurs through panhandles that are formed by these RNA molecules (Severson et al., 1999). Further, the NP has a conserved central region at, amino acid residue 175 to 217, which is responsible for the specific binding of NP to viral RNA (Xu et al., 2002; Severson et al., 2005) The NP also exhibits the ability
Figure 1-1. Trisegmented genomes of hantaviruses
The Large, Medium, and Small segment, with the proteins each segment encodes for and the WAASA site where GnGc proteins are cleaved. Untranslated regions (black), open reading frames (white) of each segment are shown.

Figure 1-2. Virion structure of hantaviruses
Hantavirus virion with tri-segmented genome encapsulated with nucleocapsid protein forming a complex with virus RNA-dependent RNA polymerase with glycoproteins (GnGc) protruding outwards of the virion.
to regulate early host antiviral responses (Ontiveros et al., 2010; Cimica et al., 2014; McAllister and Jonsson, 2014; Reuter and Krüger, 2018; Simons et al., 2019).

The virion is polymorphic with a combination of round, elongated and irregular morphologies ranging in size between 120-155 nm (Figure 1-3). The surface of the virion is covered with square grid-like pattern that comprises of the tetrameric GnGc proteins with some parts of the surface being absent of these proteins structures (Parvate et al., 2019).

**Replication Cycle of Hantaviruses**

The replication cycle of hantaviruses has seven key steps, attachment, entry, transcriptions, translation, genome replication, assembly, and egress (Figure 1-4). Entry begins when the virus glycoproteins attach to host’s cell receptors. Various host cell receptors allow for viral attachment. Early research showed that hantaviruses selectively bind to the host \( \beta_1 \) or \( \beta_3 \) integrins (Gavrilovskaya et al., 1998; Gavrilovskaya et al., 1999; Larson et al., 2005). Pathogenic hantaviruses such as *Andes orthohantavirus* (ANDV), *Hantaan orthohantavirus* (HTNV), and *Sin nombre orthohantavirus* (SNV) attach through \( \alpha_v \beta_3 \) and \( \alpha_{IIb}\beta_3 \) and the nonpathogenic hantaviruses, *Prospect Hill orthohantavirus* (PHV), binds to \( \beta_1 \) integrins. Decay accelerating receptor (DAF/CD55) has also been identified as a receptor or co-receptor for hantaviral attachment and entry. (Krautkrämer and Zeier, 2008; Buranda et al., 2010). Recently, protocadherin-1 (PCDH1) was shown to be a receptor for the New World hantaviruses but not for the Old World hantavirus (Jangra et al., 2018). The importance of PCDH1 during attachment and entry of New World hantaviruses is supported by receptor knockout studies where the depletion of \( \beta_1 \), \( \beta_3 \) integrins, and/or DAF did not affect the infectivity of Old and New World hantaviruses but that of PCDH1 resulted in lowered infectivity of New World hantaviruses (Dieterle et al., 2021).

Entry of the virus following viral attachment occurs through various mechanisms of endocytosis. The route of entry differs for Old- and New World hantaviruses (Ramanathan and Jonsson, 2008). HTNV enters through clathrin-dependent receptor-mediated endocytosis or micropinocytosis, depending on the cell type (Jin et al., 2002; Torriani et al., 2019). ANDV on the other hand uses various mechanisms ranging from clathrin-dependent and -independent, dynamin- and cholesterol-dependent pathways as well as micropinocytosis (Ramanathan and Jonsson, 2008; Chiang et al., 2016). The impact of virion ultrastructure morphology on the viral entry is yet to be confirmed but it can be assumed that the larger particles enter only through micropinocytosis.

As hantavirus glycoproteins are class II fusion proteins their fusogenic activity is pH-dependent. The lowering of pH activates the fusogenic activity in internalized cellular endosomes as the endosome traffics throughout the cell from early to late endosome. Once endosome acidification occurs, it is suggested to cause conformational changes to the glycoproteins that allow for membrane fusion and virus uncoating.
Figure 1-3. Polymorphic virion ultrastructures of Old and New World
(A) Round virion of ANDV. (B) Tubular virions of ANDV. (C) Tubular structure of SNV. (D) Tubular and irregular virions of HTNV.
Figure 1-4. Replication cycle of hantaviruses
The replication cycle of hantaviruses is comprised out of 7 steps, (1) attachment of the virus to either β₁, β₃, DAF, and PCDH1 host cell receptors by viral Gn proteins, (2) entry occurs through various mechanisms such as either clathrin-mediated or independent endocytosis, (3) transcription of viral mRNAs via viral RdRps that is initiated through host mRNA cap snatching, (4) translation of viral proteins, L and S segment mRNAs translated by free ribosomes and M-segment mRNAs by ribosomes on the ER, (5) genome replication through a cRNA intermediary, (6) assembly of virion as the virus migrates through the Golgi and (7) egress of virus via exocytosis.
The pH at which membrane fusion occurs differs between the Old World and New World hantaviruses, the pH threshold for ANDV is 6.3 and 5.8 for HTNV (Arikawa et al., 1985; Cifuentes-Muñoz et al., 2011; Kleinfelter et al., 2015).

Upon uncoating of the virus, the three RNP complexes containing the three genome segments are released into the cell’s cytoplasm. The release of the RNP complexes initiate replication of cRNA and transcription of viral mRNAs. Replication processes occur through a prime-and-realign mechanism (Garcin et al., 1995). Transcription is initiated with RdRP cleaving a 7-methylguanosine cap primer from the 5’ end of host’s mRNA, and this process is referred to as cap snatching. The cleaved primer aligns to cystine residue at the 3’ end of virus’s genomic RNA from which initial elongation occurs. After the insertion of nucleotides, the RdRp slips backwards and realigns nascent RNA to genomic viral RNA. This slippage and realignment results in the overhanging of the 7-methylguanosine cap. Following the slippage and realignment final elongation occurs to form complete viral mRNAs. Viral genome replication occurs through an antigenome or cRNA intermediary genome and replication through a similar prime-and-realign mechanism, but differences are observed in the primer that is used as instead of priming by a cap, it is performed by a guanosine triphosphate that aligns to the cystine residue which then follows the same initial and after final elongation the overhanging guanosine triphosphate is cleaved (Garcin et al., 1995; Hutchinson et al., 1996).

Following transcription, L- and S-segment mRNAs are translated by free ribosomes and M-segment mRNAs by ribosomes on the rough endoplasmic reticulum (RER). The NP is the most abundantly produces viral protein and is important during viral protein translation as the protein drives translation of viral mRNA over host mRNA by mimicking host’s cap-binding complex of Eukaryotic initiation factor 4F (eIF4F) which is responsible for host mRNA translation (Mir and Panganiban, 2008).

Translated NP traffic to the ER-Golgi intermediate complex (ERGIC) through microtubule dynein for virus assembly (Ramanathan et al., 2007; Mir et al., 2008). The translations of the M-segment mRNAs produce a precursor protein which is cleaved at a conserved amino acid motif, WAASA, this motif is located at amino acid location 264-268 in the ER (Löber et al., 2001). Cleaved proteins undergo further post-translation modification through as N- and O-linked glycosylation (Shi and Elliott, 2004). The proteins traffic to the Golgi complex to be used during assembly (Ramanathan et al., 2007).

Virus assembly occurs through the RNP complex interacting with the cytoplasmic tail of GN and budding into the Golgi to form double membrane vesicles containing the virion that are budded and migrate to the cell’s plasma membrane and egress through exocytosis. While virus assembly and egress occur through this mechanism, it is proposed that New World hantaviruses can assemble at the plasma membrane as particles of Black Creek Canal orthohantavirus (BCCV) have been observed at the extracellular
space in close proximity to plasma membrane (Goldsmith et al., 1995; Ravkov et al., 1997).

**Human Host Responses to Infection by Hantaviruses**

Infection of humans by hantaviruses occurs when virus particles in aerosolized excreta from infected reservoir hosts are inhaled into the lower respiratory system. The viruses infect epithelial cells (Andy), but the primary site of replication are the lung microvascular endothelial cells (LMVECs) that make up the pulmonary microvasculature (Zaki et al., 1995). The manner in which the virus is able to reach the LMVECs is assumed to be from basal budding from the epithelial cells where they must traverse the interstitial space or through the infection of patrolling immune cells such as macrophages (Figure 1-5) (Macneil et al., 2011). Of note, hantaviruses infection of autopsy tissues in the lung show no cell death in any infected cells (Zaki et al., 1995).

Once the virus infects other cells such as epithelial cells and LMVECs, they trigger host’s PRRs, which include TLR3 as well as RIG-I and melanoma-associated protein 5 (MDA5) (Alff et al., 2006; Handke et al., 2009). Hantaviruses induce these cascades with the end result being the expression of interferon responses (IFN) and interferon stimulated genes (ISGs) causing these responses to be a target for regulation.

Once RIG-I is triggered the cascade initiates with the activation of mitochondrial antiviral-signaling protein (MAVS) followed by TNF receptor-associated factor 3 (TRAF3), this activation leads to the recruitment of the TANK-Binding Kinase 1 (TBK1) and Inhibitor-κB kinase ε (IKKe) which forms a complex. The complex allows for the phosphorylation of Interferon Regulating Factor 3 (IRF3) and IRF7 form homo and hetero dimerization which translocate into the nucleus and bind to the promoter of interferon beta 1 (IFNB1) which allows for its expression (Alff et al., 2006; Rehwinkel and Gack, 2020). Triggering and activation of TLR3 results in the recruitment of Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) that activates TRAF3 which recruits TBK1 and IKKe, and these proteins form a complex. The complex is essential in that it phosphorylates IRF3 and IRF7, and upon their phosphorylation they form homo or heterodimers with each other and translocate across the nucleus membrane inducing IFNB1 expression (Kawasaki and Kawai, 2014). The induction of IFNs is followed by the proteins binding to the cell surface receptor interferon-α/β receptor (IFNAR) of infected and uninfected cells which triggers the Janus Kinases (Jak) and signal transduction and activators of transcription factors (STAT) pathway. Upon binding to the receptor, it triggers the phosphorylation of STAT1 and STAT2, forming a heterodimer, allows for a complex to form with IRF9. Following the formation of this complex, STAT1/STAT2/IRF9 translocate into the nucleus and induces number of ISGs, cytokines and chemokines (Bousoik and Montazeri Aliabadi, 2018).
Figure 1-5. Infection routes of hantaviruses
Hantaviruses are transmitted to humans when aerosolized excreta from infected reservoir host are inhaled. Once in the virus lungs it is suggested that the virus travels to the lungs either through infection of the epithelial layer or patrolling immune cells such as macrophages followed by the infection of the LMVECs
With the activation of host’s PRRs and the expression of IFNs, hantaviruses cause strong activations of proinflammatory immune responses. Hantaviruses regulate the antiviral responses and the ability to regulate these responses differ between virus species. The difference in this ability is proposed to be the why certain hantavirus species are pathogenic or nonpathogenic (Geimonen et al., 2002; Spiropoulou et al., 2007). These mechanisms have been studied in a number of model systems based on different cell types, e.g., cell lines and primary cells, and various assays e.g., reporter assays, gene expressions, and secreted protein analysis (Sundstrom et al., 2001; Geimonen et al., 2002; Prescott et al., 2005; Alff et al., 2006; Spiropoulou et al., 2007; Alff et al., 2008; Khaiboullina et al., 2016). One of the mechanisms proposed is the differential regulation of IFNs in human LMVECs by the nonpathogenic virus, PHV, which induce significantly higher transcript and protein levels of IFN-β as compared to the pathogenic virus ANDV which induce lower levels that are delayed. It is proposed that ANDV strongly inhibits the dimerization of IRF3 as compared to PHV, and this difference leads to the differences in IFNB1 induction and downstream of it the levels of STAT1 and STAT2; both viruses suppress STAT1 and STAT2 activation (Alff et al., 2006; Spiropoulou et al., 2007; Alff et al., 2008). The induction of ISGs also differs between viruses as gene expression analysis of human umbilical vein endothelial cells (HUVECs) revealed that PHV induces more than 20 ISGs whereas the pathogenic viruses HTNV and SNV induce one and or no ISGs, respectively (Geimonen et al., 2002).

Along with IFN induction, infection of LMVEC by hantaviruses upregulates the transcription of a number of cytokines and chemokines. In microarray studies comparing HTNV, PHV, and SNV during infection of HUVECs, C-X-C Motif Chemokine Ligand 10 (CXCL10) and CXCL11 measurements showed similar expression levels between the three viruses. HTNV and PHV infection induce high transcript levels of C-C Motif Chemokine Ligand 5 (CCL5) and CXCL1 whereas SNV did not. HTNV infection induces unique expression of interleukin-6 (IL6), granulocyte-macrophage colony-stimulating factor (GMCSF), and (GCSF) compared to PHV and SNV (Geimonen et al., 2002). Other comparative studies of pathogenic and nonpathogenic hantavirus species report that HTNV and PHV induce expression of IL-6 at a gene and protein level whereas ANDV did not (Khaiboullina et al., 2016). Infection of HLMVECs by SNV induces protein secretion of CCL5 and CXCL10 as early as 24 hpi and the levels of these proteins increase up until 120 hpi (Sundstrom et al., 2001). In summary, there is notable conflicting data in models that attempt to recapitulate the human innate immune response. In summary, there is notable conflicting data in models that attempt to recapitulate the human innate immune response.

Clinical pathology studies of HFRS and HPS patients show that these disease are associated with induce strong immune activation with the high protein levels of a number of cytokines and chemokines, IL-1, IL-6, IL-8, IL-10, IL-15, IL-18, IFN-γ, (Tumor necrosis factor- α (TNF-α), indoleamine 2,3-dioxygenase (IDO), vascular endothelial growth factor (VEGF), CCL5, and CXCL10; suggesting the role of hypercytokinemia during these disease (Borges et al., 2008; Outinen et al., 2011; Braun et al., 2014; Angulo et al., 2017; Maleki et al., 2019). Disease severity analysis of HFRS and HPS patients have suggested a number of cytokines to be correlated to disease severity. High IL-6
levels in HPS patients’ sera are correlated with disease severity but in bronchoalveolar lavage (BAL) samples of HFRS patients the protein levels are not increased (Rasmuson et al., 2016; Angulo et al., 2017) Strong correlation of VEGF protein levels in pulmonary edema fluids from HPS and disease severity is also reported suggesting the role the protein plays in causing pulmonary edema (Gavrilovskaya et al., 2012). In HFRS patients, IDO is highly activated in patients’ sera and these high levels correlate with disease severity as well as high levels of Forkhead Box P3 (FOXP3) expression in regulatory T cells indicating that IDO potentially suppresses immune responses that hinders virus clearance (Koivula et al., 2017).

Regulation of Human Host Responses by Hantaviruses

For viruses to successfully infect and replicate in host, viruses must overcome host’s responses that aim to prevent virus replication, dissemination, and ultimate clear virus from the infected host. With this, viruses have evolved mechanisms to modulate and regulate these responses. In hantaviruses, these mechanisms are driven by the interactions of the viruses’ NP and/or GP with host’s cellular components within cytoplasm (Figure 1-6). These viral proteins mostly interact with host’s PPR, immune response proteins such as IRFs, IFNs, and nuclear factor kappa B (NF-κB) and host proteins such as importins.

One mechanism by which hantaviruses regulate host’s responses is through hantaviral GP inhibiting host’s PRR signaling with that of RIG-I being of great importance. Of the two proteins that make up the GP, it is the Gn specifically its C-terminus that regulates RIG-I driven IFN responses by inhibiting TBK1 complex formation through binding to TRAF3. It is the 42 C-terminal residues within the Gn that interacts and regulates TRAF3. Interestingly, hantavirus species differ in their ability to regulate TRAF3, as only ANDV, SNV and Tula orthohantavirus (TULV) but not PHV are able to bind TRAF3 (Alff et al., 2006; Alff et al., 2008; Matthys et al., 2011; Matthys et al., 2014; Pan et al., 2015). In another study Gallo et al. reported that the cytosolic tail of the Gn protein of PHV, Puumala orthohantavirus (PUUV), and TULV are not able to inhibit RIG-I driven expression of IFN-β but that the Gn precursor of only PUUV is able to inhibit this induction (Gallo et al., 2021).

Hantaviruses are also able to regulate host’s responses through the interactions by virus NP. Through these interactions hantaviral NP blocks TNF-α driven activation of NF-κB. The inhibition of this activation is through viral NP interacting with importin-α that is responsible for the translocation of NF-κB into the nucleus. Interestingly, not all hantavirus species are able to inhibit this activation, of the species tested only Dobrava-Belgrade orthohantavirus (DOBV), HTNV and SEOV exhibited this ability and not ANDV, PUUV and SNV (Taylor et al., 2009a; Taylor et al., 2009b).

On the other hand ANDV and SNV regulate IFN-β induction and Jak/STAT signaling but the viral proteins that inhibit this induction differed between the two
Figure 1-6. Multiple mechanisms utilized by hantaviruses to suppress early innate immune responses

Hantaviral glycoproteins (GP) and nucleocapsid proteins (NP) are able to interact with multiple human host proteins that partake in innate immune responses to attempt to suppress these interactions occur during (A) PRR signaling and (B) Jak/STAT signaling.
viruses, both the NP and GP are required whereas in the case of SNV only GP is required (Levine et al., 2010). The NP of ANDV regulates RIG-I and MDA5 driven responses but not that of PHV and SNV (Cimica et al., 2014). The site within ANDV NP that is responsible for inhibiting these responses is mapped to a single amino acid, serine 386. This single amino acid residue is not observed in Maporal orthohantavirus (MAPV), which does not regulate IFN-β (Simons et al., 2019). The nonstructural (NS) proteins of PHV, PUUV, and TULV inhibit the induction of IFN-β by driven by RIG-I activation (Gallo et al., 2021). Hantaviruses also suppress immune responses associated with apoptosis leading to the inhibition of cell death. The inhibition of apoptosis occurs through viral NP sequestering NF-κB in the cytoplasm and degrading IκB in the cytoplasm blocking TNF directed NF-κB and apoptosis induction. The region of the NP that was responsible for this regulation was shown to be a 60 amino acid sequence (AA 270 to 330) (Ontiveros et al., 2010).
CHAPTER 2. COMMON THEMES IN ZOONOTIC SPILLOVER AND DISEASE EMERGENCE: LESSONS LEARNED FROM BAT- AND RODENT-BORNE RNA VIRUSES*

Introduction

Globalization, environmental and anthropogenic changes provide ample opportunities for spillover and emergence of zoonotic diseases (Dobson and Foufopoulos, 2001; Taylor et al., 2001; Childs et al., 2007b). The term zoonosis was first coined in the latter half of the 19th century by Rudolf Virchow, who noted the relationship of humans and animals in the occurrence of infectious diseases (Schultz, 2008). Today, we recognize that greater than half of all human infectious diseases are zoonotic, a majority of which originated through the cross-species transmission of RNA viruses from wildlife to humans (Jones et al., 2008; Lloyd-Smith et al., 2009; Kreuder Johnson et al., 2015; Olival et al., 2017); and, at present, we know of more than 224 RNA viruses that cause human disease with 88% of these being zoonotic in nature (Woolhouse et al., 2013; Woolhouse and Brierley, 2018). Of those zoonotic viruses that have spilled over, only a few have successfully adapted to humans and resulted in pandemics in the past two centuries, such as the 1918 H1N1 Influenza A virus, human immunodeficiency virus (HIV), hepatitis C virus (HCV), and severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). The 1918 influenza pandemic, which reached a global death toll of up to 100 million people, began as a spillover of an avian influenza H1N1 virus from a bird or some other animal such as pigs (Morens et al., 2010). In the case of HIV, which has resulted in approximately 32.7 million deaths from AIDS-related illnesses (through the end of 2019), the ancestral virus spilled over from chimpanzees to humans (Hahn et al., 2000). The original reservoir of HCV, a virus that infects over 70 million worldwide, is still unknown. Viruses belonging to the same Hepacivirus genus have been isolated from reservoirs such as dogs, rodents, and horses (Pybus and Theze, 2016). The coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 may have started with the spillover of the virus from a bat to an intermediate host (Paraskevis et al., 2020; Zhou et al., 2020); but neither the reservoir nor any intermediate host has been confirmed yet.

Most viruses that spill over from wildlife to humans do not typically result in pandemics (Kreuder Johnson et al., 2015). More commonly, following infection, humans are “dead-end” hosts, and the virus is not transmitted further from person to person. Typically, these outbreaks are limited to the geographic distribution of the reservoir, but collectively, they have resulted in hundreds of thousands of infections and case fatalities.

Some of the more notable, and geographically bound, wildlife to human outbreaks involves viruses harbored by rodents such as hantaviruses (e.g., HTNV and SNV) and arenaviruses (e.g., *Machupo mammarenavirus* (MACV), *Lassa mammarenavirus* (LASV), and *Argentinian mammarenavirus* (formerly Junin virus (JUNV))) found circulating in the Americas or Africa (*Table 2-1*). While bats have a much greater geographical range, human outbreaks of viruses carried by bats such as *Nipah henipavirus* (NiV) and *Hendra henipavirus* (HeV) are limited to the geographical range of the bat species (*Table 2-2*).

In this review, we focus on three questions regarding zoonotic viruses that originate in bats and rodents. First, we discuss biological strategies that have evolved that allow RNA viruses to reside in bats and rodents. Second, we look at some of the environmental and ecological causes that drive spillover. And third, we discuss how spillover occurs from bats and rodents to humans by highlighting some shared and unique characteristics of previous epizootic events.

**What Biological Strategies Have Evolved That Allow RNA Viruses to Reside in Bats and Rodents?**

The two most abundant and globally distributed mammalian groups are rodents (order Rodentia) followed by bats (order Chiroptera). According to the Mammal Diversity Database maintained by the American Society of Mammalogists, there are 2590 extant species within the Rodentia and 1430 extant species within the Chiroptera. Together, these represent 63% of the 6410 mammalian species known to exist today. Orders Rodentia and Chiroptera diverged approximately 96 million years ago (MYA) according to the most recent available published data (65 studies ca. December 2020) in TimeTree (Hedges et al., 2006; Hedges et al., 2015). Both mammalian orders have been cited as harboring proportionally high richness of pathogenic viruses (Olival et al., 2017), and this may be directly related to the high diversity in these two mammalian taxa. The two families known to harbor zoonotic RNA viruses within the order Rodentia are Muridae and the Cricetidae with 843 and 809 species, respectively (Carleton and Musser, 2005). Over 50% of rodent-borne zoonotic viruses are members of two families, *Hantaviridae* and *Arenaviridae* (Mollentze and Streicker, 2020). Chiroptera is the second most species-rich group of mammals with 21 families (Burgin et al., 2018). Hence, it is not surprising that over 10,000 RNA virus sequences have been identified in various bat species, several of which are known to cause human disease (Dobson, 2005; Li et al., 2005; Calisher et al., 2006; Chen et al., 2014; Hayman, 2016). Viral sequences noted in bats include *Astroviridae, Coronaviridae, Circoviridae, Adenoviridae, Filoviridae, Parvoviridae, Poxviridae, Picornaviridae*, and *Rhabdoviridae* (Li et al., 2005; Maeda et al., 2008; Ge et al., 2011; Baker and Murcia, 2014; Schountz, 2014; Hayman, 2016; Fischer et al., 2017). Of these, only a small percentage of viruses harbored by bats from the rhabdoviruses, filoviruses, paramyxoviruses, and coronaviruses have been associated with disease and outbreaks in human populations. In addition, although bats are reservoirs of several viruses that have been responsible for serious outbreaks, models have shown that viruses harbored by bats are no more likely to be zoonotic than viruses
Table 2-1. Examples of outbreaks in human populations by RNA viruses harbored by rodents

<table>
<thead>
<tr>
<th>Virus Common Name</th>
<th>Virus Species Name (ICTV)</th>
<th>Family</th>
<th>Notable Outbreaks</th>
<th>Reservoir</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapare Virus</td>
<td>Chapare mammarenavirus</td>
<td>Arenaviridae</td>
<td>2003-2004; 2019 Bolivia</td>
<td>Unknown</td>
<td>(Delgado et al., 2008; Escalera-Antezana et al., 2020)</td>
</tr>
<tr>
<td>Guanarito Virus</td>
<td>Guanarito mammarenavirus</td>
<td>Arenaviridae</td>
<td>1989 Venezuela</td>
<td>Zygodontomy brevicauda</td>
<td>(Salas et al., 1991; Fulhorst et al., 1997a)</td>
</tr>
<tr>
<td>Hantavirus</td>
<td>Hantaan orthohantavirus</td>
<td>Hantaviridae</td>
<td>1950-1953 Korea 1993 Four Corners, USA</td>
<td>Various rodent spp.</td>
<td>(Smadel, 1953; Lee et al., 1981; Centers for Disease Control and Prevention, 1993)</td>
</tr>
<tr>
<td>Junin Virus</td>
<td>Argentinian mammarenavirus</td>
<td>Arenaviridae</td>
<td>Recognized ca. 1950 Argentina</td>
<td>Calomys musculinus</td>
<td>(Parodi et al., 1958; Maiztegui, 1975)</td>
</tr>
<tr>
<td>Lassa Virus</td>
<td>Lassa mammarenavirus</td>
<td>Arenaviridae</td>
<td>Recognized ca. 1950 West Africa</td>
<td>Mastomys natalensis</td>
<td>(Frame et al., 1970; Monath et al., 1974)</td>
</tr>
<tr>
<td>Lujo Virus</td>
<td>Lujo mammarenavirus</td>
<td>Arenaviridae</td>
<td>2008 Zambia and South Africa</td>
<td>Unknown</td>
<td>(Paweska et al., 2009)</td>
</tr>
<tr>
<td>Lymphocytic Choriomeningitis Virus</td>
<td>Lymphocytic choriomeningitis mammarenavirus</td>
<td>Arenaviridae</td>
<td>Recognized ca. 1933 U.S.A.</td>
<td>Mus musculus</td>
<td>(Armstrong and Lillie, 1934; Armstrong et al., 1940)</td>
</tr>
<tr>
<td>Machupo Virus</td>
<td>Machupo mammarenavirus</td>
<td>Arenaviridae</td>
<td>1950s-60s Bolivia</td>
<td>Calomys callosus</td>
<td>(Johnson et al., 1966)</td>
</tr>
<tr>
<td>Sabiá Virus</td>
<td>Brazilian mammarenavirus</td>
<td>Arenaviridae</td>
<td>1990 Brazil</td>
<td>Unknown</td>
<td>(Coimbra et al., 1994; Ellwanger and Chies, 2017)</td>
</tr>
</tbody>
</table>
### Table 2.2. Examples of outbreaks in human populations by RNA viruses harbored by bats

<table>
<thead>
<tr>
<th>Virus Common Name</th>
<th>Virus Species Name (ICTV)</th>
<th>Family</th>
<th>Notable Outbreaks</th>
<th>Reservoir</th>
<th>Citation</th>
</tr>
</thead>
</table>
| Ebola Virus | *Sudan ebolavirus*  
West Africa | Fruit bats  
(proposed) | (World Health Organization, 1978b; Leroy et al., 2005; Koch et al., 2020)  
(Murray et al., 1995a; Murray et al., 1995b; Young et al., 1996; Halpin et al., 2000) |
| Hendra Virus | *Hendra henipavirus* | Paramyxoviridae | 1994 - present  
Australia | *Pteropus* spp. | (Siegert et al., 1968; Towner et al., 2007)  
(Zaki et al., 2012; Ithete et al., 2013; Azhar et al., 2014; Escalera-Anteza et al., 2020) |
| Marburg Virus | *Marburg marburgvirus* | Filoviridae | 1967  
Germany, Serbia | *Rousettus aegyptiacus* | (Mohd Nor et al., 2000; Olson et al., 2002) |
| MERS-CoV | *Middle East respiratory syndrome-related coronavirus* | Coronaviridae | 2012  
Middle East,  
2015 Republic of Korea | Various bat species | (Zhou et al., 2020) |
| Nipah Virus | *Nipah henipavirus* | Paramyxoviridae | 1998-1999, Southeast  
Asia,  
Sporadic in SE Asia  
Recognized since 1930's  
Africa, Asia and Americas | *Pteropus* spp. | (Hurst and Pawan, 1931; Pawan, 1959) |
| Rabies Virus | *Rabies lyssavirus* | Rhabdoviridae | Recognized since 1930’s  
Africa, Asia and Americas  
Various bat species | (Li et al., 2005) |
| SARS-CoV | *Severe acute respiratory syndrome-related coronavirus* | Coronaviridae | 2003-2004  
~24 countries | Various bat species | (Zhou et al., 2020) |
| SARS-CoV-2 | *Severe acute respiratory syndrome coronavirus 2* | Coronaviridae | 2019  
Global pandemic | Bats  
(proposed) | |
that are harbored or transmitted by rodents or other hosts (Mollentze and Streicker, 2020). For some viruses in the genus *Ebolavirus*, bats are the presumptive main reservoir, but have yet to be definitively confirmed (Leroy et al., 2005; Leendertz et al., 2016; Koch et al., 2020).

In the following section, we discuss how biological factors and lifestyle traits contribute to the ability of bats and rodents to serve as a reservoir for an RNA virus. For bat and rodent reservoirs of RNA viruses, multiple characteristics may contribute to their ability to serve as a reservoir (Brook and Dobson, 2015; Han et al., 2015; Schountz et al., 2017). The transmission of bat-borne viruses to humans through an intermediary host further complicates the biology. Moreover, various combinations of strategies have evolved that are not universal across all virus-reservoir host relationships, which include but are not limited to, limiting the pathogenesis of the viral infection, the ability of the virus-reservoir interplay to modulate the immune response such that the virus may persist, the behavioral and demographic characteristics of bats and rodents that promote endemic maintenance of the viruses they harbor, and the inherent genetic plasticity of RNA viruses. There is a lack of literature for many viruses in conjunction with their natural reservoir host in each of these areas, so we present examples of key areas of research that would benefit from continued research in the natural reservoir host and in their intermediate spillover hosts.

**Pathogenesis of the Viral Infection**

Numerous studies have reported that with some exceptions (e.g., lyssaviruses in bats, mamarenaviruses in rodents), many RNA viruses may infect and persist in bats and rodents without causing disease (Easterbrook and Klein, 2008a; Pavlovich et al., 2018; Banerjee et al., 2020; Irving et al., 2021). The ability of a mammal to carry a virus while healthy would contribute to maintenance and spread of the virus in the population. However, as stated, there are also reports of host reservoirs of viruses that experience disease or a loss in fitness. For example, pathology of bats following experimental infection with rabies viruses in the genus, *Lyssavirus*, has been reported (Begeman et al., 2018; Suu-Ire et al., 2018). However, in one report, viral antigen was found in the brain with no apparent pathology (de Araujo et al., 2014). In addition to rabies, pathology (morbidity and mortality) has been demonstrated following experimental infection of *Artibeus jamaicensis*, the Jamaican fruit-eating bat, with a high dose (not the low dose) of *Tacaribe mammarenavirus* (TCRV) (Cogswell-Hawkinson et al., 2012). TCRV has been isolated from 11 species of bats in Trinidad. However, whether Jamaican fruit-eating bat is the “real” reservoir of TCRV remains to be seen in fact, fruit bats, in general, may not be reservoirs as persistent infection with shedding has yet to be demonstrated (Malmlov et al., 2017). In studies of rodent reservoirs, there is evidence of some disease in the natural reservoir. Anemia, splenomegaly, and decreased survival have been observed in MACV-infected *Calomys callosus* (large vesper mouse) (Webb et al., 1975). In addition, Webb et al. found the female *C. callosus* infected with MACV produce only 5% of the expected number of viable offspring, although having normal estrus cycles (Webb et al., 1975). MACV does not cause illness or death in either adult or juvenile mice, and as with other arenaviruses, it infects a variety of cell types. Although infectious virus has been
recovered from most major organs, the kidney and spleen are the important target organs for arenavirus replication in rodents (Johnson, 1965; Southern, 1996). For rodent reservoirs of hantaviruses, there are examples of a loss in adult survival of animals based on ecological studies of hantaviruses in nature; for example, SNV infection decreases survival of deer mice (*Peromyscus maniculatus*) in the USA (Luis et al., 2012) and in bank vole (*Myodes glareolus*) populations harboring *Puumala or**thohantavirus* (PUUV) surveyed in 55 areas over three years in Finland (Kallio et al., 2007).

Although accepted, or presumed reservoir hosts, have an immunological response to virus infection, no apparent disease is detected in experimental or natural infections of certain hosts with viruses such as some coronaviruses, henipaviruses, some hantaviruses, paramyxoviruses, and filoviruses. In an interesting study by Watanabe et al. laboratory, Leschenault’s rousette bats (*Rousettus leschenaultii*) were experimentally infected with a wild-caught *Betacoronavirus* isolated from the lesser short-faced fruit bat (*Cynopterus brachyotis*) in the Philippines, but these animals showed no clinical signs; viral RNA was only detected in the intestines and not in the liver, kidney, lung, spleen or brain. Similarly, Munster et al. (Watanabe et al., 2010). Similarly, Munster et al. experimentally infected laboratory Jamaican fruit-eating bats with *Middle East Respiratory Syndrome‐Coronavirus* (MERS‐CoV) (Munster et al., 2016). Although they could detect shedding of virus, they also reported no clinical signs or pathology. These limited studies of coronaviruses may suggest that an acute infection with virus shedding, but no pathology, may be common in bats, regardless of whether the species is a reservoir host. In other laboratory studies, Egyptian rousette bats, *Rousettus aegyptiacus*, were infected with *Marburg marburgvirus* (MARV), and no deaths, overt signs of morbidity, or gross lesions were identified, although, microscopic pathological changes were seen in the liver of infected bats (Amman et al., 2012; Paveska et al., 2012; Schuh et al., 2017). In experimental studies by Halpin et al., wild-caught pteropid bats in Australia were infected with the henipaviruses HeV and NiV (Halpin et al., 2011). Bats showed virus shedding, but histopathology showed essentially no convincing pathology and no detection of viral antigen. Similarly, Middleton et al. infected grey-headed flying fox bats (*Pteropus poliocephalus*) with NiV and reported infection, and pathological examination of tissues did not confirm the presence of the virus with associated pathology so that lesions could not be attributed to viral infection (Middleton et al., 2007). Woon et al. also infected black flying foxes (*Pteropus alecto*) with HeV and showed virus replication in the lungs and an inflammatory response, but no viral shedding nor pathology (Woon et al., 2020). Experimental infection of Egyptian rousette bats with the paramyxovirus, *Sosuga pararubulavirus*, showed infection and virus shedding, but no morbidity nor mortality. However, they report subclinical disease in a subset of tissues (Amman et al., 2020).

Uniquely for bats, one proposed explanation for the absence of disease for some viral infections is the “flight as fever” hypothesis which posits that the high metabolic rate required for flight stimulates a fever response (O’Shea et al., 2014; Schountz et al., 2017). Daily metabolic surges during flight leads to DNA damage caused by the production of reactive oxygen species (Hanadhita et al., 2019). Evidence suggests that during evolution, bats positively selected for genes involved in DNA repair which could have influenced their antiviral immune responses (Zhang et al., 2013; Banerjee et al.,
Moreover, to avoid DNA-mediated immunopathology induced during flight, bats have an altered DNA sensing system which may limit viral replication (Xie et al., 2018; Banerjee et al., 2020). One study suggests virus replication within bat cells does not seem to be temperature dependent, suggesting that other characteristics of bats may contribute to enabling them to serve as viral reservoirs (Miller et al., 2016).

**The Host Immune Response**

The ability of bats and rodents to modulate the immune response to viral infection has been an active area of investigation and has been recently reviewed elsewhere (Schountz et al., 2017; Banerjee et al., 2020; Gorbunova et al., 2020). We highlight a few examples of how viruses interact with specific components of the bat or rodent host immune system, which results in a low level of viral infection or an ability of the host to tolerate viral replication (Schountz et al., 2007; Easterbrook and Klein, 2008a; Virgin et al., 2009; Zhou et al., 2012; Subudhi et al., 2019; Banerjee et al., 2020; Gorbunova et al., 2020). In general, immune responses of the host play a major role in controlling the viral infection and viruses can exploit these responses in at least one of three ways; (i) immune ignorance, (ii) immune escape, and (iii) immune dysfunction (or exhaustion). The first immune responses to an initial infection occur in three phases in this general order: (i) innate immunity; (ii) early induced immunity; and (iii) adaptive immunity. The goal of each of these responses is the removal of the infectious agent. The innate response plays an important role in detection of the virus during acute stages of infection, which may set the stage for the magnitude of the adaptive responses.

**Interferons (IFN) and Interferon-Stimulated Genes (ISGs).** Most viruses are indirectly sensitive to the induction of IFNs following infection as they induce the upregulation of ISGs; the proteins transcribed from the ISGs actively engage in an antiviral response that directly or indirectly thwart virus replication. Thus, many RNA viruses have evolved strategies to suppress the IFN response and studies suggest similarity of the innate immune signaling pathways in bats and rodents (He et al., 2014; Banerjee et al., 2020), although some of their counterparts in bats (Banerjee et al., 2017; Kuzmin et al., 2017) although some of their counterparts in bats and rodents may function differently. While an active area of research effort, there are few studies of the innate immune response in the many reservoir hosts with the virus they harbor.

Research on bat IFNs suggested diversity in the expression of IFN-alpha. In the black flying fox (reservoir of henipaviruses), and the lesser short-nosed fruit bat IFN-alpha is expressed constitutively (Zhou et al., 2016). This is not observed in the reservoir of MARV, the Egyptian rousette bat (Pavlovich et al., 2018). When compared to ancestral bat species, the IFN gene loci of the black flying fox have contracted whereas the loci of Egyptian rousette bats have diversified (Zhou et al., 2016; Pavlovich et al., 2018). Banerjee et al. show that IFN-beta was expressed in big brown bat, *Eptesicus fuscus*, cells stimulated with poly(I:C), however, tumor necrosis factor-alpha, TNF-alpha, was much lower, and suppressed (Banerjee et al., 2017). In one study with Egyptian rousette bats experimentally infected with MARV, Guito et al. observed a moderate elevation of innate antiviral genes, but not cytokines and adaptive immunity-related genes.
The authors suggest that Egyptian rousette bats may have evolved a mechanism to control virus replication without induction of the adaptive responses.

Two studies have examined henipaviruses in an immortalized cell line derived from the black flying fox (Virtue et al., 2011; Janardhana et al., 2012). The first study suggested that neither HeV or NiV infection results in type I and III IFN induction (Virtue et al., 2011). Both viruses also inhibit interferon signaling. In the second study, the bat cells were treated with supernatant from Chinese hamster ovary cells that contained black flying fox IFN-γ, and they showed inhibition of virus replication (Janardhana et al., 2012). Examinations of the antiviral response in rodent primary cells using the zoonotic viruses are limited. Studies of hantaviruses and their reservoirs suggest that viral strategies to antagonize the IFN response may vary (Levine et al., 2010). Permanent reservoir cell lines that have been developed for the common vole (Microtus arvalis) and bank vole for hantaviruses will provide a valuable tool to exploring virus-reservoir host interaction (Binder et al., 2019).

The Myxovirus resistance (Mx) proteins are ISGs that are well-studied in in-bred laboratory rodents and shown to directly inhibit many RNA viruses (reviewed in (Verhelst et al., 2013)). In wild caught bank voles from various endemic regions in Europe, Dubois et al. showed that bank voles from PUUV endemic regions in Europe are more tolerant to infection (higher viral load and delayed antibody production) and have lower ISG and cytokine expression (Mx2, TNF-alpha) compared to bank voles from non-endemic regions (Dubois et al., 2017; Dubois et al., 2018). The Mx1 gene was cloned from three families of bats: Pteropodidae, Phyllostomidae, and Vespertilionidae (Fuchs et al., 2017). Examination of the ability of each of the bat Mx proteins confirmed their ability to reduce replication of Zaire ebolavirus (ZEBOV), vesicular stomatitis virus and Rift Valley fever phlebovirus (RVFV) (Fuchs et al., 2017). However, the Mx from common pipistrelle, Pipistrellus pipistrellus did not show in vitro antiviral activity against RVFV, suggesting functional differences may exist among bats. Direct examination of immune signaling in primary bat cells from a reservoir with the virus they harbor has not been reported for any of these viruses.

B and T cells of bats and rodents. Bats potentially have more VDJ germline gene segments when compared to other mammals and with this lies the potential for bats to have a much larger naive immunoglobulin repertoire. For example, the naive B cell repertoire of the little brown bat (Myotis lucifugus) may potentially be more than 70,000 specificities (Bratsch et al., 2011). Having a much larger repertoire suggests that the bat has a much lower need for somatic hypermutation as well as affinity maturation, leading to lower titers of antibody (Bratsch et al., 2011). The larger repertoire, along with other host responses such as having constitutively expressed IFN reducing viral load, is suggested to explain why bats develop non-robust antibody responses during viral infection allowing for virus replication within the host (Zhou et al., 2016; Schountz et al., 2017).

For some hantaviral infections, the differences in immune responses between reservoir and nonreservoir host are evident in the cytotoxic T lymphocytes (CTL)
response (see reviews (Kruger et al., 2011; Terajima and Ennis, 2011; Yasuda et al., 2021)). Seoul orthohantavirus (SEOV) infection of animals without a functional T cell response (e.g., nude rat model) succumb rapidly to infection and diseases suggesting that cell-mediated immunity plays an important role in controlling infection (Dohmae et al., 1994). Mouse models of transient and persistent infection for HTNV (Araki et al., 2003) were used to analyze the immune response of virus specific CD8+ T cells with MHC tetramers (Taruishi et al., 2007). They showed that N-specific CTLs are strongly regulated and suppressed in this persistently infected mouse model by an unknown mechanism, whereas they are upregulated in the transient model (Taruishi et al., 2007).

Viral replication in immune cells such as monocytes, macrophages, or T cells can interfere with or actively suppress immunity and cause persistence (Easterbrook and Klein, 2008b; Au et al., 2010; Li and Klein, 2012). Taruishi et al., proposed that the infection of immune cells in the spleen early in infection may result in the suppression of the CTL response (Taruishi et al., 2007). In their persistent animal model experiments, the infection of the spleen correlates with changes in CTL responses. Consequently, due to the downregulation of the CTLs, some of the endothelial cells may remain infected, resulting in a persistent infection in the natural reservoir. In addition, it is hypothesized that the regulatory T cells (Treg) are activated early in the infection process, resulting in decrease in the CTL response (de Araujo et al., 2014; Suu-Ire et al., 2018). Treg responses are suggested to enable a persistent infection by suppressing innate immunity, proinflammatory, and effector T cell activity in SEOV-infected Norway rats (Rattus norvegicus) and SNV-infected deer mouse (Botten et al., 2003; Easterbrook et al., 2007; Schountz et al., 2007; Li and Klein, 2012; Schountz et al., 2012). In these studies, Forkhead box P3 gene expression and TGF-β1 –expressing b1 Treg cells were elevated during persistence, potentially interfering with viral clearance and limiting pathology (Easterbrook and Klein, 2008b).

As with hantaviruses, the outcome of arenavirus infection is mediated by CTL responses (Saeidi et al., 2018; Laura M. McLane et al., 2019; Sandu et al., 2020). During chronic lymphocytic choriomeningitis virus (LCMV)-Cl13 infection, CTLs become functionally unresponsive or exhausted and are unable to kill virally infected cells or produce antiviral cytokines (Zajac et al., 1998). Following infection, the functional capacity of CTLs is lost in a hierarchical manner based upon the amount of available antigen (Wherry et al., 2003). When there is little virus present, CTLs maintain their functional activity, but as viral load increases, all CTL effector functions are lost, including IL-2, TNF-α, and IFN- γ production (Wherry et al., 2003). Exhausted LCMV-Cl13-specific CTLs have been shown to upregulate several inhibitory receptors including programmed death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), CD39, and CTL-associated antigen 4 (CTLA-4) (Wherry et al., 2007; Richter et al., 2010; Odorizzi and Wherry, 2012). It has been suggested that these distinct transcriptional signatures may attenuate signaling through the T cell receptor (TCR), facilitating virus persistence (Wherry et al., 2007). Recently, the transcription factor Thymocyte Selection Associated High Mobility Group Box (TOX) was identified as the master regulator of CTL exhaustion during chronic LCMV infection (Yao et al., 2019). Moreover, the phenotypic landscape of virus specific CTLs was shown to differ by tissue indicating that tissues,
with a higher percentage of functionally exhaustive CTLs may provide a niche for viral persistence (Shawn D. Blackburn et al., 2010; Sandu et al., 2020).

For bats, less is known about the T cell responses following viral infection due to the lack of available immunological reagents; however, some groups have begun phenotypically and functionally characterizing bat lymphocyte subsets (Martínez Gómez et al., 2016; Periasamy et al., 2019). Gomez et al. (Martínez Gómez et al., 2016) identified CTL and T helper cell populations in the black flying fox using commercially available anti-human/mouse antibodies. They observed an unusually high proportion of CTLs in the spleen, and the T cell populations within the spleen expressed IL-17A, IL-22, and TGF-β, indicating polarization toward Th17 and Treg cell subsets (Martínez Gómez et al., 2016). These findings suggests that the bat immune system may be armed and ready to thwart viral infection. However, this may not be generalizable across bat species as differences in lymphocyte populations have been reported (Periasamy et al., 2019). Therefore, it would be of great interest to examine the steady state T cell responses across different wild bat species.

**Behavioral and Demographic Characteristics**

Unlike most rodents, most species of bats are social, roosting in groups which may range from a few individuals to colonies of many millions of individuals, including the largest single-species aggregations of mammals on earth except for Homo sapiens. Some, such as the straw-colored fruit bat (*Eidolon helvum*, Family Pteropodidae) are tree-roosters and long-distance migrators. However, the majority of massively colonial species are cave-roosters, where they congregate in maximally close proximity (Kunz, 1982). Examples include several species of Egyptian rousette bats, (*Rousettus* spp., Family Pteropodidae), bent-winged bats, *Miniopterus* spp. (Miniopteridae), Egyptian slit-faced bats, (*Nyteris thebaica*, family Nycteridae), and several species of free-tailed bats (e.g., *Chaerephon plicatus*, Mormopterus spp., and *Tadarida brasiliensis*, Family Molossidae)) (Leu, 2000; Ruiz, 2002; Esmailka, 2005; Richter and Cumming, 2006; Schiefelbein, 2013; McFarlane et al., 2015).

Bat species that live in social groups, whether in small groups or in massive colonies with close contact, must play a key role in viral maintenance in the population. In large permanent colonies, viral transmission can potentially follow the birthing cycle of pups, in which an increase in population size with naive (newborn) members reduces herd immunity, allowing for viruses to be reestablished cyclically within a colony (Plowright et al., 2011; Plowright et al., 2016). Recently, individuals of the Egyptian rousette bat were found to self-isolate when immunologically challenged, a behavior which presumably would reduce pathogen contagion within the colony (Moreno et al.). Notably, the bat species which have been implicated most frequently as primary reservoirs of zoonotic RNA viruses include *Pteropus* spp. (NiV, HeV, *Menangle pararubulavirus, Tioman pararubulavirus*) and *Rhinolophus* spp. (SARS-CoV related viruses) (Wang and Anderson, 2019). These bats are colonial, although not massively so.
Calisher et al. list and discuss many of the viruses associated with bats, as well as several unique features of bats which may enhance their suitability as viral reservoirs (Calisher et al., 2006). Bats’ capacity for flight (mobility, escape from predators), their long evolutionary history and broad taxonomic diversity, diverse population structure (discussed above) and social/colonial strategies, wide geographical distribution and ability to migrate (virus dispersal and spread), their distinctively high longevity, and seasonal hibernation in some temperate species (viral persistence) may all contribute to making them suitable reservoir hosts (Calisher et al., 2006; Kerth, 2008; Geiser and Stawski, 2011; O’Shea et al., 2014; Peixoto et al., 2018; Fleming, 2019; Andreassen et al., 2021).

As with bats, the behavioral and demographic characteristics of rodents may contribute to their capacity to serve as reservoirs for harboring and maintaining viruses. In comparison, trade-offs between reproduction and lifespan constitute the life-history of rodents. Rodents generally exhibit an r-selected life history, characterized by early sexual maturity and large litter sizes (Han et al., 2015), which leaves them vulnerable to resource depletion or climatic variation. Consequently, rodent populations fluctuate with seasonality and climate change, which affects the availability of food resources and, therefore, reproductive activity (Andreassen et al., 2021). This, in turn, impacts the prevalence of some RNA viruses in the population. For example, in areas with large seasonal variations such as the temperate forests of Central Europe, beech mast seedings have been shown to trigger population surges of the bank vole in the following year and correlate with human PUUV outbreaks (Reil et al., 2015). Conversely, during periods of drought, when food resources are limited, *Mastomys natalensis*, natal multimammate mice, the reservoir of LASV, are found in high abundances within human dwellings, which likely drives the higher rate of human LASV infections (Arruda et al., 2021). Even on a local scale, significant hantaviral seroprevalence differences have been observed in deer mice (Calisher et al., 2007), and microhabitat preferences have been reported to vary seasonally and interannually in two sympatric reservoirs for hantaviruses in Paraguay (Owen, 2021). Moreover, habitat associations of seropositive individuals differed from the general population of *Akodon montensis* (Owen et al., 2010). In general, for a virus to persist in a reservoir with short generation time and rapid population turnover, it must be maintained once the virus reaches a persistent and low level of replication. Calisher et al. found evidence that although deer mice individuals typically lived less than a year, a few long-lived persistently infected individuals are the principal trans-seasonal reservoir of SNV (Calisher et al., 2001).

Some viruses within the *Arenaviridae* and *Hantaviridae* persist for the lifetime of the reservoir host, with the viruses present at relatively low levels in the host (Meyer and Schmaljohn, 2000; Kane and Golovkina, 2010). However, persistent infection as a mechanism of maintenance in the reservoir population may depend on the age of infection. For arenaviruses such as *Guanarito mammarenavirus*, JUNV, LASV, LCMV, MACV, and Morogoro virus, studies have shown an age-dependency in the duration of infection among reservoir hosts (Justines and Johnson, 1969; Vitullo et al., 1987; Walker and Murphy, 1987; Vitullo and Merani, 1990; Charles F. Fulhorst et al., 1999; Borremans et al., 2015; Kang and McGavern, 2017; Hoffmann et al., 2021). Neonates develop long-
term viral persistence, while adults can clear infection hosts (Justines and Johnson, 1969; Vitullo et al., 1987; Walker and Murphy, 1987; Vitullo and Merani, 1990; Charles F. Fulhorst et al., 1999; Borremans et al., 2015; Kang and McGavern, 2017; Hoffmann et al., 2021). The mechanism by which virus persists or is cleared is an area in need of additional investigation. Experimental infection studies of captive bats have shown that some viruses (e.g., HeV and MARV) have relatively short acute infections lasting days to months, followed by the virus being cleared from the host due to the animal creating antibodies against the virus (Halpin et al., 2011; Schuh et al., 2017).

Regardless of whether the virus persists in its reservoir host, the maintenance of virus in the population depends on the value of the basic reproduction number, $R_0$, which represents the average number of secondary infections possible from a primary infected individual (Ciupe and Heffernan, 2017). When the value of $R_0$ falls below 1.0 and remains below 1.0 for several generations, then the virus will eventually be eliminated from the population. We have limited information on the $R_0$ of RNA viruses in bat and rodent populations and how it is affected by physiological and environmental factors as well as age, the duration of infection, and animal behavior.

The Inherent Genetic Plasticity of RNA Viruses

All the viruses discussed herein replicate and transcribe their genomes using an RNA dependent RNA polymerase (RdRp) encoded in their genomes. The RdRp and other viral proteins that support replication do not usually correct misincorporation of nucleosides – an exception being coronaviruses and other members of its family that can proofread mismatched base pairings during replication. Thus, replication and transcription have a high rate of error ranging from $10^{-3}$ to $10^{-5}$ per round of RNA synthesis (Drake and Holland, 1999; Sanjuan et al., 2010; Grande-Pérez et al., 2016). For a 10,000 nt genome, a $10^{-4}$ rate of misincorporation results in, on average, one error per round of replication. As discussed by Peck and Lauring (Peck and Lauring, 2018), the measurement of mutation rates is complex, situational and may be biased against lethal mutations, which may result in an underestimate of the mutation rate (Peck and Lauring, 2018). Genetic mutations arise randomly through error-prone viral replication machinery and may be selected for in the subsequent cycle of infection and replication if they confer some advantage in the reservoir. Upon infection, RNA viruses infect as quasispecies (a population of heterogenous virions) (Domingo, 1997; Klein and Calisher, 2007; Jonsson et al., 2010; Lauring and Andino, 2010; Grande-Pérez et al., 2016). Emergence of a zoonotic virus within a naive, nonreservoir host species may follow a bottleneck where only a few genotypes survive, and even these may require additional genetic changes within the viral population for successful transmission and adaptation to the new host species (e.g., by suppressing innate immunity as discussed above) (Childs et al., 2007b; Wolfe et al., 2007; Parrish et al., 2008; Lloyd-Smith et al., 2009).

Examples of genetic mutations in viruses harbored by wildlife reservoirs that emerge and play a role in promoting reservoir-to-human and sustained human-to-human transmission are few but often involve the binding and/or entry of the virus to host cells,
enhanced replicative ability in the new hosts, and/or suppression of innate immunity.
Mutations enhancing reservoir-to-human transmission were reported in the first SARS-CoV spillover, where mutations in the virus’s spike protein increased the protein’s binding affinity to angiotensin converting enzyme 2 (ACE2) receptor of either the intermediary host, Asian palm civets (Paradoxurus hermaphroditus) or humans (Wu et al., 2012). Mutations occurring after initial spillover of EBOV from its reservoir host into humans were observed in the 2013 West Africa outbreak where a mutation in the virus’s host cell-binding glycoprotein resulted in increased infectivity (Diehl et al., 2016). The mutation was primate specific, and ultimately, the mutation was associated with an increase in human mortality.

A Look at Environmental Factors That Drive Spillover of Viruses in Bat and Rodent Populations

From the first recognition of virus spillover from nature, scientists have endeavored to understand the drivers behind the emergence of new viruses. Factors such as climate change, land use change (i.e., logging, burning, agriculture), human expansion into habitat (i.e., recreation, farming), and land fragmentation (i.e., roads, buildings, towns) are some of the key drivers which seemingly alter wildlife populations and hence the balance of the virus in reservoir populations and drive spillover (Smolinkski et al., 2003; Patz et al., 2005; Childs et al., 2007b; Horby et al., 2014). Each of these extrinsic pressures may impact the animal population by alteration of habitat (e.g., size, composition, fragmentation), resource availability (e.g., water, food, refuge), and/or community structure (e.g., species richness and diversity, population density and demographics) (Hjelle and Yates, 2001; Childs et al., 2007b; Armien et al., 2009; Goodin et al., 2009; Owen et al., 2010). Any one of these, or a combination thereof, could influence host well-being and contact rates and thus may drive spillover of infectious agents into new host species. Complicating matters further, habitat preferences of abundant rodent species may be seasonally and interannually variable, resulting in probable temporal variation in interaction rates among reservoir and nonreservoir species (Owen, 2020). Similarly, human activities may vary seasonally, altering their exposure and risk. Additionally, human cases may result from seasonal increases in rodent population densities and the movement of rodents into homes and barns during the colder seasons (Childs et al., 2007b; Jonsson et al., 2010).

The ecological drivers of outbreaks by emerging and re-emerging zoonotic viruses are not well understood due to the challenges in designing controlled field studies to understand how each of these specific factors drive pathogen presence and prevalence in wild animal populations, and during an outbreak, surveillance is usually not conducted with this in mind. For example, during the outbreak of hantavirus pulmonary syndrome (HPS) in the southwestern USA in 1993 researchers surveyed animals in the vicinity of the trailers and homes of infected persons. This resulted in the identification of the deer mouse, as the reservoir of the causative agent, SNV. In nature, the prevalence of hantaviruses in rodent populations can vary from undetectable to 40% in the primary rodent reservoir (Calisher et al., 2007) (Ahlm et al., 1997; Calisher et al., 1999; Calisher
et al., 2001; Armién et al., 2009; Medina et al., 2009). A persistent conundrum of rodent-borne virus outbreaks that occur over large areas (e.g., southwestern USA, southern Argentina) is that cricetid and murid rodents live in relatively small habitats and conduct limited travel during their life span (usually < two km²) (Meserve, 1977). This raises the question of whether all the mice in a particular outbreak zone respond similarly to an ecological or environmental change in situ or if viruses carried by the reservoir move across rodent populations as a traveling wave as one model suggests (Abramson et al., 2003). Retrospective analyses on the environmental characteristics of sites where people were infected with those at sites where people were not infected suggested that the areas impacted by El Niño-Southern Oscillation (ENSO) had a greater risk (Glass et al., 2000). Nearly 20 years later, we have no concrete evidence, other than this, as to what drove the outbreak in the desert southwest. A second strong ENSO occurred in 1997-1998, and HPS rose 5-fold in the southwestern USA suggesting it as a clear factor (Hjelle and Glass, 2000).

It has been suggested that the most important drivers of spillover of bat-borne RNA viruses to humans is the anthropogenic encroachment into natural habitats of bats through urbanization and agriculture (Chua, 2003; Plowright et al., 2011; Pulliam et al., 2012). For example, anthropogenic deforestation as well as climate anomalies are proposed to have played a role in the outbreak of NiV. Large forest areas were burnt producing extensive haze, which drove the bats to follow an irregular migration. The area was also undergoing a severe drought caused by an ENSO event. These two factors combined drove bats carrying the virus to move into areas where pig barns were located, thereby transmitting NiV to pigs (Chua et al., 2002a). Outbreaks of filoviruses and henipaviruses correlate to birthing pulses of pups in the bat population (Plowright et al., 2011; Hayman, 2015; Schuh et al., 2017).

Clearly, reservoir population density and the prevalence of the virus in the population contribute to the probability of increased prevalence of the virus in its reservoir and the probability of spillover. In general, however, very little is known for many viruses in terms of how these viruses are maintained within their natural reservoirs, the normal prevalence of virus in wildlife, genetic variation within host and communities, and what drives increased spread in reservoir populations. In addition to natural history study of reservoir-virus populations in their native habitats, experimental field studies would help identify factors that change virus-host population dynamics.

**How Do Viruses Spillover from Bats or Rodents to Humans?**

The majority of spillover events from a wild rodent (Table 2-1) or bat (Table 2-2) species that results in infections of humans begin with direct or indirect contact (Figure 2-1) (Kreuder Johnson et al., 2015). Direct contact between humans and rodent or bat reservoir hosts may occur through interactions such as touching animals shedding virus, activities associated with hunting and harvesting bushmeat, human consumption of bushmeat or contaminated food, or being bitten. Indirect contact with a virus from a rodent reservoir to a human mainly occurs through inhalation of aerosolized excreta from
Figure 2-1. Major routes of spillover transmission of viruses harbored by bats and rodents

Three of the most common routes of exposure from bats or rodents to humans are depicted. As illustrated, more than one route may occur. Abbreviations: RABV, rabies viruses; HeV, Hendra henipavirus; NiV, Nipah henipavirus; MARV, Marburg marburgvirus; SARS-COV, severe acute respiratory syndrome coronavirus; EBOV, Ebolavirus species.
infected rodents (Childs et al., 2007b). Exposures to excreta have been associated with human activities such as farming, camping, or outdoor military exercises in areas that are contaminated with the reservoir excreta. Similarly, inhalation of bat guano has been suggested as a potential route of exposure as suggested by human cases of Pteropine orthoreoviruses, coronaviruses, filoviruses and others that have occurred following visits to workplaces or caves where bats roost (Chua et al., 2007; Timen et al., 2009). Many exposures are associated with housing or other buildings which rodents have invaded and occupied or have left excreta (Childs et al., 1987; Childs et al., 1991; Childs et al., 1992; Armstrong et al., 1995; Glass et al., 1997; Cline et al., 2010). Direct or indirect contact may also occur via interactions with an intermediate spillover host (Figure 2-1).

In the following, we briefly review the above scenarios of spillover that resulted in human cases of disease and characteristics of these events.

**Spillover Associated with Direct Animal Reservoir Contact**

*Rabies lyssavirus*, genus *Lyssavirus*, was the first bat-borne virus discovered, which in retrospect is unsurprising because 50% of the currently known viruses carried by bats are rhabdoviruses (Pawan, 1959; Mollentze and Streicker, 2020). The first reported outbreak of rabies happened in 1929 in Trinidad when 17 fatal human cases were reported along with a similar disease in nearby cattle (Hurst and Pawan, 1931). Dr. Pawan, the island’s bacteriologist, found that rabies virus was transmitted from the bite of vampire bats to humans and cattle (Hurst and Pawan, 1931). In the subsequent decade, Pawan further showed that insectivorous and frugivorous bats can harbor the virus as well (Pawan, 1959). Later in the United States, in addition to insectivorous bats, raccoons, skunks, and foxes were identified as additional reservoirs of rabies virus variants (Gilbert, 2018). According to the WHO, there are approximately 59,000 human deaths from rabies in over 150 countries each year. Currently, 99% of all rabies cases comes from one intermediate host, dogs, through bites. In the US, rabies in dogs has been eradicated, and hence while the cases are low, rabies cases originate in bats, coyotes, raccoons, and skunks (Pieracci et al., 2019). In addition to bites from rabies-infected animals, other animal bites are also associated with human cases of disease for several additional viruses (Douron et al., 1984; Schultze et al., 2002; Childs et al., 2019).

Human cases of infection have been associated with consumption of their reservoirs for both bats and rodents. In certain areas, eating rodent bushmeat is considered a delicacy; therefore, human transmission can also occur by exposure to their bodily fluids (e.g., viremia in blood (Schuh et al., 2017; Childs et al., 2019)) during hunting and food preparation (Ter Meulen et al., 1996; Bonwitt et al., 2016). Hence, it is not clear whether the consumption or the hunting was the major risk factor. Several cases of LASV have been associated with the capture and consumption of rodents (Ter Meulen et al., 1996).
Spillover Associated with Direct Contact: Intermediate Hosts

Some outbreaks in humans involving paramyxoviruses, coronaviruses, and filoviruses have occurred through contact with an intermediate host that acts as an amplifier host (Murray et al., 1995a; Murray et al., 1995b; Chua, 2003). For example, palm civets and raccoon dogs (Nyctereutes procyonoides) from live markets may have played a crucial role in outbreaks of human diseases such as those caused by SARS-CoV (Guan et al., 2003; Wang and Eaton, 2007). Since coronaviruses in nature were very poorly sampled until the 2002–2003 SARS outbreak, there is no data that definitively shows whether direct or indirect transmission is more plausible (Hu et al., 2017). The bat reservoir for SARS-CoV-2 has yet to be identified and an intermediate host has yet to be confirmed. In both instances, it has been suggested that a spillover occurred from a bat to an animal sold for human consumption in a live wildlife market (Guan et al., 2003; Zhou et al., 2020). In the case of MERS-CoV, research supports that spillover occurred from bats to dromedary camels, and camels transmit the virus to humans presumably through contact with nasal discharge (Ithete et al., 2013; Azhar et al., 2014; Corman et al., 2014; Fehr et al., 2017).

MARV emerged in 1967 in Marburg and Frankfurt, Germany, as well as in Belgrade, Serbia, when 31 cases were reported of an unknown disease to which seven patients succumbed (Siegert et al., 1968). Epidemiological evaluation of the three outbreaks reported that those infected were working with vervet monkeys (Chlorocebus pygerythrus) that were imported from Uganda (Smith et al., 1967; Kissling et al., 1968). The search for the reservoir of MARV was a lengthy process, and in 2009 the putative reservoir was identified through molecular detection methods as the Egyptian rousette bat (Swanepoel et al., 2007; Towner et al., 2007; Towner et al., 2009).

In September 1976, Zaire ebolavirus (ZEBOV) emerged in Zaire (now the Democratic Republic of Congo) and resulted in 318 cases with a mortality rate of 88% (World Health Organization, 1978b). The first case occurred in a village near the Ebola River in Zaire. The man was an instructor at the Mission School, who, while traveling, purchased fresh and smoked antelope and monkey meat but only ate the antelope (World Health Organization, 1978b). Outbreaks of EBOV occurred throughout Central and Western Africa in 1994 and 2000, and the largest outbreak occurred during 2013–2016 with sporadic outbreaks continuing. Extensive searches have been conducted to identify the reservoir, but it remains elusive (Leirs et al., 1999; Reiter et al., 1999). Bats have been proposed to harbor the viruses but have not been proven to be the reservoir (Leroy et al., 2005; Koch et al., 2020). While the reservoir for the virus has yet to be identified, numerous outbreaks have occurred following humans handling animals such as primates (chimpanzees and gorillas) and antelopes (duikers) and their carcasses (Le Guenno et al., 1995; Georges et al., 1999; Leroy et al., 2004). The presence of EBOV in a number of human bodily fluids enables very efficient person-to-person transmission (Bausch et al., 2007).

There are two species of the Henipavirus associated with outbreaks of human disease, HeV and NiV; these viruses are carried by multiple species of the bats.
commonly referred to as flying foxes (*Pteropus* spp.) (Young et al., 1996; Halpin et al., 2000; Wang et al., 2000b; Chan et al., 2001). HeV first emerged in Brisbane, Australia in 1994 when two human and 21 horse cases of an unidentified respiratory disease were reported, resulting in one human fatality and 14 horses being euthanized (Murray et al., 1995a; Murray et al., 1995b). Since the initial outbreak, cases have been reported in Australia from interactions of humans with horses infected with the virus (Rogers et al., 1996; Hanna et al., 2006; Field et al., 2011). Although transmission of HeV from flying foxes to horses has yet to be demonstrated, it is hypothesized that transmission occurs when horses encounter urine from infected bats (Williamson et al., 1998; Edson et al., 2015).

The first outbreak of NiV occurred in Malaysia during 1998 in pigs and humans, with 265 human cases (105 fatal) and the culling of nearly one million pigs (Centers for Disease Control and Prevention, 1999; Mohd Nor et al., 2000). The earlier outbreak of HeV suggested bats as a reservoir, and ultimately, seroprevalence surveys of bats showed that multiple species of bats had neutralizing antibodies to NiV, and the virus was isolated from the urine collected from island flying foxes (Yob et al., 2001; Chua et al., 2002b; Olson et al., 2002). Spillover of NiV to pigs is thought to occur when pigs consume fruits that have been partially eaten by bats or via exposure to the excreta of infected bats (Pulliam et al., 2012). In addition to contact with pigs, bat-to-human spillover is thought to occur through the consumption of date palm sap that has been contaminated with NiV from bat excreta (Khan et al., 2010). Outbreaks of NiV with 40–70% fatality have since been reported in Bangladesh, India, and the Philippines (Hsu et al., 2004; Ching et al., 2015). Significantly, in Bangladesh and India, epidemiological evidence also indicates human-to-human transmission through close contact.

**Spillover Associated with Indirect Animal Reservoir Contact**

Epidemiological evidence suggests the indirect mode of transmission (aerosolized excreta) from rodents or bats to humans is similar for many zoonotic viruses. The literature supports indirect contact via inhalation of rodent excrement or direct contact with rodents such as rodent bites (discussed above) as the main routes of transmission to humans (Childs et al., 2007b). These exposures occur during certain types of human activities that are often in rural areas or in the natural environment or habitat of the reservoir. We will highlight seminal examples of specific human activities associated with outbreaks in human populations.

**The role of agriculture.** In South America, human cases of HPS, Argentine hemorrhagic fever (AHF), and Bolivian hemorrhagic fever (BHF) have been associated with agricultural activities. In the following we briefly discuss two notable outbreaks of arenaviruses associated with agricultural activity. Seven species of arenaviruses are known to cause hemorrhagic fever in humans in South America and Africa, presumably through the inhalation of aerosolized infectious excreta (Walker and Murphy, 1987; Enria and Pinheiro, 2000; Peters, 2002). After its isolation in 1959, JUNV was the second arenavirus (following LCMV in 1933 in the USA) recognized to cause human illness, AHF (Parodi et al., 1958; Childs et al., 2007a). The rodent reservoirs of JUNV, *Calomys*...
*C. musculinus* (dry lands vespertine mouse) and *C. laucha* (small vespertine mouse), are endemic to the Humid Pampas, a farming region in central east Argentina (Mills et al., 1992). *C. musculinus* is believed to be the primary reservoir of JUNV, although antibody to the virus is also detected in *C. laucha* (Mills et al., 1992). Rodent population density is positively correlated with JUNV seroprevalence. From these field studies of the reservoir, the main route of transmission is likely horizontal (fewer infections of juveniles and subadults and there is higher prevalence among males). The emergence of AHF is hypothesized to have occurred due to intensive deforestation and agriculture practices that promoted contact between humans and the rodent reservoirs. Since the first recognition of AHF in 1958, annual epidemics have been reported ranging from several hundred to 3,500 cases, with most cases occurring between April and July (late fall and early winter) (Maiztegui, 1975; Childs et al., 2007a). These outbreaks coincide with the corn harvesting season, which is thought to cause an increase in rodent population densities and to promote human-rodent contact. In fact, AHF mainly affects male rural workers.

Around the same time as JUNV was recognized, human outbreaks of MACV associated with Bolivian hemorrhagic fever (BHF) were reported in Bolivia (Mackenzie, 1965). The reservoir host of MACV is *C. callous* (Kuns, 1965). Outbreaks of BHF emerged in Beni Department, Bolivia during 1959, following political instability that led families living in rural areas to rapidly transition to subsistence agriculture (Mackenzie, 1965). It was reported that an increase in the use of DDT on crops for insect control coupled with abnormally low rainfall led to a decline in cats, resulting in an increase in rodent numbers. After 1959, there were multiple outbreaks in rural communities in Bolivia, which continued until 1964 with the implementation of rodent control measures (mouse traps and poisoning with zinc phosphide) (Mercado, 1975). Rodent control programs developed in San Joaquín during the outbreak of BHF in 1962 killed ca. 3000 *C. callosus* in a span of 4 months, demonstrating that this rodent can acquire high population densities around human habitations. During 18 months of research in Beni, Bolivia cases of BHF were reported in every month of the year, with a distinct increase from January to May a period of intense agricultural activity (rainy season) (Mackenzie, 1965). It was not until 1963 that MACV was isolated from the spleen of a patient who succumbed to disease (Johnson, 1965). Since its emergence, there have been sporadic outbreaks of BHF which have been related to agricultural occupational exposure. Cases are highest between April and July, which are the late rainy and early dry seasons, as *C. callosus* invades homes during the rainy seasons. However, only a handful of cases have occurred since the 1960’s. In 2007-2008, an outbreak of five cases was reported among farmers in Bolivia (Aguilar et al., 2009).

**The role of caves, rural workplaces, and homes.** After the first cases of MARV through direct contact with nonhuman primates, several other large outbreaks occurred throughout central and southern Africa, however, in contrast to the interaction with an intermediate nonhuman primate host discussed above, these cases were associated with patients visiting caves (Gear et al., 1975; Bausch et al., 2006; Towner et al., 2006). The first human cases of *Sudan ebolavirus* (SUDV) likely originated in a cotton factory in Nudar, Sudan in June 1976. In the five months that followed, 284 cases
were reported with a mortality rate of 53%. It is suggested that exposure occurred via excreta from bats roosting in this factory (World Health Organization, 1978a).

Cases of Lassa fever were recognized in the 1950s; however, the virus causing the illness, LASV, was not identified until 1969 when three nurses died from it in a mission hospital in Nigeria (Frame et al., 1970; Monath, 2019). Many of the early outbreaks were localized within hospital wards, where nurses and doctors became exposed to patient bodily fluids (Hambliion et al., 2018; Dan-Nwafor et al., 2019). Now, nosocomial infections are uncommon, and human transmission occurs mainly in village homes through direct contact with rodents or indirectly by consumption of contaminated food products, exposure to surfaces contaminated with rodent excreta, or inhalation of aerosolized virus (Joseph B. McCormick et al., 1987). Additionally, several cases have been recognized to be imported from individuals traveling from West Africa (Overbosch et al., 2020; Wolf et al., 2020). In West African villages where LASV is endemic, homes are usually devoid of food storage spaces such as cabinets (cupboards), so food is stored in plastic buckets or large flour bags on the ground or hung from ceilings or walls (Bonwitt et al., 2017). During the dry season (January–March) when there are limited food resources, *M. natalensis* tends to aggregate in homes in search for food (Arruda et al., 2021). These rodents excrete virus in their urine; therefore, an infected rodent can indirectly transmit the virus by urinating on food products (Walker and Murphy, 1987). More recently, the African wood mouse, *Hylomyscus pamfi*, in Nigeria and Guinea multimammate mouse, *Mastomys erythroleucus*, in both Nigeria and Guinea were recognized as additional hosts (Olayemi et al., 2016).

In the first outbreak of SNV in the summer of 1993 through 1995 in the Four Corners states of the southwestern USA, 69% of the hantavirus pulmonary syndrome (HPS) cases had exposures closely associated with peridomestic activities in homes that showed signs of rodent infestation (Armstrong et al., 1995). A retrospective analysis of the outbreak of SNV during 1998-1999 noted that most HPS case patients reported indoor exposure to deer mice (Hjelle and Glass, 2000). In the fall of 2012, a cluster of cases occurred in Yosemite in deer mice infested tent cabins in Curry Village (Núñez et al., 2014). Similar observations were made in outbreaks in Paraguay, while in Argentina most cases over a 13-year period were associated with agriculture (Martinez et al., 2010), although there are reports suggesting peridomestic exposure (Wells et al., 1997). The role of war. The first recorded outbreak of hemorrhagic fever with renal syndrome (HFRS) associated with the hantavirus, HTNV occurred in soldiers during the Korean War in the early 1950’s and probably resulted from outdoor activities such as sleeping in tents and performing military exercises (Song et al., 2009b). Similarly, cases of HFRS occurred in soldiers during the war in Bosnia (Hukic et al., 1996) and in military training exercises of USA soldiers in Germany (Clement et al., 1996). As noted in ecological surveillances of military sites in Korea (Klein et al., 2011b; Klein et al., 2015), reservoir mice harboring hantaviruses are common in training areas with mortar ranges and troop maneuver/assembly areas and pose a threat for exposure.
**Person-to-person transmission following spillover.** Most human infections with hantaviruses and arenaviruses are associated with indirect exposure of rodent excreta. However, viruses in each genus have had suspected or documented cases of person-to-person transmission. In one report of illness from MACV, a 20-year-old female nursing student living in Cochabamba, Bolivia became ill following travel to her hometown in Fortaleza in Beni Department in December 1970 (Peters et al., 1974). Upon return to Cochabamba, she was hospitalized and died January 28. Subsequently, five individuals who had direct contact with her in the hospital became ill and all died, including her father, nurses, and the pathologist who conducted her autopsy. More recently in 2008, a case of hemorrhagic fever caused by *Lujo mammarenavirus* involved a 36-year-old female travel agent who worked on a peri-urban farm near Lusaka, Zambia. Following illness, she was flown to Johannesburg, South Africa to be treated at a private hospital. There, four healthcare workers were exposed and became ill and three died (Simulundu et al., 2016). Among the hantaviruses, there have been documented cases of person-to-person transmission only for the South American *Andes orthohantavirus* (ANDV) which have occurred in Argentina and Chile (Enría et al., 1996; Wells et al., 1997; Padula et al., 1998; Ferres et al., 2007; Martínez et al., 2020). In 1996, the first human-to-human outbreak of ANDV was reported in southwest Argentina among 18 people (Wells et al., 1997). The outbreak began on September 22, 1996 and lasted until December 5, 1996. The index case was a 41-year-old male who lived in the rural town El Bolsón. Twenty days after he became ill, his doctor became ill, followed by his 70-year-old mother a day later. Subsequently, several cases were reported from small household clusters, nosocomial infections, and from a car trip to the funeral of the 70-year-old mother. No evidence of nosocomial infections has been reported in Chile where ANDV is endemic (Castillo et al., 2004). However, transmission occurs mainly through household contacts and risk is especially high among sexual partners (Ferres et al., 2007).

**Conclusions**

The number of novel or reemerging RNA viruses which cause infections in humans increases each year. While most of these zoonotic infections do not lead to a pandemic, the global public health burden of all combined infections is high. The 2002-2003 SARS-CoV epidemic resulted in more than $40 billion in losses despite causing fewer than 1000 deaths (Institute of Medicine Forum on Microbial, 2004). Predicting the next outbreak, epidemic or pandemic caused by an RNA virus will remain an on-going challenge for the foreseeable future. Predictive models will require a deep understanding of the intrinsic and extrinsic drivers of virus spillover in their native habitat and of how these viruses are maintained in their native reservoir. Whether a virus can persist for the lifetime of a reservoir host or can be cleared, the mechanisms that drive these outcomes is an area in need of expanded investigation, as there is a lack of literature for many reservoir species and viruses in this regard. Experimental animal reservoir models are essential for understanding these host-virus dynamics, and even conservative efforts to expand the taxonomic diversity of experimental models would prove worth-while. Alternative rodent and bat experimental models of infection have provided important information as highlighted herein. In addition to expanding reservoir models, well-
designed, targeted, empirical surveillance studies that incorporate immunological and pathological examinations may provide crucial information about the effect of natural infections. The principal challenges of field studies are largely technical; for example, identification of specific stages of infection of viruses in animals in nature while in the field in is unlikely, and the ability to isolate or detect virus in a persistent infection in the laboratory may be difficult if below the limit of detection, requiring more samples than in standard, controlled laboratory studies. Metagenomic approaches have improved in recent years and may eventually overcome technical limitations of detecting virus in field-sampled, persistently infected reservoirs. However, integration and interpretation of this information with immune response and pathology specific to the virus of interest will be challenging, as many animals collected in the field harbor other pathogens. Hence this underscores the need to establish laboratory colonies of the natural reservoirs to benefit understanding. The difficulties of establishing alternative, reservoir models are numerous and challenging, but important for advancement of understanding biological mechanisms.

In addition to the natural reservoirs, intermediate spillover hosts (e.g., agricultural livestock and wildlife species that are kept as pets or taken for food consumption) play an important role in the transmission network of viruses to humans. As such, intermediate hosts acting as a bridge between natural reservoirs and humans warrants greater attention for advancing our understanding of virus transmission and maintenance from these hosts. In addition to surveillance of bats, efforts need to focus on intermediate hosts as many bat viruses’ transmission to humans relies on these amplifier hosts. Metagenomic surveillance of potential intermediate hosts would provide valuable information on circulating and newly introduced viruses. Maintaining such samples in tissue biorepositories for wildlife specimens in museums is critical for retrospective analyses.

In areas where reservoirs and intermediate hosts are known, control measures should be used to keep these reservoirs in check, such as preventing bats and rodents from contaminating food sources. Controlling the further spread of outbreaks is an important aspect of study to prevent isolated outbreaks from progressing into epidemics or pandemics. One essential feature to controlling outbreaks is understanding transmission routes to limit further spread e.g., aerosol with SARS-CoV-2 and exposure to infected bodily fluids in the case of EBOV. Controlling virus transmission during outbreak scenarios depends on virus population dynamics within the reservoir and intermediate hosts. Understanding virus populations and their variants within reservoir and intermediate hosts are important as inherent mutations and mutations acquired through transmission may lead to an increase in viral fitness, allowing for spillover and increased transmission, as seen as in the case of SARS-CoV and the Asian palm civet. Additionally, host genetics needs to be investigated as host genetic background can influence infection outcome. Some host population members can have a higher likelihood of transmitting a virus compared to others within the same population (e.g., superspreaders). Efforts focused on virus surveillance, virus-host interaction, and virus population changes can be complicated as reservoirs, intermediate hosts, and the viruses they harbor lack necessary reagents for their study, so development of these reagents are essential. In summary, we have addressed 1) why zoonotic viruses reside in bat and rodent reservoirs, 2) environmental and ecological causes driving spillover, and 3) shared
and unique characteristics of previous epizootic events. Further investigation of these topics is a long-term process that will take vigorous effort, but continuous investigation will provide an effective means to understand the relationship between spillover and emergence of bat- and rodent-borne RNA viruses in their hosts.
CHAPTER 3. PREVALENCE OF HANTAVIRUSES HARBORED BY MURID RODENTS IN NORTHWESTERN UKRAINE*

Introduction

In the family Hantaviridae, order Bunyavirales, more than 47 distinct species of hantaviruses have been identified in rodents, moles, shrews, and bats across the globe (Walker et al., 2020). Of these, some species within the genus Orthohantavirus, harbored by rodents, cause serious disease when transmitted to humans. In Europe and Asia, human cases of hemorrhagic fever with renal syndrome (HFRS) occur following aerosol transmission of excreta with hantaviruses carried by two rodent genera, Apodemus and Myodes. Hantaan orthohantavirus (HTNV) is carried by Apodemus agrarius and is found east of the Ural Mountains, in China, and Korea; while Dobrava-Belgrade orthohantavirus (DOBV), is harbored by A. agrarius (striped field mouse) and A. flavicollis (yellow-necked mouse), in numerous European countries (Avsic-Zupanc et al., 1995; Avsic-Zupanc et al., 2000; Golovljeva et al., 2000; Klempa et al., 2005; Klingström et al., 2006; Papa et al., 2006; Jakab et al., 2007; Klempa et al., 2008). In Europe, Puumala orthohantavirus (PUUV) harbored by Myodes glareolus (bank vole) causes a less severe form of HFRS, which is referred to as nephropathia epidemica (NE) (Lähdevirta et al., 1984). In some years, PUUV has caused up to 10,000 human cases in northern Europe and Russia. However, in contrast to HFRS which has a case fatality rate (CFR) of up to 10%, NE disease has a lower CFR (<1%) (Hjertqvist et al., 2010).

Given the geographical location of Ukraine and prior surveillance of hantaviruses in humans and wildlife (Demchyshyna et al., 2020; Lozynskyi et al., 2020), we hypothesized that DOBV and PUUV, or even perhaps novel strains of hantaviruses, circulate in wild rodent populations in Ukraine. We previously reported the presence of antibodies to hantaviruses in the A. agrarius, A. flavicollis, and M. glareolus in a western region of Ukraine (Demchyshyna et al., 2020). In this prior study, however, we did not have specimens available for the genetic identification of the species circulating in Ukraine. We launched a field survey in September and October of 2019 of wild rodents in a rural region of the Volyn Oblast where we had detected antibodies to hantaviruses in rodents collected ten years prior (Figure 3-1).

The goal of this study was to identify the viral species and strains of hantaviruses circulating in western Ukraine. Toward this goal, we conducted a field survey of 424

Figure 3-1. **Survey location in northwestern Ukraine**
The survey was conducted in northwestern Ukraine (top) at two distinct locations (lower left panel), which were ten km from each other: site 1, lines A–D and site 2, lines E–I (lower right panel).
captured small mammals in northwestern Ukraine in order to obtain and identify hantavirus species and the genotypes circulating in Ukraine. We report a high seroprevalence in our sampling region, eight mammalian species were identified with antibodies to hantaviruses, with the highest number of antibody positive mammals being *A. agrarius* (33.9%), *A. flavicollis* (20.5%), and *M. glareolus* (15.7%).

Materials and Methods

Small Mammal Collection

During two weeks in September and October 2019, fifty (Site 1, lines A-D) and twenty-five (Site 2, lines E-I) Sherman traps (7.6 x 8.9 x 22.9 cm, Sherman Trap Company, Tallahassee, FL) were set, 10 m apart, along 500 or 250 m transects. Sites 1 and 2 were located 10 km apart from one another (Figure 3-1). These were staggered across nine collecting sites as effectively as possible (Supplemental Capture Location Coordinates). We ran each line (except line H) for up to 5 nights, for a total of 167 line-nights, or 8,350 trap-nights with various success rates per line (Supplemental Capture Line Success Rate).

Each small mammal was identified morphologically to species and weight, total length, tail, ear, and hind foot lengths were measured. Species were identified in the field. The sex and categorical age (juvenile, adult) were also recorded (Supplemental Capture Small Mammal Database). Lung, heart, kidney, muscle, and blood were harvested and stored immediately in liquid nitrogen, prior to transport to the Virology Department at the Public Health Center in Kyiv, where the samples were stored at -80°C until processing.

Immunofluorescence Assay (IFA)

An indirect IFA was used to screen blood samples for antibodies that cross-reacted with PUUV, HTNV or DOBV grown in Vero E6 cells as described previously (Chu et al., 2003; Demchysyn et al., 2020). Briefly, whole blood samples were diluted 1:10 in PBS in a 96-well plate and incubated for 30 min at 37°C on acetone-fixed, 10-spot slides. Slides were washed copiously in PBS. Twenty-microliters of a 1:500 dilution of Alexa Fluor 488 F (ab`)2 Fragment rabbit anti- mouse IgG (H+L) (Invitrogen) was added and incubated for 30 min at 37°C. Slides were washed copiously in PBS and each well was scored as positive or negative using a 20x objective with a Nikon epifluorescence microscope. Positive blood samples were titrated to end-point by a two-fold serial dilution of blood from 1:32 through 1:8192 and tested and scored microscopically for the characteristic punctate staining of hantaviruses.
Statistical Analyses

Descriptive statistics were used to describe the distribution of captured rodents and prevalence of hantavirus infection using GraphPad (Prism 9 version 9.1). We analyzed the prevalence of hantaviruses detected by hantaviral Ab/RNA with sex, age, or weight as independent variables using the Fishers Exact Test to calculate the p-value and the odds ratio and its confidence interval.

Ethics Statements

All animal procedures were approved (ACUP No. 18-108) by the UTHSC Institutional Animal Care and Use Committee (IACUC), which follows the 8th Edition of the Guide for the Care and Use of Laboratory Animals (Guide), NRC 2011, and the Animal Care and Use Committee guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. The study did not involve endangered or protected species. The study was conducted as part of the routine rodent surveillance program of the Ministry of Health.

Results

Distribution of Small Mammal Species

A total of 424 small mammals were captured along nine distinct lines (A-I) where Sherman trap lines were set at 10 meters apart. Lines A-C were set in a forest and line D was set adjacent to a four-lane roadway in shrubs, the roadway separated lines A and B from line C and D (Figure 3-1). Lines E, F, and I were placed in a forest and lines G and H were placed in shrubs and grasslands near the forest’s edge nearby small agricultural farms (Figure 3-1). The collection was completed over two weeks in the last week of September and the first week of October 2019 and over that period we captured small mammals representing seven genera and nine species (Table 3-1 and Figure 3-2). These include species from the orders, Carnivora, Eulipotyphla, and Rodentia. Line D had majority of captures with 120 animals (28%; 95%CI: 24.22%-32.77%), followed by line C with 92 animals (22%; 95%CI: 18%-25%). Only one rodent was captured from line H during the first night of trapping therefore, this line was moved to another field site and was omitted from diversity studies, where mentioned. The M. glareolus, 45% (95%CI: 40.38%-49.81%) was the most captured small mammal in total followed by the A. flavicollis (29%; 95%CI: 24.89%-33.50%) and then A. agrarius (15%; 95%CI:11.58%-18.30%).
Table 3-1. Distribution of captured small mammal species by line

<table>
<thead>
<tr>
<th>Species</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apodemus agrarius</em></td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>20</td>
<td>3</td>
<td>0</td>
<td>21</td>
<td>1</td>
<td>2</td>
<td>62</td>
</tr>
<tr>
<td><em>Apodemus flavicollis</em></td>
<td>19</td>
<td>16</td>
<td>18</td>
<td>42</td>
<td>7</td>
<td>13</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>123</td>
</tr>
<tr>
<td><em>Microtus arvalis</em></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td><em>Microtus subterraneus</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Muscardinus avellanarius</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Myodes glareolus</em></td>
<td>9</td>
<td>24</td>
<td>51</td>
<td>39</td>
<td>24</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>17</td>
<td>191</td>
</tr>
<tr>
<td><em>Sorex minutus</em></td>
<td>5</td>
<td>0</td>
<td>10</td>
<td>15</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>40</td>
<td>40</td>
<td>92</td>
<td>120</td>
<td>37</td>
<td>39</td>
<td>29</td>
<td>1</td>
<td>26</td>
<td>424</td>
</tr>
</tbody>
</table>

Figure 3-2. Distribution of captured small mammal species by line

Small mammals were captured from one of nine capture lines between two capture locations. *Apodemus agrarius* (blue), *Apodemus flavicollis* (red), *Microtus arvalis* (green), *Microtus subterraneus* (purple), *Mus musculus* (orange) *Muscardinus avellanarius* (black), *Mustela nivales* (brown), *Myodes glareolus* (navy), *Sorex minutus* (dark purple).
Evidence and Distribution of Antibodies to Hantavirus by Line

Capture lines varied amongst each other based on the number of captured animals, and the number of hantavirus-positive animals (Tables 3-2 and Figure 3-3). Molecular diagnostics showed that 79 of the animals tested positive for the presence of hantavirus by serology. Of the small mammals that were captured and tested by IFA for the presence of hantavirus antibodies, only mammals from the orders Eulipotyphla and Rodentia tested positive. Antibody-positive animals were captured on every line except for line B. The most hantavirus seropositive animals were captured on line C with 23 animals (29%; 95%CI: 20%-40%) followed by line D with 16 animals (20%; 95%CI: 13%-30%). There was no difference between the number of captured male and female animals that were positive for hantavirus antibodies, (Odds ratio: 1.087; 95%CI: 0.6475-1.819; p = 0.7905). Adults (83%) made up the largest age group of small mammals captured with only 73 (17%) being juvenile (data not shown). The distribution of the endpoint reciprocal titer of the antibody-positive animals is presented in (Table 3-3 and Figure 3-4). IFA reciprocal titers showed a vast distribution ranging from 1:32 to 1:8192 indicating that recent reservoir infections are occurring. In addition to the *Myodes* and *Apodemus* species, we identified one *Sorex minutus*, one *Microtus arvalis*, and one *Mus musculus* that were antibody positive.

Rodent Diversity Within Each Line

The Shannon index was calculated for each line as a measure of the species diversity (Table 3-4 and Figure 3-5). Line A had the highest Shannon index value (1.46) and had a proportion of hantavirus (HV) value of 0.2 which was the average for all the lines. Line D had the second highest Shannon index value (1.44) but had the lowest proportion of HV at 0.1 and the line where most small mammals were captured. There was no relationship found between Shannon index and the number of HV positive animals ($R^2=0.25$) or the proportion of HV positive animals ($R^2=0.31$).

Association of Population Structure and Prevalence of Antibody to Hantavirus in *Myodes glareolus* and *Apodemus* Species

The distribution of the presence of antibodies to hantavirus was stratified by categorical age and sex (Table 3-5 and Figure 3-6). Amongst the three most abundantly captured rodents neither sex nor age was associated with the rodents being hantaviral positive. We noted a similar level of antibody in juvenile and adults in both male *Apodemus* species. This trend did not hold in the female as the numbers of juveniles captured were much lower and so no comparative results can be drawn. Overall a similar level of antibody presence was measured in male or female adults within each species.

The weight class of the three small mammal species that had the greatest number of animals that were hantavirus seropositive were 11-15 g for *A. agrarius* and 21-25g for
Table 3-2. Distribution of antibody-positive small mammal species by line

<table>
<thead>
<tr>
<th>Species</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apodemus agrarius</em></td>
<td>2(4)</td>
<td>0</td>
<td>3(11)</td>
<td>3(20)</td>
<td>1(3)</td>
<td>0</td>
<td>11(21)</td>
<td>1(1)</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td><em>Apodemus flavicollis</em></td>
<td>3(19)</td>
<td>0</td>
<td>5(18)</td>
<td>8(42)</td>
<td>3(7)</td>
<td>4(13)</td>
<td>0</td>
<td>0</td>
<td>2(6)</td>
<td>25</td>
</tr>
<tr>
<td><em>Microtus arvalis</em></td>
<td>0</td>
<td>0</td>
<td>1(2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Microtus subterraneus</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>1(1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Muscardinus avellanarius</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Mustela nivalis</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Myodes glareolus</em></td>
<td>2(9)</td>
<td>0</td>
<td>14(51)</td>
<td>4(39)</td>
<td>5(24)</td>
<td>3(25)</td>
<td>0</td>
<td>0</td>
<td>2(17)</td>
<td>30</td>
</tr>
<tr>
<td><em>Sorex minutus</em></td>
<td>0</td>
<td>0</td>
<td>1(10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>8</td>
<td>0</td>
<td>23</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>11</td>
<td>1</td>
<td>4</td>
<td>79</td>
</tr>
</tbody>
</table>

Figure 3-3. Distribution of hantavirus seropositive small mammal species
Distribution of hantavirus antibody negative (black) and positive (magenta) small mammals based on (A) capture lines and (B) species.
Table 3-3. Distribution of IFA reciprocal titers in rodent reservoir species of PUUV and DOBV

<table>
<thead>
<tr>
<th>Species</th>
<th>1:32</th>
<th>1:64</th>
<th>1:128</th>
<th>1:256</th>
<th>1:512</th>
<th>1:1024</th>
<th>1:2048</th>
<th>1:4096</th>
<th>1:8192</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apodemus agrarius</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Apodemus flavicollis</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Microtus arvalis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myodes glareolus</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>9</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sorex minutus</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3-4. Distribution of IFA reciprocal titers in rodent reservoir species of PUUV and DOBV

The blood from each captured small mammal was used to screen for antibodies that cross-reacted with PUUV, HTNV, or DOBV to determine IFA reciprocal titers 1:32 (blue), 1:64 (red), 1:128 (green), 1:256 (purple), 1:512 (orange) 1:1024 (black), 1:2048 (brown), 1:4096 (navy), 1:8192 (dark purple) for each species.
Table 3-4. Overall Hantaviral Ab status and Shannon diversity in captured rodents by line

<table>
<thead>
<tr>
<th>Line</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>HV-Negative</td>
<td>32</td>
<td>40</td>
<td>69</td>
<td>104</td>
<td>28</td>
<td>32</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>HV-Positive</td>
<td>8</td>
<td>0</td>
<td>23</td>
<td>16</td>
<td>9</td>
<td>7</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Total No. Rodents</td>
<td>40</td>
<td>40</td>
<td>92</td>
<td>120</td>
<td>37</td>
<td>39</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>Proportion of HV</td>
<td>0.2</td>
<td>0.0</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>SHANNON (H)</td>
<td>1.46</td>
<td>0.67</td>
<td>1.24</td>
<td>1.44</td>
<td>1.00</td>
<td>0.75</td>
<td>0.95</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Figure 3-5. Relationship between proportion of seropositive animals at each capture line and Shannon Index
Table 3-5.  *A. agrarius*, *A. flavicollis*, and *M. glareolus* captured according to age, sex, and Ab prevalence

<table>
<thead>
<tr>
<th>Age/Sex</th>
<th>Total No.</th>
<th>Total No. Ab Pos</th>
<th>% Pos</th>
<th>Total No.</th>
<th>Total No. Ab Pos</th>
<th>% Pos</th>
<th>Total No.</th>
<th>Total No. Ab Pos</th>
<th>% Pos</th>
</tr>
</thead>
<tbody>
<tr>
<td>MALE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>3</td>
<td>1</td>
<td>33.3</td>
<td>22</td>
<td>4</td>
<td>18.2</td>
<td>35</td>
<td>6</td>
<td>16.7</td>
</tr>
<tr>
<td>Adult</td>
<td>31</td>
<td>11</td>
<td>35.5</td>
<td>60</td>
<td>14</td>
<td>23.3</td>
<td>108</td>
<td>16</td>
<td>15.0</td>
</tr>
<tr>
<td>FEMALE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juvenile</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>14.3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adult</td>
<td>26</td>
<td>9</td>
<td>34.6</td>
<td>33</td>
<td>6</td>
<td>18.2</td>
<td>47</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>21</td>
<td></td>
<td>122</td>
<td>25</td>
<td></td>
<td>191</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-6.  Distribution of male and female hantavirus antibody positive small mammals
Number of hantavirus antibody positive small mammals based on sex, male (black) and female (magenta).
A. flavicollis and 16-20g for the M. glareolus (Figure 3-7). This weight range suggests that most of these were subadults or adults.

Discussion

The prevalence of HFRS cases in humans remains unknown for Ukraine, although recent research reported a 1.6% seroprevalence of hantaviral antibody in 966 healthy individuals within a small locality in Lviv Oblast, Ukraine (Lozynskyi et al., 2020). The lack of information on diseases caused by hantaviruses is two-fold, firstly there have been few surveillance efforts on the reservoirs of viruses that cause these diseases, and secondly, there is lack of molecular tools for-diagnostic identification of the causative virus strains in the region. The prevalence of reservoir hosts of hantaviruses and the incidence of cases in neighboring countries suggests that hantaviruses are circulating within the country although there has only been one previous study which reported the seroprevalence within humans and the rodent reservoir (Demchyshyna et al., 2020; Lozynskyi et al., 2020). Our previous work studying antibody prevalence in in Volyn during spring and autumn reported a seroprevalence of 2.2% (9/404) in A. agrarius and 7% (31/433) in M. glareolus. Our current 2019 survey conducted in autumn showed a seroprevalence of 33.9% (21/62) in A. agrarius, 20.5% (25/122) in A. flavicollis, and 15.7% (30/191) in M. glareolus. With this study, we show that for people living in Western Ukraine, there is a high potential for HFRS caused by PUUV. Our results will enable the development of qRT-PCR assays to confirm diagnosis in febrile patients with symptoms suggestive of the spectrum of illness caused by hantaviruses.

Apart from our previous study that suggested hantaviruses are present in Ukraine, little is known about the species or associated disease. All of the countries surrounding Ukraine (Belarus, Hungary, Moldova, Poland, Russia, Romania, and Slovakia) have reported cases of HFRS, but the species or genotype of the viruses that caused the disease or the reservoir of the virus is not known in some cases (Bilcíková et al., 1989; Mikhaïlenko et al., 1994; Maftei et al., 2012; GeurtsVanKessel et al., 2016; Tkachenko et al., 2019). Most hantavirus identifications reported in these studies have focused on humans.

Only a small number of field trapping surveys have been conducted in surrounding countries. In a large field survey spanning multiple districts southwest of where this study was conducted in the Subcarpathia region in Poland, 194 rodents were captured with the most abundantly captured rodents consisting of A. agrarius, A. flavicollis and M. glareolus as was in this study (Michalski et al., 2014). From the 194 captured rodents 17 showed evidence of hantavirus infection by PCR and/or IFA for DOBV, PUUV, and TULV. In this study the percentage of positive rodents were half of the current study. In another field survey in western Poland which captured 106 small mammals, the most abundantly captured rodents (A. agrarius, A. flavicollis and M. glareolus) were the same as our study but only five of the animals tested positive for RNA and these animals tested positive for DOBV and Seewis orthohantavirus (SWSV) (Lee et al., 2020). We not only identified PUUV in M. glareolus but we also identified
Figure 3-7.  Weight range of rodents and percent hantavirus for *A. agrarius*, *A. flavicollis* and *M. glareolus*
Number of animals that were hantavirus antibody positive (magenta) and negative (black) based on their weight classes (g).
PUUV sequences within the reservoir host of DOBV. Our study shows that reservoir hosts can act as spillover hosts to harbor other species of hantaviruses. Lastly in Northeast Poland a field survey was conducted that led to the first reported isolation of PUUV in Poland, where three captured rodents out of 45 tested positive for PUUV (Ali et al., 2014).

This study describes the circulation of PUUV in multiple rodent species in Ukraine which is likely an important driver of HFRS cases. This work emphasizes the necessity of field surveys to identify viruses that will allow for the development of more accurate molecular diagnostic tools for suspected HFRS cases and to provide a further understanding of the impact these viruses have on human health.
CHAPTER 4.  THE IMMUNE RESPONSE OF HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELLS TO PATHOGENIC AND NONPATHOGENIC ORTHOHANTAVIRUS INFECTIONS

Introduction

The RNA viruses within the genus Orthohantavirus, subfamily Mammantaviridae, family Hantaviridae are negative-sense, single-stranded RNA viruses with tri-segmented genomes (Schmaljohn and Dalrymple, 1983; Maes et al., 2018; Laenen et al., 2019). Hantaviruses are harbored by reservoir hosts from the order Rodentia and Eulipotyphla which persist for the life of the animal (Jonsson et al., 2010; Plyusnin and Sironen, 2014). Transmission and infection of humans from rodents occurs predominantly from the inhalation or consumption of excreta containing virus during peridomestic activities. While not all hantaviruses cause human disease, several species may cause one of two different syndromes, hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS) (Lee et al., 1978; Nichol et al., 1993; Avšič-Županc et al., 2019). During the early stages of illness, HFRS and HPS cases present with high fever and hypercytokinemia caused by robust activation of the immune system, extensive vascular leakage, and thrombocytopenia (Duchin et al., 1994; Linderholm et al., 1996; Borges et al., 2008; Macneil et al., 2011; Saksida et al., 2011; Outinen et al., 2016). Immune responses in patient blood sera shows a robust activation of the innate immune responses with high levels of interferons (IFN-β and IFN-γ) and other cytokines and chemokines including, interleukin-1 (IL-1), IL-6, IL-10, tumor necrosis factor-α (TNF-α), C-C Motif Chemokine Ligand 5 (CCL5), C-X-C Motif Chemokine Ligand 10 (CXCL10), CXCL11, and vascular endothelial growth factor (VEGF) (Gavrilovskaya et al., 2012; Wang et al., 2012; Morzunov et al., 2015; Angulo et al., 2017; Maleki et al., 2019). The innate immune response is followed by an influx of cytotoxic T lymphocytes (CTLs) to the sites of infection which correlates with disease severity (Lewis et al., 1991; Kilpatrick et al., 2004; Saksida et al., 2011). Natural killer (NK) cells are highly activated during infection, and their cell numbers in sera remain high for a long period (60 days) after the onset of disease symptoms (Björkström et al., 2010; Braun et al., 2014). Strong humoral responses are activated following infection as high viral protein specific Immunoglobulin M (IgM) and IgG antibodies titers are observed during the acute phase of infection (Lundkvist et al., 1993; Groen et al., 1994; Bostik et al., 2000; MacNeil et al., 2010) Death in HFRS cases is usually attributed to renal failure which occurs in up to 40% of the cases (Kim et al., 2007; Turčinov et al., 2013). In autopsies of HPS and HFRS cases, hantaviral antigen is prevalent in the lower respiratory tract in epithelial cells, and microvascular endothelial cells in the kidneys (HFRS) and the lungs (HPS) (Poljak and Županc, 1994; Zaki et al., 1995; Settergren et al., 1997).

The mechanisms used by hantaviruses to modulate the innate response in humans has been an active area of study. To probe virus-host interactions, surrogate cell types have been employed including A549 lung epithelial cells (Handke et al., 2009; Witkowski et al., 2016), human embryonic kidney (HEK) 293 cells (Alff et al., 2006;
Taylor et al., 2009a), primary human umbilical cord vein endothelial cells (HUVECs) (Geimonen et al., 2002; Prescott et al., 2005; Alff et al., 2006; Alff et al., 2008; Khaiboullina et al., 2016) and primary human lung microvascular endothelial cells (HLMVEC) (Sundstrom et al., 2001; Spiropoulou et al., 2007). Together, the research suggests the hantaviral glycoproteins (GnGc) and the nucleocapsid (N) protein play key roles in the downregulation of the antiviral immune responses. The Gn-tail of the glycoproteins (GnGc) and the nucleocapsid (N) protein have been implicated in inhibition of Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) cascades at multiple steps such as blocking TANK Binding Kinase 1 (TBK1) complex formation (Alff et al., 2006; Matthys et al., 2014), nuclear factor kappa B (NF-κB) (Ontiveros, Li, & Jonsson, 2010) and interferon regulatory factor (IRF) nuclear translocation (Alff et al., 2006; Taylor et al., 2009a; Ontiveros et al., 2010; Matthys et al., 2014). The inhibition of pattern recognition receptor (PRR) signaling by the virus is essential for replication because hantaviruses are sensitive to IFN responses (Alff et al., 2006). In contrast, pathogenic viruses such as Andes Orthohantavirus (ANDV), Hantaan Orthohantavirus (HTNV), and Sin Nombre orthohantavirus (SNV) have been shown to delay the proinflammatory host response by blocking PRR signaling (Geimonen et al., 2002; Alff et al., 2006; Spiropoulou et al., 2007; Alff et al., 2008; Matthys et al., 2014).

As stated above, prior studies have contributed greatly to our understanding of hantaviral-host interactions, however, all studies have only examined two to three time points post-infection and have used numerous cell types. Therefore, understanding the dynamics of host responses remains an important gap in our knowledge. To further advance insight into the dynamics of the proinflammatory and anti-inflammatory responses of primary HLMVEC following infection with pathogenic and nonpathogenic hantaviruses, we made an extensive evaluation of the innate immune responses every 12 hours following infection with ANDV, HTNV, or PHV. We evaluated 39 genes at six timepoints. Few showed upregulation and included CCL5, CXCL10, CXCL11, IDO1, IRF7, TLR3 and IFNB1. We confirmed these results by examining secreted proteins levels for CCL5, CXCL10, CXCL11, IDO, and IFN-β. To further validate our findings, we expanded our studies to one additional male and two female donors. Notably, female donors produced higher levels of CXCL10, IDO and IFN-β than males. Moreover, the magnitude of the response was not similar across ANDV, HTNV, and PHV. Statistical pairwise analysis of protein levels suggests that virus species and individual HLMVEC characteristics, together, contribute to the outcomes. Lastly, we examined the hantaviral suppression immune responses involved with cell death for all three viruses and report that only ANDV inhibited cell death in HLMVECs.

Materials and Methods

Cells and Viruses

HLMVECs (Cell Applications Inc., or Promo Cell) were cultured in HMVEC Growth Medium (Table 4-1). All experiments were performed with cells passaged four
### Table 4-1. Characteristics of HLMVEC donors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>2559</th>
<th>2572</th>
<th>2551</th>
<th>438Z013.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Age</td>
<td>42</td>
<td>19</td>
<td>34</td>
<td>53</td>
</tr>
<tr>
<td>Race</td>
<td>Caucasian</td>
<td>Hispanic</td>
<td>Hispanic</td>
<td>Caucasian</td>
</tr>
</tbody>
</table>
to six times. Primary HLMVECs were seeded at a density of 40,000 cells/cm² on plates coated with collagen as specified by the manufacturer. All experiments were performed 12-16 h after seeding. ANDV (strain Chile-9717869), HTNV (strain 76-118) and PHV were a kind gift of Connie Schmaljohn at the United States Medical Research Institute of Infectious Diseases. Viruses were amplified in Vero E6 cells (ATCC CRL-1586) in Earls’ Modified Essential Medium (EMEM, Corning) with 10% FBS (Gibco), 5 mM L-glutamine (Corning) and 1% Penicillin-Streptomycin (Gibco). Virus titers were determined by plaque assay (Jonsson and Yong K. C. Ontiveros, 2011). Cells and viruses were checked for Mycoplasma spp. using LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich). All work with virus was conducted at the University of Tennessee Health Science Center (UTHSC) Regional Biocontainment Laboratory (RBL) in BSL-3 containment.

**Immunofluorescence Microscopy**

HLMVECs, donor 2559, were seeded on glass coverslips in a 24-well plate and infected ANDV, HTNV, or PHV at an MOI of 2 for 1 h and rocked every 15 min, after infection cells were washed twice with Hank’s balanced saline solution (HBSS, Cell Applications Inc.) to remove residual virus from each well and incubated at 37°C, 5% CO₂. At 24 and 72 hpi, cells were washed with HBSS and fixed for 15 min at RT with 4% paraformaldehyde and 0.25 M sucrose, paraformaldehyde solution was neutralized by washing cells three times with 50 nM ammonium chloride. Cells were permeabilized with a 0.2% Triton-X, 1% bovine serum albumin (BSA) solution and blocked for 1 h with 5% goat calf serum, 0.2% Saponin. Cells were incubated with primary antibody in 0.2% Saponin, 1% BSA for 2 h at RT or overnight at 4°C. For ANDV and HTNV, a rabbit polyclonal antibody against their N protein was used and for PHV, a mouse monoclonal antibody (10R-2502, Fitzgerald) Puumala N protein was used. Cells were incubated with anti-secondary antibody conjugated AlexaFlour 488 (Invitrogen) for 1 h at RT. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) for 2 minutes. Coverslips were mounted and visualized using a Zeiss 710 at the UTHSC Imaging Center and analyzed using Zeiss Zen (Black edition, version 2.3).

**Gene Expression Assay**

A custom QuantiGene Plex Assay (Invitrogen) consisting of 41 analytes containing two endogenous controls was used to measure gene transcript levels. The genes included in the panel were IRF3, IRF7, IFNA1, IFNB1, IFNG, NFKB1, TLR3, TLR7; proinflammatory cytokines, IL1A, IL1B, IL6, IL8, IL12A, IL15, TNF; proinflammatory chemokines, CCL2, CCL5, CCR7, CXCL10, CXCL11; proinflammatory costimulatory molecules, CD11B, CD14, CD200R1, CD80; proinflammatory metabolites ARG1 and NOS2; anti-inflammatory cytokines IL1RN, IL10, TGFβ1; anti-inflammatory chemokine CCL22; anti-inflammatory costimulatory molecule, CD274; anti-inflammatory metabolites IDO1 and PPARγ; apoptosis and endothelial cell signaling; CASP3, CASP7, CASP8, VEGFA and the endogenous controls HPRT1 and TBP.
HLMVECs, donor 2559, were seeded in 48-well plates precoated with collagen (Cell Application Inc.). HLMVECs, donor 2559, were infected with ANDV, HTNV, or PHV at an MOI of 2 for 1 h, rocked every 15 minutes, washed twice with HBSS to remove residual virus from each well, and incubated at 37°C, 5% CO₂. Virus and mock infected cell monolayer were collected at 12, 24, 36, 48, 60, and 72 hpi using lysis mixture solutions (Invitrogen) and stored at -80°C until completing the assay which was performed following manufacturer’s instructions. Assay endpoints were measured on a MagPix (Luminex). Raw results were analyzed by subtracting values measured from water substituted samples from all experimental sample values to which a constant value was added. All samples were normalized to endogenous controls and the mean from the biological repeats were calculated which was used to calculate fold change.

Multiplex Immunoassay

A custom ProcartaPlex immunoassay (Invitrogen) consisting of CCL5, CXCL10, CXCL11, IDO and IFN-β five analytes were purchased Invitrogen. HLMVECs, from four donors (Table 4-1), were seeded in collagen (Cell Application Inc.) coated 48-well plates and infected with ANDV, HTNV, or PHV at an MOI of 2 for 1 h and rocked every 15 min, after infection cells were washed with HBSS to remove residual virus and incubated at 37°C, 5% CO₂. At 48 hpi and 60 hpi supernatant was removed from wells and centrifuged for 5 min at 220 x g to which Halt Protease Inhibitor Cocktail (Thermo Fisher) was added and the assay was performed immediately following collection following manufacturer’s instructions. The assay was read on a MAGPIX instrument (Luminex).

Caspase-3/7 Glo Activity Assay

HLMVECs, donor 2559, were seeded in collagen coated 96-well plates and infected with ANDV at an MOI of 2 for 1 h and rocked every 15 min, after initial infection cells were washed with HBSS to remove residual virus from each well and incubated at 37°C, 5% CO₂. At 60 hours post-infection, 1 µM staurosporine (Sigma-Aldrich) was added and incubated for six h. After incubation Caspase-3/7 Glo was added to each well and incubated at RT for 30 min from which luminescence was read on an EnVision 2104 Multilabel Reader (PerkinElmer).

Results

Infection of a Male HLMVEC Donor by ANDV, HTNV, or PHV Hantaviruses

As microvascular endothelial cells serve as the primary site of replication of hantaviruses, we obtained primary HLMVECs from a male, Caucasian 42-year-old, male donor 2559 to evaluate hantavirus replication. Our preliminary studies with this donor
suggested that a seeding density of $4 \times 10^5$ cells/well and MOI of 2 was optimal for infection. Based on these results, we evaluated infection of ANDV, HTNV, and PHV in HLMVEC donor 2559, microscopically. We used microscopy as we wanted to evaluate whether the number of cells infected were at a similar levels as this is required for comparative studies of the host response to ANDV, HTNV, or PHV. Cells were plated in triplicate, infected at an MOI of 2. At 24 and 72 hpi, cells were assayed microscopically by immunostaining for viral N protein using primary polyclonal antibodies to N protein (Figure 4-1A) (Ramanathan and Jonsson, 2008). Total number of cells were counted by counting DAPI stained nuclei and cells that had the characteristic punctate and/or perinuclear staining for the N protein were counted as infected (Figure 4-1B). At 24 hpi, all wells showed a similar number of cells with N protein with ANDV having infected 50% of the cells whereas both HTNV and PHV infected 45% of the cells. A similar percentage of cells in all wells were infected at 72 hpi with no change seen from 24 hpi with ANDV having infected 46% of the cells, HTNV having infected 44% of the cells, and PHV having infected 39% of the cells. We evaluated the results of each well using T-test analysis, which showed there was no significant differences in the percent cells infected at 24 and 72 hpi for ANDV ($p = 0.22$), HTNV ($p = 0.72$), or PHV ($p = 0.68$). These results suggested that the donor would be appropriate for the proposed comparative studies of host response.

**Gene Expression Profiles of a Male HLMVEC Donor Following Infection by ANDV, HTNV, or PHV**

A great deal of knowledge has been gained using surrogate cell-based models with hantaviruses (Matthys and Mackow, 2012; McAllister and Jonsson, 2014). However, as most studies have evaluated only two to three timepoints, the dynamics of these responses are not known. This level of resolution is critical for revealing the magnitude and timing of the differences over time. We selected 39 genes to capture events during infection such as pathogen recognition (i.e., TLRs), innate immune responses (i.e., IFNs, IL6) and endothelial cell activation (i.e., VEGF, CD274).

We infected HLMVEC, donor 2559 with ANDV, HTNV, or PHV at an MOI of 2. At 12, 24, 36, 48, 60, and 72 hpi, cells were resuspended in lysis buffer mixture and processed for QuantiGene Plex assay analyses. Results were normalized to an endogenous controls and average fold change calculated. The fold changed results revealed that mRNA levels were significantly upregulated for TLR3, IRF7, IFNB1, CCL5, CXCL10, CXCL11, and IDO1 following infection by ANDV, HTNV, and PHV (Figure 4-2). No increases in mRNA transcript levels were observed for IFNA1, IFNG, IRF3, NFKB1, TLR4, TLR7, IL1A, IL1B, IL6, IL8, IL12A, IL15, TNF, CCR5, CD14, CD200R1, CD80, CD86, ITGAM, ARG1, NO2, IL1RA, IL10, TGFBI, CCL22, CD274, PPARG, CASP3, CASP7 and VEGFA. Fold change results of each gene by each virus were assessed using a principal component analysis (PCA) which suggested clusters based on the measurement timepoint and virus species (Figure 4-3).
HLMVECs infected by ANDV, HTNV, or PHV at 24 hpi and 72 hpi
HLMVECs from donor 2559 were infected with ANDV, HTNV, or PHV with an MOI of 2 (n=3). At 24 hpi and 72 hpi infected HLMVECs (n=3) were fixed and immunofluorescent stained for cell’s nuclei with DAPI and virus NP antibody and visualized on a confocal microscope (A) HLMVECs infected by ANDV, HTNV, or PHV at 24 hpi stained for virus NP and cell nuclei (20x). (B) Percent HLMVECs infected by ANDV, HTNV, or PHV at 24 and 72 hpi. No differences were observed between the percentage of cells infected between viruses and timepoints. Error bars are mean and standard deviation.
Figure 4-2. Selected PRR, cytokines and chemokines showing upregulation at 12, 24, 36, 48, 60, and 72 hpi in HLMVECs following infection with ANDV, HTNV, or PHV. HLMVECs from donor 2559 were infected with ANDV (black), HTNV (magenta), or PHV (teal) with an MOI of 2 (n=3). At each time point mRNA levels were measured and analyzed as relative fold change to mock infected cells. Error bars are mean and standard deviation.
Figure 4-3. Principle component analysis of normalized mRNA levels of 39 genes in HLMVECs at 12, 24, 36, 48, 60, and 72 hpi following infection with ANDV, HTNV, or PHV.

HLMVECs from donor 2559 were infected by ANDV (circles), HTNV (triangles), or PHV (squares) with an MOI of 2. At 12 (green), 24 (pink), 36 (blue), 48 (gold), 60 (orange), and 72 hpi (teal) mRNA levels were measured and normalized to endogenous controls and analyzed by principal component analysis (n=3).
We used an ANOVA to test for differences in levels of TLR3, IRF7, IFNB1, CCL5, CXCL10, CXCL11, and IDO1 upregulated by each virus for the six time points. At 60 hpi, TLR3 mRNA levels induced by HTNV-infected cells were significantly higher than those of ANDV-infected cells (p = 0.005). However, there was no significant difference at 60 hpi between HTNV and PHV (p = 0.45). TLR3 levels at 72 hpi and were significantly higher for HTNV-infected cells in compared to ANDV- and PHV-infected cells (p<0.0001 and p = 0.017, respectively).

As expected, because IRF7 is upregulated via TLR3, mRNA levels of IRF7 were significantly higher at 36 hpi in ANDV- and HTNV-infected cells compared to PHV-infected cells (p = 0.039 and p = 0.0001, respectively). IRF7 levels were also significantly higher in HTNV-infected cells compared to ANDV- and PHV-infected cells at both 60 hpi (p <0.0001 and p = 0.0005, respectively) and 72 hpi (p <0.0001 and p <0.0001, respectively).

CCL5 mRNA levels in ANDV-infected cells was significantly higher compared to PHV at 48 hpi (p = 0.0051). In HTNV-infected cells at 72 hpi levels of CCL5 were significantly higher compared to ANDV- and PHV-infected cells (p <0.0001 and p <0.0001, respectively).

The mRNA levels of CXCL10 differed significantly at 36 hpi where levels were significantly higher in ANDV-infected cells as compared to HTNV- and PHV-infected cells (p = 0.006 and p = 0.0075, respectively). However, at 72 hpi CXCL10 levels were significantly higher in HTNV-infected cells as compared to PHV-infected cells (p = 0.279).

CXCL11 mRNA levels were significantly lower in ANDV-infected cells compared to HTNV- and PHV-infected cells at 60 hpi (p = 0.0054 and 0.0278, respectively) and 72 hpi (p<0.0001 and p = 0.006, respectively).

IDO1 mRNA levels in PHV-infected cells were significantly lower compared to the levels of IDO1 in ANDV-infected cells at 36 hpi (p = 0.0227). Interestingly, IDO1 mRNA levels at 48 hpi in PHV-infected cells were significantly lower compared to ANDV- and HTNV-infected cells (p = 0.0007 and p = 0.073, respectively).

IFNB1 mRNA in ANDV-infected cells were significantly higher compared to HTNV- and PHV-infected cells at both 36 hpi (p = 0.00011 and p = 0.0126, respectively) and 48 hpi (p = 0.0005 and 0.0088, respectively).

Immune Responses of Male and Female Donors Following Infection

We hypothesized that genetic differences in donors may potentially confound host response to ANDV, HTNV, and PHV. Case studies of HFRS and HPS patients show that immune responses can differ between patients such that females have higher mortality rates compared to males. Hence, we obtained HLMVECs from two female and one additional
male donor, resulting in two donors from males ages 19 and 42, and two donors from female ages 34 and 53 (Table 4-1). Based on the prior results reported above, we chose to measure protein levels of CCL5, CXCL10, CXCL11, IDO and IFN-β at 48 and 60 hpi. Each donor was infected with ANDV, HTNV, or PHV at an MOI of 2.

Secreted protein measured during infection of each of the four HLMVEC donors by ANDV, HTNV, or PHV showed that each of the five measured proteins were upregulated at 48 hpi and 60 hpi (Figure 4-4). Principle component analyses of the protein levels secreted by each donor with each virus, suggested that there was no clear clustering based on either donors’ characteristics or virus species (Figure 4-5). ANOVA of these data suggested the donor characteristic and virus species contribute to the heterogenous immune responses observed during infection of the four donors during infection by each hantavirus species. Stratifying measured protein levels by donor sexes and analyzing by ANOVA revealed in female donor cells, specific virus species induced higher levels of CXCL10 (ANDV at 48 HPI), IDO (ANDV at 48-60 hpi; PHV at 60 hpi) and IFN-β (ANDV, HTNV at 60 hpi) (Figure 4-6) in female donor cells. Lastly, these results confirm that the upregulated mRNA expression of IFNB1, CCL5, CXCL10, CXCL11, and IDO1 (Figure 4-2) correlates with protein levels.

**ANDV Infection Inhibits Cell Death**

Based on our findings, and those of others (Geimonen et al., 2002; Connolly-Andersen et al., 2014; Safronetz et al., 2014), where a highly dysregulated proinflammatory immune response is accompanied by high IDO, we hypothesized that these viruses may suppress cell death and/or apoptosis. To test this hypothesis, HLMVECs from donor 2559 were infected with BS (mock) or ANDV. At 60 hpi, cells were treated with vehicle (1% DMSO) or staurosporine to induce apoptosis and, at 6 h post-staurosporine addition, caspase 3/7 activity was measured (Figure 4-7). In staurosporine-treated HLMVECs a four-fold increase of caspase activity was observed, and the cells were visibly dying. ANDV caspase activity was reduced three-fold compared to the staurosporine-treated HLMVECs. The identical experiment was conducted for HTNV and PHV, but no suppression of caspase 3/7 activity was observed.

**Discussion**

In this study, we examine the early hantaviral-host interaction in HLMVECs following infection of by pathogenic (ANDV and HTNV) and nonpathogenic (PHV) hantaviruses. The understanding of these early virus-host interactions is critical because it is hypothesized that they drive differential disease outcomes between pathogenic and nonpathogenic hantaviruses. This hypothesis is an active area of hantavirus research, but current research models are either based on surrogate cell models or only study a limited number of host markers and/or time points using different hantavirus species. To advance the understanding of hantaviral-host interactions, we examined the dynamics of
Figure 4-4. Secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β at 48 hpi and 60 hpi by four HLMVECs donors following infection with ANDV, HTNV, or PHV. HLMVECs from two male (circles, 2559 (black), 2572 (magenta)) and two female donors (square, 2551 (teal), 438Z013.1 (purple)) differing in age were infected with ANDV, HTNV, or PHV at an MOI of 2 (n=3). Supernatants were collected at 48 and 60 hpi then secreted protein levels measured. Error bars are mean and standard deviation.
Figure 4-5. Principle component analysis of the protein levels of CCL5, CXCL10, CXCL11, IDO, and IFNB-β secreted at 48 and 60 hpi by four HLMVEC donors following infection with ANDV, HTNV, or PHV.

HLMVECs from four donors 2551 (orange), 2559 (green), 2572 (teal), or 438Z013.1 (purple) were infected with ANDV (circles), HTNV (triangles), or PHV (squares) at an MOI of 2 (n=3). Supernatants were collected at 48 hpi (small symbols) and 60 hpi (large symbols) and secreted protein levels of measured and analyzed by principal component analysis.
Figure 4-6. Secreted protein levels of CCL5, CXCL10, CXCL11, IDO, and IFN-β at 48 hpi and 60 hpi by male and female HLMVECs donors following infection with ANDV, HTNV, or PHV. HLMVECs from four donors, two males (black circles) and two females (magenta squares), were infected with ANDV, HTNV, or PHV at an MOI of 2 (n=3). Supernatants were collected at 48 hpi and 60 hpi then secreted protein levels were measured. Error bars are mean and standard deviation.
Figure 4-7. The inhibition of cell death of ANDV-infected HLMVECs following staurosporine treatment
HLMVECs, donor 2559, was either mock or ANDV-infected at an MOI of 1.0 and at 60 hpi, cells were treated with 1 μM staurosporine (ST) and after six hours caspase 3/7 activity was measured. Error bars are represented by mean and standard deviation.
proinflammatory and anti-inflammatory responses at multiple time points during infection with ANDV, HTNV, or PHV. These findings are of interest because they can be used to examine the difference in immune response in HLMVECs following hantaviral infection, and the time-resolved data is useful within mathematical models that can aid interpretation of the data.

In our established hantavirus HLMVEC-hantavirus model, we measured mRNA levels of 39 genes consisting of genes that are involved in proinflammatory and anti-inflammatory response during hantavirus infection. Our gene expression analysis revealed only TLR3, IRF7, IFNB1, CCL5, IDO1, CXCL10, and CXCL11 were upregulated however mRNA levels differed in temporal dynamics and/or magnitude between virus species. These findings suggest a highly dysregulated immune response with a number of proinflammatory genes, such as IL6, IFNG, and TNF, that were not upregulated in addition to the upregulation of the immunosuppressive gene, IDO1.

Hantaviruses are able to trigger both TLR3- and RIG-I-mediated responses during infection (Alff et al., 2006; Handke et al., 2009; Matthys et al., 2014), but this RIG-I was not included in the current study. Interestingly, not all hantavirus species trigger these PRRs as RIG-I is not required for activation of immune response during infection of HuH7.5 cells with PHV. Similarly, TLR3 is required for MxA activation in A549 cells infected with HTNV but not PHV (Handke et al., 2009). The activation of TLR3 is also observed in other primary endothelial cells models such as HUVECs during hantaviral infection (Prescott et al., 2005).

Within TRL and RIG-I cascades are the IRF proteins. This family of proteins is critical because they regulate the expression of a number of genes involved with immune responses. Of the IRF proteins, IRF3 has been implicated in driving differing immune response activation observed during pathogenic and nonpathogenic hantavirus infection. Some studies show pathogenic hantaviruses ANDV (Spiropoulou et al., 2007) and SNV (Alff et al., 2006; Alff et al., 2008) are able to inhibit IRF3 nuclear translocation upstream of the protein at multiple locations to a greater extent compared to nonpathogenic hantaviruses, such as PHV. Of note, IRF7 levels were not measured in these studies, which may be of importance because a number of genes’ are regulated by either/both IRF3 and IRF7. In addition, these two proteins can form homo- and heterodimers with themselves and translocate across the nucleus. In contrast to past studies, we did not observe upregulated mRNA levels of IRF3 but did observe an increase in IRF7. Similar results were observed in both ANDV- and HTNV-infected Syrian golden hamsters (Brocato et al., 2021). (Brocato et al., 2021). Interestingly, IRF3 and IRF7 proteins are expressed in SNV-infected HLMVECs. However, neither IRF3 nor IRF7 is required for the activation of immune responses in human hepatoma cell lines (Prescott et al., 2007).

As expected, given our observation that TLR3 and IRF7 are upregulated, levels of IFNB1 were increased as well during hantaviral infection of HLMVECs. Hantaviruses are sensitive to type I interferons, which may suggest why hantaviruses have evolved mechanisms to control their expression as discussed above. Past research on IFN-β
activation during hantavirus infection reported that HLMVECs infected with ANDV does not activate expression of IFNB1 as strong or as early compared to PHV-infected cells. In contrast to these findings, our measurements revealed IFNB1 was significantly higher in ANDV-infected cells compared to PHV-infected cells at multiple time points (Spiropoulou et al., 2007).

Of the chemokines we measured in our gene expression panels, only CCL5, CXCL10, and CXCL11 mRNA levels were increased. All three genes are upregulated through IRF7 expression and/or NF-kB binding to interferon stimulated response element (ISRE) in their promoters (Génin et al., 2000; Antonczyk et al., 2019) The upregulation of the genes may be critical to the infiltration and activity of various immune cells during human hantaviral infection as suggested in the Syrian golden hamster model with ANVD. Syrian golden hamsters infected with ANDV or HTNV, only infection by ANDV results in increased mRNA levels of CCL5 and CXCL10 that coincides with the influx of macrophages, dendritic cells, CTLs and NK cells (Brocato et al., 2021). In other comparative studies of hantavirus species in HUVECs, CCL5 mRNA levels are similar between HTNV- and PHV-infected cells. The same study reported, CXCL10 and CXCL11 mRNA levels are higher in HTNV-infected cells compared to that of PHV-infected cells (Geimonen et al., 2002). In our current study, we observed similar trends in HTNV-infected HLMVECs which had higher mRNA levels of CCL5, CXCL10, and CXCL11 at later stages of infection compared to PHV-infected HLMVECs. The upregulated levels of CCL5, CXCL10, and CXCL11 are observed in the sera of HPS patients (Morzunov et al., 2015).

A novel finding in this study is the upregulation of IDO in hantavirus infected HLMVECs. The presence of IDO has only been previously reported during human cases of nephropathia epidemic (NE), a milder form of HFRS caused by Puumala orthohantavirus (PUUV) infections (Outinen et al., 2011; Koivula et al., 2017). High levels of IDO are reported in the sera of NE patients and IDO levels increases as disease phases progress. These studies report that high IDO levels correlates with disease severity (Outinen et al., 2011), and correlated with the infiltration of regulatory T cells expressing forkhead box P3 (FOXP3) (Koivula et al., 2017) The authors suggest IDO levels may suppress immune responses and potentially hindering viral clearance. IDO activity is noted during infection for a number of RNA viruses, such as Dengue virus, Hepatitis-C virus, and influenza A virus (Larrea et al., 2007; Becerra et al., 2009; Lepiller et al., 2015; Lin et al., 2020). IDO inhibition of Influenza A virus infected mice results in increased levels of IFN-γ secreted by CD4+ and CD8+ T-cells, higher rates of Th1 responses and improved repair of lung tissues (Sage et al., 2014).

A second finding in our model system was the lack of upregulation of mRNA levels of proinflammatory cytokines (i.e., IFNG, IL6, or TNF) suggesting a highly dysregulated immune response in hantavirus infected HLMVECs. This correlated with a lack of a NFKB1 response which regulates a number of proinflammatory cytokines. Previous studies on NF-kB mediated responses during hantavirus infection revealed hantavirus N protein sequesters NF-kB in the cytoplasm and inhibiting its nuclear translocation through interactions with host importins (Taylor et al., 2009a; Taylor et al.,
The inhibition of NF-κB translocation, potentially inhibits the mRNA expression of proinflammatory cytokines that are observed in human HPS and HFRS patients.

The dysregulated immune response and the upregulation of IDO showing suppressed immune responses in hantavirus-infected HLMVECs suggests hantaviruses can potentially suppress other host responses such as those involved in cell death. Our group has previously shown that HTNV-infected Vero E6 cells inhibit cell death induced by staurosporine treatment (Ontiveros et al., 2010). Cell death inhibition was determined to be caused by the virus N protein interacting with host’s effector caspases as well as inhibiting NF-κB nuclear translocation. This current study shows similar cell death inhibition in ANDV-infected HLMVECs following treatment with staurosporine whereas PHV-infected cells were not able to.

In our study of the host immune responses to hantavirus infection in HLMVECs from multiple donors, differing in age and sex, we showed that HLMVEC donor characteristics and hantavirus species together contribute to differences observed in these responses. Another finding of great interest in these studies were that female HLMVECs produced higher secreted protein levels of CXCL10, IDO, and IFN-β. However the upregulation of these proteins was unique to hantavirus species. In clinical case studies of hantavirus diseased patients report that mortality rates are higher for females with HRFS in China and with HPS in Argentina (Martinez et al., 2010; Klein et al., 2011a). These findings show the need for research studies on host immune responses to include multiple donors in study models.

In conclusion this study shows, hantaviruses that drive different diseases in humans cause a highly dysregulated immune responses with immune suppression and IDO upregulation in HLMVECs during infection. While the infection of these viruses results in upregulation of the same genes at the same time points, they differ in their temporal dynamics and/or magnitude expression. Expansion of our study models to multiple donors show that donor characteristics can drive different immune responses during infection. Together these findings advance the understanding of the immune responses activated during hantavirus infection.
CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

The understanding of how zoonotic diseases emergence and cause outbreaks caused by RNA viruses through zoonotic spillover events is of great importance from a public health standpoint, as zoonotic pathogens can potentially have a large impact and burden on public health. One of these emerging zoonotic pathogens are hantaviruses. Hantaviruses comprise of a large number of species of viruses and their spillover into human populations cause diseases globally. The impact of this family of viruses is evident with more than 100,000 cases that are diagnosed each year in Europe and Asia alone. This number can only increase with the discovery of more hantavirus species, the environmental factors that drive spillover of these viruses and improved clinical diagnostic tools. Subsequently, there is a critical need to understand how spillover occurs, how these viruses interact with their host during infection and to identify these viruses where they are found circulating.

In Chapter 2, the themes that are observed during the spillover and emergence of RNA viruses harbored by bats and rodents into humans that have caused outbreaks in humans are discussed. The focus of RNA viruses harbored by bats and rodents that cause zoonotic diseases in humans are of importance as these are the two largest groups of mammals on earth. To better understand how RNA virus spillovers occur, I identified the factors that allow these viruses to be so successful in their reservoirs, what the drivers of spillover of these viruses are from bats and rodents, and lastly how past spillover events occur from reservoirs to humans and resulted in outbreaks. For each of the factors, the main components that are at play and how these components interact with each other and allow for spillover, are identified. RNA viruses which use bats and rodents as their reservoirs take advantage of multiple biological strategies that have been acquired through evolution. These biological strategies include, but are not limited to, how these viruses interact with their reservoir hosts during infection. The evolution of these viruses has allowed for common strategies, such as causing limited-to-no pathology in these hosts and how this phenomenon is achieved is through viruses being able to modulate host immune responses allowing hosts to tolerate viral infection. However, this is not observed in all reservoir infections, as there are a number of consequences related to these virus infections including lowered fecundity or decreased lifespan.

The biological strategies utilized by viruses include limiting immune responses through the modulation of early immunity, such as that of interferons and adaptive immunity such as B and T cells. These strategies gained through evolution help maintain virus populations in their reservoir hosts but are not solely responsible for causing spillover of zoonic viruses, as environmental factors play a critical role as well. Environmental factors cause viruses through their reservoir host to come in close proximity with humans and allow for the occurrence of spillover. One of the largest drivers of virus spillover is the anthropogenic encroachment of reservoir habitats, such as the forever expansion of farmlands and urbanization and other factors such as climate change. These environmental factors and biological strategies have a combinatorial effect allowing for spillover and have been studied in past outbreaks of RNA zoonotic diseases.
to help determine the role they played in these outbreaks. The study of past spillover events is crucial to better understand what factors may have driven spillover events and with this gained knowledge this can help to prevent these events from occurring in the future. This chapter brings to the fore the gaps in knowledge in understanding spillover and what is needed to fill these gaps. It shows the importance of making use of proactive surveillance efforts such as those conducted in Chapter 3 to preemptively prevent or predict outbreaks by determining what viruses are circulating in their reservoir species and developing experimental reservoir and human hosts models to study how these viruses interact with these hosts as performed in Chapter 4.

In Chapter 3, the prevalence of hantaviruses that are circulating in their reservoir hosts in northwestern Ukraine is investigated through a surveillance study. In this field collection surveillance effort, we collected 424 small mammals from their natural habitat at two sites, with each site consisting of multiple trapping lines (Figure 3-1 and Table 3-1). Each of these trap lines were located in distinct habitats that included forest, shrubs and grasslands, with some of these trapping lines being in close proximity of human dwellings or farmlands. The small mammal collection encapsulated a large diversity of species with nine different species from three mammal orders (Table 3-1 and Figure 3-2). The most abundant species were *Myodes glareolus* (bank vole, 46%), *Apodemus flavicollis* (yellow-necked mouse, 29%) and *Apodemus agrarius* (striped field mouse, 14.6%). These three species are of great importance, as they are reservoirs of hantavirus species with the *M. glareolus* harboring *Puumala orthohantavirus* (PUUV), which results in nephropathic epidemic (NE) during human infections and the *Apodemus* spp. harboring *Dobrava-Belgrade orthohantavirus* (DOBV) which results in hemorrhagic fever with renal syndrome (HFRS) during human infection. From the 424 small mammals captured, 79 were determined to be hantavirus seropositive by immunofluorescent assay (IFA) (Table 3-2 and Figure 3-3). It was found that only rodents and shrews were seropositive for hantaviruses. *M. glareolus* was the species of which tested specimens were antibody positive, followed by *A. flavicollis* and *A. flavicollis*. Interestingly, a captured Eurasian pygmy shrew (*Sorex minutus*) and house mouse (*Mus musculus*) was also seropositive, indicating that spillover of these viruses from reservoir hosts to nonreservoir hosts is occurring. IFA reciprocal tires showed a large distribution of IgG titers ranging 1:32 to 1:8192, indicating that new infections of these host are occurring (Table 3-3 and Figure 3-4). The study of the capture locations showed that there were differences between the locations of the trap lines based on the proportion of captured animals that were seropositive and species diversity based on Shannon index of each location (Table 3-4 and Figure 3-5). I found that there was no relationship between either the number of seropositive animals and species diversity nor the proportion of seropositive animals and species. Focusing on the age and sex of the three most abundantly captured rodent species that were seropositive, it was found that neither sex nor age was associated with being seropositivity, as similar numbers of males and female were seropositive, and that similar number of adults and juvenile were seropositive (Table 3-5 and Figure 3-6). Differences were observed between males and females when ages were studied as there were similar numbers of seropositive adult and juvenile males, but this was not true for adult and juvenile females, as not a large number
of juvenile females were captured. Weight classes of each of these three rodent species suggest that most of these rodents that were seropositive were subadults or adults.

As discussed in Chapter 2, field capture surveillance studies such as these are of great importance and play a crucial role in preventing virus spillover into human populations. Surveillance efforts are critical as they aid in determining whether a virus is found circulating within a particular region. These studies can aid in the prediction of zoonotic RNA virus spillover occurrences, as it can be an early indicator of changes of not only virus populations, but also that of reservoir hosts. Population structure changes have been determined to be the cause of outbreaks such as in the case of masting years of food sources that are consumed by reservoirs, which results in an explosion of populations allowing for more carriers of a zoonotic virus.

The knowledge of identifying that a virus is circulating can be built upon through the use of molecular tools to determining similarities between viruses and determine whether the development of improved diagnostic tools is needed to better diagnose human infection cases. Diagnostic tools are crucial from a public health standpoint, as it allows for better treatment of infected patients that would have been circumvented if patients were misdiagnosed.

In Chapter 4, I examined human host’s antiviral and innate immune responses during hantavirus infection. These responses were studied during infection by hantavirus species that are classified as pathogenic, *Andes orthohantavirus* (ANDV) and *Hantaan orthohantavirus* (HTNV), and nonpathogenic, *Prospect Hill orthohantavirus* (PHV) in primary human lung microvascular endothelial cells (HLMVECs), a biological relevant model.

To study the early antiviral and innate immune responses to hantaviruses following infection of HLMVECs, I made examined host’s gene expression and protein secretion level. Through microscopy, I show HLMVECs from a 42-year-old is an appropriate model system to study hantaviral-host immune responses as ANDV, HTNV, and PHV infected the donor cells and similarly number of cells infected by each of the three viruses were similar (Figure 4-1). Based on these findings, I examined the mRNA levels of 39 genes in the same HLMVECs donor infected by ANDV, HTNV, or PHV at 12, 24, 36, 48, 60, and 72 hpi. Gene expression analysis revealed only seven genes, *CCL5, CXCL10, CXCL11, IDO1, IFNB1, IRF7,* and *TLR3* were upregulated (Figure 4-2). These findings suggest a highly dysregulated and suppressive immune response in HLMVECs. Principle component analysis of the mRNA levels of each of the 39 gene showed groupings based on virus species as well as measurement time points (Figure 4-3). The examination of the seven upregulated genes, revealed that mRNA levels of the seven genes differed significantly at time points based on virus species. mRNA expression of the regulated genes was confirmed by measuring secreted protein levels of *CCL5, CXCL10, CXCL11, IDO,* and *IFN-β* (Figure 4-4). To further my gene expression findings, I expanded my study model to include more HLMVEC donors with the addition of HLMVECs from another male and two females. I measured secreted protein levels of *CCL5, CXCL10, CXCL11, IDO,* and *IFN-β,* secreted by each
HLMVEC donor following infection by ANDV, HTNV, or PHV (Figure 4-4). The measured secreted protein levels revealed that each of the proteins were up regulated in each donors following infection by ANDV, HTNV, or PHV. Principle component analysis of the measured secreted protein levels revealed the results were heterogenous with no apparent grouping based on donors or virus species (Figure 4-5). Pairwise analysis of the measured protein levels suggests that donors’ characteristics and virus species together drive these outcomes. Interestingly, comparison of secreted protein levels measurements based on the sexes of the donors revealed that female donors had significantly higher levels of CXCL10 (ANDV at 48 hpi), IDO (ANDV at 48-60 hpi; PHV at 60 hpi) and IFN-β (ANDV, HTNV at 60 hpi) (Figure 4-6). These finding highlights the importance of incorporating multiple donors and how it can aid in the better understanding of virus-host interactions. With the upregulation of IDO and dysregulated and suppressed immune responses during hantavirus infection, I examined the suppression of cell death by hantaviruses in HLMVECs. These studies showed that staurosporine treated ANDV infected HLMVECs is capable of suppressing cell death greater when compared to uninfected HLMVECs (Figure 4-7).

To advance the understanding of the interactions between hantaviruses and human hosts during early infection, there is a need to use biologically relevant models, such as HLMVECs. While research studying the immune response to hantavirus infection in surrogate models based on cell lines or primary cells have generated a wealth of knowledge, findings from these studies can be contradicting at times and makes the translation of this research complicated. Models that better mimic a natural human infection, create a basic foundation that can be built and further expanded upon. There is a need to understand what roles the genes and proteins found in Chapter 4 have on other cells during hantavirus infection. To address this need, host immune cells such as macrophages, dendritic cells, natural killer cells, and cytotoxic t cells (CTLs) need to be incorporated into study models through combinatorial model systems. These studies will be able to tease apart how HLMVECs interact with immune cells and how each cell contributes to disease outcomes observed in humans. These models are essential, as currently there is no small mammal model that mimics disease progression observed during HFRS or HPS. While there is no small mammal model that induces similar disease outcomes observed in humans, great strides have been made through models, such as Syrian Golden hamsters (Mesocricetus auratus). A shortcoming of this infectious model is that ANDV is the only hantavirus that is able to cause disease without manipulating the infection through immune suppression by which other viruses such as Sin Nombre orthohantavirus (SNV) are able to cause mortality of infected hamsters. A compounding factor with the study of the use of small mammal infection models is that these infection studies are restricted to only biosafety level (BSL) 4 laboratories, which are limited. These small mammal models are also not well established making them expensive and difficult to acquire. One aspect of these models that can be of use is the isolation of HLMVECs of these small mammals that can be studied in a similar manner as was performed in Chapter 4. A constraining factor of not using established models is the lack of basic knowledge, which is taken for granted in other models, such has not having a complete genome mapped, making studies such a gene expression analysis complicated. Additionally, many non-established models lack the necessary reagents that
are compatible with these models. Lastly, during hantaviral infection of each virus’ reservoir hosts, the responses induced differ greatly to what is observed in humans. Thus, results that are obtained from these models are complicated to translate from one model to another.

In conclusion, this work reveals the importance of zoonotic diseases caused by RNA viruses. The work identifies the drivers of spillover of these pathogens into human populations and cause diseases. It shows how the emerging zoonotic pathogen, hantaviruses, interact and activate the early innate immune responses in humans through the use of infection models as well as the ecology of these viruses in their reservoir hosts. Lastly it shows what future efforts are needed to build upon current knowledge.
LIST OF REFERENCES


Becerra, A., Warke, R.V., Xhaja, K., Evans, B., Evans, J., Martin, K., De Bosch, N., Rothman, A.L., and Bosch, I. (2009). Increased activity of indoleamine 2,3-dioxygenase in serum from acutely infected dengue patients linked to gamma interferon antiviral function. *J Gen Virol* 90, 810-817. doi: [https://doi.org/10.1099/vir.0.004416-0](https://doi.org/10.1099/vir.0.004416-0)


Cline, B.J., Carver, S., and Douglass, R.J. (2010). Relationship of human behavior within outbuildings to potential exposure to Sin Nombre virus in western Montana. *Ecohealth* 7, 389-393. doi: https://doi.org/10.1074/s10393-010-0318-x


Fischer, K., Pinho Dos Reis, V., and Balkema-Buschmann, A. (2017). Bat Astroviruses: Towards Understanding the Transmission Dynamics of a Neglected Virus Family. *Viruses* 9, 34. doi: [https://doi.org/10.3390/v9020034](https://doi.org/10.3390/v9020034)


Gu, S.H., Nicolas, V., Lalis, A., Sathirapongsasuti, N., and Yanagihara, R. (2013). Complete genome sequence and molecular phylogeny of a newfound hantavirus harbored by the Doucet’s musk shrew (Crocidura douceti) in Guinea. *Infection,*


Justines, G., and Johnson, K.M. (1969). Immune Tolerance in *Calomys callosus* infected with Machupo Virus. *Nature* 222, 1090-1091. doi: [https://doi.org/10.1038/2221090a0](https://doi.org/10.1038/2221090a0)


hemorrhagic fever, hantavirus pulmonary syndrome. *J Immunol* 172, 3297-3304. doi: [https://doi.org/10.4049/jimmunol.172.5.3297](https://doi.org/10.4049/jimmunol.172.5.3297)


Indoleamine-2,3-Dioxygenase in Hepatitis C Virus Infection. *Journal of Innate Immunity* 7, 530-544. doi: [https://doi.org/10.1159/000375161](https://doi.org/10.1159/000375161)


Lundkvist, Å., Hörling, J., and Niklasson, B. (1993). The humoral response to Puumala virus infection (nephropathia epidemica) investigated by viral protein specific immunoassays. Archives of Virology 130, 121-130. doi: https://doi.org/10.1007/BF01319001


fatal disease in horses and humans. Science 268, 94-97. doi: https://doi.org/10.1126/science.7701348


Odorizzi, P.M., and Wherry, E.J. (2012). Inhibitory Receptors on Lymphocytes: Insights from Infections. The Journal of Immunology 188, 2957-2965. doi: https://doi.org/10.4049/jimmunol.1100038


Overbosch, F., De Boer, M., Veldkamp, K.E., Ellerbroek, P., Bleeker-Rovers, C.P., Goorhuis, B., Van Vught, M., Van Der Eijk, A., Leenstra, T., Khargi, M., Ros, J.,


Owen, R.D., Goodin, D.G., Koch, D.E., Chu, Y.-K., and Jonsson, C.B. (2010). Spatiotemporal variation in Akodon montensis (Cricetidae: Sigmodontineae) and hantaviral seroprevalence in a subtropical forest ecosystem. *Journal of Mammalogy* 91, 467-481. doi: [https://doi.org/10.1644/09-mamm-a-152.1](https://doi.org/10.1644/09-mamm-a-152.1)


Peters, C.J. (2002). Human infection with arenaviruses in the Americas. *Curr Top Microbiol Immunol* 262, 65-74. doi: [https://doi.org/10.1007/978-3-642-56029-3_3](https://doi.org/10.1007/978-3-642-56029-3_3)


Prescott, J.B., Hall, P.R., Bondu-Hawkins, V.S., Ye, C., and Hjelle, B. (2007). Early innate immune responses to Sin Nombre hantavirus occur independently of IFN regulatory factor 3, characterized pattern recognition receptors, and viral entry. *J Immunol* 179, 1796-1802. doi: [https://doi.org/10.4049/jimmunol.179.3.1796](https://doi.org/10.4049/jimmunol.179.3.1796)


Rogers, R.J., Douglas, I.C., Baldock, F.C., Glanville, R.J., Seppanen, K.T., Gleeson, L.J., Selleck, P.N., and Dunn, K.J. (1996). Investigation of a second focus of equine morbillivirus infection in coastal Queensland. *Aust Vet J* 74, 243-244. doi: [https://doi.org/10.1017/S000490900001306X](https://doi.org/10.1017/S000490900001306X)


Smadel, J.E. (1953). Epidemic hemorrhagic fever. *Am J Public Health Nations Health* 43, 1327-1330. doi: [https://doi.org/10.2105/ajph.43.10.1327](https://doi.org/10.2105/ajph.43.10.1327)


Wu, K., Peng, G., Wilken, M., Geraghty, R.J., and Li, F. (2012). Mechanisms of Host Receptor Adaptation by Severe Acute Respiratory Syndrome Coronavirus. *Journal of Biological Chemistry* 287, 8904-8911. doi: [https://doi.org/10.1074/jbc.m111.325803](https://doi.org/10.1074/jbc.m111.325803)


key regulator of CD8+ T cell persistence in chronic infection. Nature Immunology 20, 890-901. doi: https://doi.org/10.1038/s41590-019-0403-4


constitutive expression of IFN-alpha in bats. *Proc Natl Acad Sci U S A* 113, 2696-2701. doi: [https://doi.org/10.1073/pnas.1518240113](https://doi.org/10.1073/pnas.1518240113)


VITA

Evan Peter Williams was born in Knysna, South Africa in 1991. He attended Paarl Gimnasium and graduated in 2008. Evan graduated in 2013 with Bachelor of Science in Molecular Biology and Biotechnology from Stellenbosch University, Stellenbosch South Africa. Following graduating from Stellenbosch University he earned a Bachelor of Science in Biology from Kennesaw State University, Kennesaw, GA in 2015. In 2015, he was accepted to the University of Tennessee, Knoxville as graduate student in the Department of Microbiology, here he joined the laboratory of Dr. Colleen B. Jonsson, researching hantaviruses. The Jonsson laboratory transferred in 2017 to the University of Tennessee Health Science Center in Memphis, Tennessee in the Department of Microbiology, Immunology and Biochemistry. Evan expects to graduate with a Doctor of Philosophy degree in the Integrated Biomedical Sciences Program in December 2021.